Blood and Hemoglobin: The Evolution of Knowledge of Functional Adaptation in a Biochemical System

PART I: THE ADAPTATION OF CHEMICAL STRUCTURE TO FUNCTION IN HEMOGLOBIN

JOHN T. EDSALL

Harvard University Biological Laboratories
Cambridge, Massachusetts

The circulating blood, while passing through the lungs, discharges carbon dioxide and takes on oxygen. As it flows through the capillaries of the tissues, it releases oxygen and takes up carbon dioxide. By the late nineteenth century the basic nature of this respiratory function of the blood was clearly recognized. In the transport of oxygen the central role of hemoglobin, the iron-containing protein of the red blood cells, was also apparent. However, the adaptation of structure to function in the hemoglobin molecule, and in blood regarded as a physicochemical system for the transport of oxygen and carbon dioxide, was in fact far more subtle and more efficient than any investigator could have anticipated in the year 1900. During the first thirty years of the twentieth century a few investigators succeeded in characterizing these complex and subtle interactions that demonstrated the biochemical basis of a remarkable biological adaptation. By 1930 the fundamental picture of blood as a highly organized system for transport of the gases involved in respiration was largely complete. Much has since been learned, and in the last forty years our knowledge of the detailed structure and function of the hemoglobin molecule has increased immeasurably. However, the central picture of blood as a functioning system, set forth by Lawrence J. Henderson in his Silliman Lectures[1] on "Blood" in 1928, still stands in its main outlines as a definitive achievement. My central theme here is to trace the steps that led to this comprehensive understanding, which was largely the work of Christian Bohr and August Krogh in Copen-

1. L. J. Henderson, *Blood: A Study in General Physiology* (New Haven: Yale University Press, 1928).

Journal of the History of Biology, vol. 5, no. 2 (Fall 1972), pp. 205–257.

hagen, of John Scott Haldane in Oxford, of Joseph Barcroft and A. V. Hill at Cambridge, of Lawrence J. Henderson at Harvard and of Donald D. Van Slyke at the Rockefeller Institute in New York. At a crucial period, the work of G. S. Adair in Cambridge and of The Svedberg at Uppsala, on the size of the hemoglobin molecule, and the work of Adair on its interactions with oxygen, were of central importance. We shall be concerned not only with the achievements of these men, but with their errors and their failure to perceive clues that later seemed obvious, and in some cases with the philosophical outlook that conditioned their research, or grew out of their experimental work.

This, the first of two papers, deals primarily with the hemoglobin molecule, and the growth of knowledge concerning its role in the transport of oxygen and carbon dioxide in the blood. The following paper will deal with the organization of blood as a system, involving not only the transport of oxygen and carbon dioxide, but the flow of water and electrolytes between the red cells and the plasma during the respiratory cycle. It will also deal with the kinetics of uptake and release of oxygen and carbon dioxide, and with the direct role of hemoglobin in combining with carbon dioxide. The period under consideration in these two papers is from the early nineteenth century to 1930 or slightly later. The more recent advances in our knowledge of hemoglobin, fundamental and dramatic as they are, are perhaps too close to be seen in historical perspective. First, however, we must consider, somewhat briefly, the accumulated knowledge of blood and hemoglobin as it stood at the end of the nineteenth century.

EARLY WORK ON THE CHEMISTRY OF HEMOGLOBIN

In the following discussion we may think of blood, as Henderson did, in terms of a simplified model system composed of red blood cells, some five million of them per cubic millimeter of blood, occupying 35–40% of the total blood volume, and of plasma, occupying the rest of the blood volume. The plasma contains some 7 grams of various proteins per 100 cc, but the plasma proteins, though of the greatest physiological importance, are far less important for our present concerns than the hemoglobin of the red cells. We may neglect the white cells, so important in immune reactions and in other ways, but relatively few in number, and unrelated to the transport of the respiratory gases. The blood coagulation process, except for the practical necessity to remove the clot if it forms, or to inhibit its formation by adding salts such as sodium oxalate or citrate,

or in other ways, may also be neglected. Physiologists and chemists studying the respiratory function of the blood concentrated their thoughts and experiments essentially on this sort of simplified model system, although to my knowledge only Henderson (1) explicitly formulated this concept of a model system as a basis for thinking about the respiratory function of the blood.

The pigment that was responsible for the intense color of blood, and of red blood cells, had already been obtained in crystalline form, from one or two species of animals, as early as 1840. By 1850 hemoglobin from several species of animals had been crystallized.[2,3] It was soon apparent that this hemoglobin from certain species—for instance rats, guinea pigs, mice, and squirrels—crystallized with great ease and almost spontaneously. It was only necessary to render the red cells permeable to hemoglobin—as, for instance, by adding distilled water, with rupture of the membrane by osmotic inflow of water—and to acidify the resulting solution slightly with carbon dioxide, in order to obtain rapid formation of crystals. Horse hemoglobin also crystallized with relative ease. Other hemoglobins, such as those of man and cattle, required much more careful adjustment of conditions before crystals were obtained. In 1871 appeared the remarkable monograph of Wilhelm Preyer (1841–1897),[4] who described the crystallization of hemoglobin from more than forty species of mammals, amphibia, birds, fishes, and reptiles. He described in detail many of the properties of the crystals, and methods of obtaining them.

The discovery that hemoglobin contained iron came early. In 1853 Teichmann,[5] by treating blood with glacial acetic acid, and heating, obtained reddish brown prismatic crystals—often referred to as Teichmann's crystals during the next generation—of a substance containing iron, and later known as hemin. It soon became clear that hemin was derived from hemoglobin and that its removal from the hemoglobin molecule left behind a colorless iron-free protein, later known as globin.[6,7]

2. These early developments are well described in the monograph by F. N. Schulz, *Die Krystallisation von Eiweisstoffen und ihre Bedeutung für die Eiweisschemie* (Jena: Gustav Fischer, 1901).

3. See also J. T. Edsall, "Proteins as Macromolecules: An Essay on the Development of the Macromolecule Concept and Some of its Vicissitudes," *Arch. Biochem. Biophys.*, Supplement I (1962), 12–20.

4. W. Preyer *Die Blutkrystalle* (Jena: Mauke's Verlag, 1871).

5. Teichmann, *Z. rat. Med.*, 3 (1853), 875; 8 (1858), 141. This reference is taken from Gamgee (see n. 11 below), p. 252.

6. The name "hemoglobin" was first proposed by the eminent German physiological chemist Felix Hoppe-Seyler (1825–1895), and was soon

Under normal conditions, in neutral solution, however, the hemoglobin molecule remained a functional unit, the large protein component and the smaller iron-containing component forming a conjugated molecule that remained stable under physiological conditions. Early analytical determinations indicated that hemoglobin contained roughly 0.4 per cent iron; but all earlier work was superseded by the beautiful analyses obtained, in the laboratory of Gustav von Bunge in Basel, by Zinoffsky[8] on horse hemoglobin in 1885, and by Jaquet[9] on dog and chicken hemoglobin four years later. Zinoffsky obtained an iron content of 0.335% and Jaquet's value was 0.336% for dog and 0.3353% for chicken hemoglobin. The agreement was certainly within experimental error, although such agreement was not necessarily to be expected, since the hemoglobins were derived from two different species. Zinoffsky calculated the minimum molecular weight of the hemoglobin molecule if it contained only one atom of iron. Taking the atomic weight of iron as 55.85, the minimum molecular weight of hemoglobin must be $55.85 \times 100/0.335$ or 16,670. If the molecule contained n atoms of iron, the molecular weight must be n times as great as this. Plainly hemoglobin must be a very large molecule even at the minimum estimate, almost incredibly large by the standards of the chemists of the late nineteenth century. Zinoffsky's value for sulfur in horse hemoglobin indicated, with high precision, that the atomic ratio of sulfur to iron was 2 to 1, and this ratio is indeed confirmed by the most modern determinations of the complete structure of the molecule.[10] Thus the work

universally adopted. See *Virchows Arch. path. anat. Physiol.*, 29 (1864), 223.

7. For the purposes of this article, it is not necessary to consider the great advances made in the twentieth century in the chemistry of the iron porphyrins and other metalloporphyrins, of which the hemin (in its reduced state now known as heme, or haem) derived from hemoglobin is one. The most notable work in determining the complete structures of these compounds was that of Hans Fischer and his associates in Munich, from about 1920 to 1940. For a survey of the field see, for example, R. Lemberg and J. W. Legge, *Haematin Compounds and Bile Pigments: Their Constitution, Metabolism and Function* (New York: Interscience Publishers, 1949).

8. O. Zinoffsky, "Ueber die Grösse des Hämoglobinmoleküls," *Hoppe-Seylers Z. physiol. Chem.*, *10* (1886), 16–34.

9. A. Jaquet, "Beiträge zur Kenntniss des Blutfarbstoffs," *Hoppe-Seylers Z. physiol. Chem.*, *14* (1889), 289–296.

10. See, for instance, the tabulations in M. Dayhoff, ed., *Atlas of Protein Sequence and Structure* (Silver Spring, Md.: National Biomedical Research Foundation, 1969). Data for horse hemoglobin are on pages D–43 and D–56. We should note incidentally that Zinoffsky's carbon and hydrogen determinations were apparently much less accurate than his values for iron and sulfur.

of Zinoffsky and Jaquet was in harmony with the view that hemoglobin was a definite chemical molecule, in spite of its great size. Later work by Gustav von Hüfner on ox hemoglobin gave values for iron content in very close agreement with the data for horse, dog, and chicken.[11]

THE SPECTROSCOPY OF HEMOGLOBIN, OXYHEMOGLOBIN AND CARBONMONOXYHEMOGLOBIN

Apparently Felix Hoppe-Seyler, a notable and versatile pioneer in biochemical research and the founder of *Hoppe-Seyler's Zeitschrift für Physiologische Chemie,* was the first to report that he had observed the beautiful absorption spectrum of blood, and of hemoglobin, in the visible region.[12] In the presence of oxygen he saw two intense and characteristic absorption bands, one in the region near 535 nanometers (nm), the other at a longer wavelength near 560 nm. The full significance of these observations, however, became apparent only two years later, in a major contribution[13] by the eminent mathematical physicist, George Gabriel Stokes (1819–1903). Stokes removed oxygen from the hemoglobin solution with a reducing solution composed of ferrous sulfate and tartaric acid, the solution being made somewhat alkaline by adding ammonia or sodium carbonate. In the presence of the reducing agent, the two-banded spectrum disappeared and was replaced by a broad single band, centered nearly midway between the two bands of the oxygenated solution; and the color of the solution changed from scarlet to purple. On exposing the purple solution to air in a shallow dish, the scarlet color and the two-banded spectrum reappeared. Addi-

11. These and other data are summarized in one section of the comprehensive review by Arthur Gamgee, "Hemoglobin: Its Compounds and the Principal Products of its Decomposition," in *Textbook of Physiology,* E. A. Schäfer, ed., (Edinburgh and London: Young J. Pentland, 1898), I, 185–260. For the discussion of analytical data see pp. 197–203. Gamgee's review gives a valuable picture of the status of knowledge of hemoglobin at the end of the nineteenth century, and I shall have occasion to refer to it again.

12. F. Hoppe, "Uber das Verhalten des Blutfarbstoffes im Spectrum des Sonnenlichtes," *Virchow's Arch. path. anat. Physiol.,* 23 (1862), 446–449. Hoppe, whose father had died when he was nine years old, changed his name to Hoppe-Seyler in 1864, when he was formally adopted as a son by his guardian and brother-in-law, Dr. Seyler. See E. Baumann and A. Kossel, "Zur Erinnerung an Felix Hoppe-Seyler," *Z. physiol. Chem., 21* (1895–96), i–lxii. This biographical memoir also appeared, in essentially identical form, in *Ber. Deut. chem. Ges., 28* (1896), 1147–1192. I have referred to him as Hoppe-Seyler in the text.

13. G. G. Stokes, "On the Reduction and Oxidation of the Colouring Matter of the Blood," *Proc. Roy. Soc. Lond. 13* (1864), 355–364.

tion of more reducing agent, if the solution were protected from air, produced the purple solution again. The process could be repeated any number of times. Stokes concluded "that the colouring matter of blood, like indigo, is capable of existing in two states of oxidation, distinguishable by a difference of colour and fundamental difference in the action on the spectrum. It may be made to pass from the more to the less oxidized state by the action of suitable reducing agents, and recovers its oxygen by absorption from the air." [14] Stokes termed the blood substance, in its two states of oxidation, "scarlet cruorine" and "purple cruorine"; these names were soon replaced by Hoppe-Seyler's terms, hemoglobin (or reduced hemoglobin) and oxyhemoglobin respectively.[15] Stokes inferred correctly that the blood, in passing from the lungs to the tissues, gives up some of its oxygen, so that a fraction of the "scarlet cruorine" becomes reduced to "purple cruorine." He noted that "it is only a rather small proportion of the cruorine present in venous blood which exists in the state of purple cruorine under normal conditions of life and health" and that "this may be inferred, not only from the colour, but directly from the results of the most recent experiments." [16]

This work of Stokes remains a landmark in the study of the spectroscopy of hemoglobin, and of the striking changes pro-

14. Ibid., p. 357.

15. More than sixty years later, in the light of modern electronic concepts of valence, J. B. Conant, in "An Electrochemical Study of Hemoglobin," *J. Biol. Chem.*, 57 (1923), 401–414, showed that the combination of oxygen with hemoglobin was not an oxidation, as the term is understood today, but an oxygenation, the iron remaining in the ferrous state even after the attachment of oxygen. The true product of oxidation is methemoglobin (ferrihemoglobin), in which the iron is in the ferric state. Methemoglobin in fact does not contain bound oxygen, as earlier workers, from Hoppe-Seyler on, had supposed; the essential change is the loss of an electron from the iron atom during the oxidation. However, we are here concerned with the change in light absorption and in other properties that occurs when hemoglobin binds oxygen reversibly. The earlier use of an incorrect terminology need not mislead us in considering the observed phenomena.

16. Stokes here cites Funk's *Lehrbuch der Physiologie* (1863), vol. 1, sec. 108. I have not seen this reference, but Stokes's statement shows that physiological studies on the deoxygenation of blood, as it passes from the arteries to the veins, had already made significant progress. Stokes's paper is a lucid and masterly presentation of some significant experiments and of the conclusions drawn from them. His grasp of the physiological significance of his chemical spectroscopic experiments is impressive, and shows the breadth of his interests. Stokes was Lucasian Professor of Mathematics in the University of Cambridge, and is notable for his law of motion of falling bodies in a viscous medium, for his important work on fluorescence, and for a fundamental theorem in vector analysis, among many other contributions.

duced by adding oxygen to, or removing it from, the hemoglobin molecule. We need not trace the details of further developments here; the progress in the spectroscopy of hemoglobin and its derivatives during the late nineteenth century was well portrayed by Rollett[17] in 1880, and especially by Gamgee[18] in 1898. Gamgee laid particular stress on the work of Gustav von Hüfner (1840–1908) in Tübingen, who was clearly a major contributor of important work in this field. Shortly we shall encounter other, and more controversial, aspects of Hüfner's work on hemoglobin.

UPTAKE AND RELEASE OF OXYGEN IN BLOOD; EARLY QUANTITATIVE STUDIES

Since the time of Lavoisier it had been apparent that oxygen passes into the blood as it flows through the lungs, and carbon dioxide passes out. Inevitably the question arose: how much oxygen and carbon dioxide will the blood carry, when equilibrated with a given partial pressure of the gas in question? Early in the nineteenth century, Henry formulated his law for the solubility of gases in pure liquids: the amount of gas dissolved in the liquid, at equilibrium, is directly proportional to the partial pressure of the gas phase above the liquid. For oxygen and carbon dioxide in blood, however, no such simple relation held. As early as 1799 Humphrey Davy had heated blood at 93°C. and measured the oxygen and carbon dioxide given off. A series of other investigators during the next forty years studied this same problem of gases in blood, with highly confusing results. Some denied altogether the presence of free gases in the blood; others reported the presence of carbonic acid, but could find no oxygen.[19] The work of Gustav Magnus (1802–1870) in 1837, represented a substantial advance toward precision in such measurements. The question was then still under debate, whether the carbon dioxide released from the blood into the lungs was formed in the lungs themselves by oxidation, or

17. A. Rollett, "Physiologie des Blutes und der Blutbewegung," in *Handbuch der Physiologie,* L. Hermann, ed. (Leipzig: F. C. W. Vogel. Verlag, 1880), IV, pt. 1, 1–340. For the spectroscopy of hemoglobin see pp. 45–71.

18. See above, n. 11.

19. For references to this early work, one may consult the admirable chapter by Nathan Zuntz, "Blutgase und Respiratorische Gaswechsel," in L. Hermann's *Handbuch der Physiologie,* (1882), IV, (pt. 2), 3–162; in this connection see especially pp. 24ff. Zuntz himself had contributed, and in later years continued to contribute, many fundamental observations in this field, and this review of the subject was a masterly portrayal of the problems as they then stood. We shall have more to say of Zuntz's work below.

whether it was transported to the lungs by the blood, and discharged there. Magnus obtained data that gave decisive evidence for the latter alternative. He showed clearly that both arterial and venous blood contained large quantities of both oxygen and CO_2 and that venous blood contained more CO_2 and less oxygen than arterial blood.[20] Even so, however, the accuracy of his measurements was inadequate for determination of quantitative relations. Hemoglobin had not yet been discovered at the time of his work, so that the chemical basis for the large oxygen-binding capacity of blood was still obscure. The technical problems of equilibrating blood with known mixtures of gases, transferring the blood without further gas exchange into a closed vessel, removing the oxygen and CO_2 by heating, or by the use of a vacuum pump, and analyzing the gases so released, were formidable. Numerous workers—Lothar Mayer, Carl Ludwig and J. Setschenow, E. Pflüger and others—contributed to such technical advances over the next four decades after Magnus.[21] The central role of hemoglobin in the uptake of oxygen became apparent in the years after 1840; after the work of Stokes and others (see above) it was natural to believe that all the oxygen carried in the blood was bound to hemoglobin, except for the small amount in physical solution. Numerous analyses indicated that hemoglobin bound oxygen in a simple ratio: one atom of hemoglobin iron corresponded to one molecule of oxygen (O_2) when the oxygen partial pressure was high enough to saturate the hemoglobin. Hüfner in 1884 gave the figure of 1.34 cc of gas per gram of hemoglobin,[22] which does in fact correspond fairly closely to the best modern data, and to the composition of hemoglobin as reported by Zinoffsky[23] and by Jaquet.[24] However,

20. G. Magnus, "Ueber die im Blute enthaltenen Gase, Sauerstoff, Stickstoff und Kohlensäure," *Poggendorffs Ann. Phys. Chem.*, *40* (1837), 583–606. Magnus was eminent in his time, and is the subject of an extensive biographical article by his friend August Wilhelm von Hofmann: *Zur Erinnerung von vorangegangene Freunde*, 3 vols. (Braunschweig, 1888–89), I, 45–194. Magnus's work on blood is discussed on pp. 89–97.

21. For details of the techniques developed, see the article by Zuntz (n. 19), pp. 24–32.

22. G. Hüfner, "Ueber das oxyhämoglobin das Pferdes," *Hoppe-Seyler's Z. physiol. Chem.*, *8* (1884), 338–365. The volume of gas is given for standard conditions, i.e., 0°C and 1 atmosphere pressure. In this particular study Hüfner actually measured the combination of carbon monoxide, rather than oxygen, with hemoglobin; but the figure for the two gases should be the same. The value of 1.34 cc of gas per gram of hemoglobin corresponds to 32 grams of oxygen per 16,700 grams of hemoglobin, i.e., one gram mole of oxygen per gram atom of iron in hemoglobin.

23. See above, n. 8.

24. See above, n. 9. G. Hüfner, "Neue Versuche zur Bestimmung der

as we shall see, this simple and correct conclusion was doubted by important later workers, and had to be reestablished, a generation later, by the work of R. A. Peters in Barcroft's laboratory. One important investigator, Christian Bohr, whose later work is central to our theme, held that blood contains several different forms of hemoglobin with different chemical compositions and different capacities for combining with oxygen.[25] Others also remained doubtful whether the relation between oxygen and hemoglobin could be described in simple terms of molar equivalence.

In any case this was only the beginning of the physiological problem. The hemoglobin in the arterial blood might be—indeed, it normally was—practically saturated with oxygen; but its function, as the blood flowed through the capillaries, was to release that oxygen to the tissues, where the partial pressure of oxygen was very low or nearly zero. How did the amount of oxygen bound by hemoglobin vary with the partial pressure of the gas with which it was in equilibrium? This question had been studied by Paul Bert, the most distinguished of the pupils of Claude Bernard. He recognized the important fact that the affinity of hemoglobin for oxygen decreases with rising temperature, and found that dog blood, after saturation with oxygen, gave up approximately half its bound oxygen when the oxygen pressure fell to about 25 mm of mercury, at 40°C.[26] However, the best-known work during this period, which was widely accepted at the time, came from Hüfner,[27] who started with the assumption that one molecule of hemoglobin would

---

Sauerstoffcapacität des Blutfarbstoffs," *Arch. Anat. Physiol. Leipzig* (1894), 130, 176. He criticized the views of Bohr, *Zentbl. Physiol.*, 4 242–252, who held that there were several oxyhemoglobins differing in elementary composition and in O_2 combining capacity, even in a single sample of blood.

25. Gamgee (above, n. 11, p. 192) refers to these views of Bohr, only to dismiss them with the statement that Hüfner's later work (1894) had completely refuted Bohr. In the light of present day knowledge, Gamgee's statement appears justified. This was not the last time that Hüfner's conclusions clashed with those of Bohr. In a still more important disagreement between them, shortly to be recounted, Hüfner was wrong and Bohr was right.

26. P. Bert *La Pression Barometrique: recherches de physiologie experimentale* (Paris: G. Masson, 1878), pp. 683–697.

27. G. Hüfner, "Ueber das Gesetz der Dissociation des Oxyhämoglobins und über einige daran sich knüpfenden wichtigen Fragen aus der Biologie," *Arch. Anat. Physiol. (Physiol. Abtheilung)* (1890), 1–27; "Neue Versuche über die Dissociation des Oxyhämoglobins," *Arch. Anat. Physiol., Supplement* 5 (1901), 187–217.

combine with one molecule of oxygen, according to the equation (denoting hemoglobin as Hb):

$$Hb + O_2 \rightleftharpoons HbO_2 \qquad (1)$$

From the law of mass action, this leads to the equation for an equilibrium constant k':

$$k' = [HbO_2]/[Hb]\ [O_2] \qquad (2)$$

or

$$k = [HbO_2]/[Hb]\ p \qquad (2')$$

The symbols in brackets denote concentrations, which may be expressed in moles per liter. However, since the concentration of free oxygen in the blood, or hemoglobin solution, is proportional, by Henry's law to the partial pressure of oxygen, p, the equation may be written either in the form (2) or (2'). Since most investigators have used the latter form, we shall use it here.

It is convenient to describe the results of measurement in terms of the fractional saturation of the hemoglobin—that is, the fraction of the total hemoglobin, [Hb] + [HbO_2], that is combined with oxygen, i.e., the quantity y:

$$y = [HbO_2]/([Hb] + [HbO_2]) \qquad (3)$$

Thus y may have any value between zero and unity.[28] The relation of y and p, from equations (2') and (3), is readily found to be:

$$y = kp/(1 + kp) \qquad (4)$$

If this relation holds, the value of k is obviously equal to the reciprocal of the oxygen pressure at which the hemoglobin is half saturated with oxygen ($y = 0.5$). The curve defined by equation (4) is a rectangular hyperbola; at very low values of p, when $kp<<1$, y rises linearly with p, with a slope equal to k. When $kp>>1$, y approaches its limiting value of unity.

Hüfner assumed the validity of equation (4) (he wrote it in a somewhat different but equivalent form), made a series of measurements on various hemoglobin solutions, and constructed a hyperbolic curve. He found his hemoglobin solutions to be half-saturated when the oxygen partial pressure was only 4 to 5 mm Hg, a value far below that reported by Paul Bert. Hüfner's

28. Many authors have defined y as the percentage saturation of the hemoglobin with oxygen, in which case a factor of 100 must be inserted on the right hand side of equations (3) and (4). These units are used in Fig. 1 later in this article.

data appear to have been widely accepted by his contemporaries;[29] yet they proved to be incompatible with fundamental facts of chemistry and physiology, as the work of Bohr and others was to show.

CARBON DIOXIDE IN BLOOD

It was soon apparent that the transport of carbon dioxide in blood was a more complicated problem than the transport of oxygen. Large quantities of carbon dioxide, up to 40 or 50 volumes per 100 volumes of blood, could be released by pumping off the gas with a vacuum pump, or by treating the blood with acid. The amount so obtainable from venous blood was naturally greater than from arterial blood; but, unlike oxygen, CO_2 did not appear to be held in combination with any specific constituent of the blood. Bound oxygen, moreover, is found entirely in the hemoglobin of the red cells; the plasma carries only a small amount of dissolved oxygen, slightly less than would be dissolved by the same volume of water under the same conditions. In contrast, bound carbon dioxide is present in large amounts in both cells and plasma. From the first it was apparent that CO_2 must, to a large extent, react with basic constituents of the blood to form bicarbonate, and must be carried in that form. In 1867 Nathan Zuntz (1847–1920), a young investigator in the laboratory of Eduard Pflüger in Bonn, noted that the red cells (cruor), when separated from serum and exposed to a given pressure of CO_2, took up two or three times as much CO_2 as the same volume of serum separated from the cells. Yet when whole blood was exposed to CO_2 at the same pressure, and cells and serum were then separated and analyzed, much more bicarbonate was found in the serum than in the cells.[30] Zuntz

29. See, for example, the chapter by M. S. Pembrey on "Chemistry of Respiration" in E. A. Schäfer's *Textbook of Physiology* (Edinburgh and London: Y. J. Pentland, 1898), I, 692–784. Hüfner's data are discussed on p. 775.

30. N. Zuntz, "Ueber den Einfluss des Partialdrucks der Kohlensäure auf die Vertheilung dieses Gases im Blute," *Centralbl. med. Wiss.* 5 (1867), 529–533; *Beiträge zur Physiologie des Blutes,* Inaugural Dissertation (1868), University of Bonn. Alexander Schmidt, *Ber. sächs. Akad. Wiss. 19* (1967), 30, made similar observations independently, as Zuntz noted in his later review (n. 19). Zuntz was a major figure in physiology for the next fifty years. In 1910, with Joseph Barcroft and others, he took part in the expedition to Teneriffe to study respiration at high altitudes. I have already referred (n. 19) to his important article in Hermann's *Handbuch.* For biographical sketches by his associates, see A. Loewy, *Berl. klin, Wschr.,* 57 (1920), 433–435, and A. Durig, *Wien klin Wschr.,* 33 (1920), 344–345.

perceived therefore that most of the bicarbonate derived from CO_2 must form by an initial reaction in the cells, and must then migrate across the red cell membrane from cells to serum. He postulated that hemoglobin, and to a lesser extent the serum proteins also, must contain what he, in the terminology of his time, called "bound alkali"; that is, they must exist largely as sodium or potassium salts of the proteins. These therefore function as bases, neutralizing carbonic acid while releasing their "bound alkali" to form sodium or potassium bicarbonate. This then could migrate across the red cell membrane to the serum, explaining the observed results.[31]

Zuntz's proposal showed great insight, but in one crucial respect it failed to correspond to the facts. In 1879 Hermann Nasse,[32] a distinguished physiologist of the older generation, reported that, when he increased the partial pressure of CO_2 in equilibrium with blood, water and chloride moved *into* the cells from the serum. (He referred to an inward movement of salt [Kochsalz] rather than of chloride, but clearly he had no direct evidence that sodium entered the cells.) He offered no explanation for these interesting facts; the phenomenon was forgotten until H. J. Hamburger[33] in 1891 demonstrated the effect of

31. For the clearest and most comprehensive statement of Zuntz's views, see his review in Hermann's *Handbuch*, pp. 64–83.

For the reader familiar with modern chemistry, one may formulate Zuntz's proposal in the following equations. Hemoglobin and other proteins, under physiological conditions, are negatively charged; we may denote them by the general symbol P^-. To balance their negative charge, equivalent cations (mostly K^+) must be present. When CO_2 dissolves, it becomes hydrated to H_2CO_3. Then $H_2CO_3^- + P^- \rightleftharpoons HCO^-_3 + HP$. The protein ions thus act as bases, accepting protons from carbonic acid. Zuntz's hypothesis would them assume a joint migration of K^+ and HCO_3–ions from cells to serum. In fact, of course, the K^+ ions cannot cross the membrane freely, and electrical neutrality is maintained by inward migration of chloride ions to balance the outward migration of bicarbonate, as discussed below.

It was of course impossible for Zuntz in 1867, or in his review, in 1882, to formulate the problem in such terms. It was not until 1887 that Arrhenius published his theory of electrolytic dissociation of acids, bases, and salts, and it required a decade or more after that for physiologists and biochemists to assimilate his views.

32. H. Nasse, "Untersuchungen über den Austritt und Eintritt von Stoffen (Transudation und Diffusion) durch die Wand der Haargefässe," *Pflügers Arch. ges. Physiol., 16* (1878), 604–634. Nasse had actually reported the essential facts in 1874 at a meeting in Marburg, but noted in his later paper that the findings had aroused little interest. Unfortunately his later paper also was generally ignored.

33. H. J. Hamburger, "Ueber den Einfluss der Atmung auf die Permeabilität der Blutkörperchen," *Z. Biol.*, 28 (1891), 405–416. Hamburger (1859–1924), who was Professor in Groningen, played an important role

increasing CO_2 tensions in causing chloride to enter the cells, the so-called "Hamburger chloride shift." Hamburger also showed that the effect was reversible; on passing air through the system, and driving off CO_2, chloride migrated out into the serum again. This was a confirmation of Nasse's work, of which Hamburger—like almost everyone else—was apparently unaware. However, it was now possible to think in terms of ions as the constituents of electrolytes, following the work of Arrhenius, and to visualize an exchange of bicarbonate ions, passing out of the cells, with chloride ions passing in.

R. von Limbeck in Vienna, however, noted that Hamburger had not taken account of the volume changes that occur, on saturation with CO_2, due to the flow of water from serum to cells. He described these in detail, noting that the cells increase in volume, in chloride content, in content of nitrogenous substances, and especially in water content, when exposed to CO_2.[34] Here again his observations repeated, while also greatly extending and refining, the earlier work of Nasse, of which he, like Hamburger, was evidently ignorant.

One crucial fact was revealed a little later by the work of Gürber.[35] He confirmed Zuntz's finding that serum, when blood is saturated with CO_2, gives a "more strongly alkaline reaction," by which he meant an increase in titratable bicarbonate. However, by analytical determinations, he disproved Zuntz's assumption that "alkali"—that is, sodium or potassium ions—crossed the cell boundary in conjunction with bicarbonate. When he allowed for the change in relative volume of cells and serum that accompanied exposure to CO_2 he found no change in the sodium and potassium content of either serum or cells. He definitely confirmed the inward shift of chloride into the cells, and concluded that this must counterbalance the outward shift of bicarbonate into the serum. He wrote, however, as if he were

---

in introducing the new developments in the physical chemistry of solutions into physiological research. L. J. Henderson's *Blood* rather surprisingly makes no mention of Hamburger, although Henderson does refer to all the other authors I have discussed here. Hamburger reported on this phenomenon further in a series of later papers.

34. R. von Limbeck, "Ueber den Einfluss des respiratorischen Gaswechsels auf die rothen Blutkörperchen," *Arch. exp. Path. Pharm.*, 35 (1894), 309–334.

35. A. Gürber, "Ueber den Einfluss der Kohlensäure auf die Vertheilung von Basen und Säuren zwischen Serum und Blutkörperchen," *Jber. Fortschr. Tierchem (Maly's Jahresbericht)*, 25 (1896), 164–167. Gürber, unlike the other investigators mentioned, was aware of Nasse's work and made reference to it.

unaware of the ionic dissociation theory of Arrhenius (1887) since, to explain his findings, he invoked an improbable picture of carbonic acid reacting with sodium chloride to give hydrochloric acid, which then crosses the red cell membrane. His experimental work, however, was abundantly confirmed by later workers, and led to the postulate, which was accepted for the next forty years, that the normal red cell membrane is impermeable to cations like sodium and potassium but readily permeable to anions like chloride or bicarbonate.[36]

Thus the study of carbon dioxide uptake in blood had led to the establishment of a variety of interesting but largely isolated facts—notably the exchange of chloride and bicarbonate ions, and the flow of water, across the red cell membrane. No comprehensive conceptual scheme was available, into which the facts would fit. Essential elements for such a scheme were still lacking. One additional fact of great importance, however, had early been perceived by Zuntz and others. On exposing whole blood, or red cells, to the vacuum pumps used by Pflüger and others, all the CO_2 present could be pumped off—that is, just as much as could be released by acidification. From serum, however, it was possible with the vacuum pump to drive off only about half the CO_2 that could be released by acid.[37] Zuntz[38] clearly recognized the implications of these facts. Not only could the materials in the red cells function as bases in taking up CO_2, as the CO_2 pressure was increased, but they could also function as acids in driving off CO_2 as its pressure fell. The constituent responsible for these acidic and basic functions must be hemoglobin; no other known constituent could serve. All later experience justified this assumption. Since hemoglobin, in driving off CO_2 from blood, appeared to act as a weak acid, was it not possible that its strength as an acid was affected by its combination with oxygen? If so, might not the oxygenated hemoglobin be a stronger acid than the deoxygenated form, the process of oxygenation thereby assisting in driving off more

36. This postulate was eventually shown to be incorrect, as we shall see later in Part II of this study. However, it was so close to the truth, for the practical purposes of the workers of the following generation, that it was never challenged until the rise of isotope labeling techniques after 1940.

37. An exact analysis shows that, if the partial pressure of carbon dioxide actually falls to zero, it should be possible to pump off all the potential CO_2, even from a sodium carbonate solution. See for instance, J. T. Edsall and J. Wyman, *Biophysical Chemistry* (New York: Academic Press, 1958), I, 561–571. However, in practice the available vacuum pumps were obviously unable to approach this theoretical limit.

38. See above, n. 19.

CO_2 in the lungs? In 1863, Holmgren, a young investigator working in Carl Ludwig's laboratory in Vienna, reported that he had observed such an effect of oxygen in driving off CO_2 from blood and estimated that the amount of CO_2 driven off in the presence of oxygen could be two to three times as great as that released in the absence of oxygen.[39] However, Ludwig himself, on repeating and extending the experiments, concluded that the effect was zero, or at least negligibly small.[40]

Zuntz, however, considered that the effect reported by Holmgren might be real and stated the problem clearly. "The significance of hemoglobin as a weak acid . . . must lead to the question whether its strength as an acid is influenced by its combination with oxygen. One has generally thought of its action as promoting the release of CO_2 in the lungs, therefore as increasing the avidity of hemoglobin"[41] [that is as an acid].

Thereafter there were, from time to time, further reports claiming such an effect of oxygenation, but in no case was the evidence decisive to carry conviction to the scientific world. So matters stood until the new developments of the early twentieth century.

CHRISTIAN BOHR: HIS OUTLOOK AND CONTRIBUTIONS THE INFLUENCE OF CARBON DIOXIDE ON OXYGEN AFFINITY

A new epoch in the study of blood and hemoglobin began in 1904 with the publication of a paper by Christian Bohr, K. A. Hasselbalch, and August Krogh on the effect of carbon dioxide on the binding of oxygen in blood. Before turning to their work, however, we may consider more generally the character and attitude of Bohr, the leader of the Copenhagen school of physiology from 1885 until his death.[42]

39. F. Holmgren, "Ueber den Mechanismus des Gasaustausches bei der Respiration," *Sitz. Akad. Wiss. Wien., 48* (1863), 614–648; C. Ludwig, *Wien. Med. Jahrbuch,* 1865, p. 159. The latter paper I have not seen but it is cited by Zuntz (above, n. 19), p. 81 who gives an excellent short discussion. Further references to early work on this subject are given in the notable 1914 paper by Christiansen, Douglas and Haldane (see n. 112), which is discussed in detail later in this article.

40. Carl Ludwig was at this time still in Vienna, where he had been professor for ten years, but in 1865 he moved to Leipzig, which was the home of his famous school of physiology for the next twenty years or more.

41. Zuntz (above, n. 19), p. 81. Translated by the author.

42. For Bohr's career and scientific achievements, see Robert Tigerstedt, "Christian Bohr: Ein Nachruf," *Skand. Arch. Physiol., 25* (1911), ix–xviii; also a briefer but valuable article by L. S. Fridericia in *Prominent Danish Scientists,* V. Meisen, ed. (Copenhagen, 1932), pp. 173–176.

Christian Bohr (1855–1911) was the son of H. G. Bohr the headmaster of a school. As a boy he developed early an ardent love of natural science, and later wrote: "I am quite sure that I had this love in my ninth year in essentially the same form as it still dominates my life today." [43] He took his medical degree at the University of Copenhagen in 1880, having already done research as a student with Professor P. L. Panum on the secretion and composition of milk. In the following year he went to Leipzig, to the famous laboratory of Carl Ludwig, where he worked on tetanic contractions in muscle. Although his later work lay in very different field, his approach in this early study was characteristic of the attitude he had learned in Ludwig's laboratory—he formulated empirical relations to describe the experimental data he had obtained, relations that should hold, if the experimental facts were correctly observed, independently of any theory of muscular contraction. This attitude of respect for the experimental facts regardless of theory Bohr maintained throughout his career; it was an essential element in the making of his greatest discovery, while it also involved his failure to perceive one of the most important implications of that discovery. Nor could this attitude be a sure protection against error; in a field so full of technical pitfalls and complexities as the physiological chemistry of blood and respiration—Bohr's chosen field of research from 1883 on—it was easy to draw wrong conclusions even from the results of diligent and carefully planned experiments. As we shall see, some of Bohr's major conclusions were later rejected by the work of other investigators.

With all his respect for the primacy of the experimental facts, Bohr retained an abiding concern for the philosophical understanding of the nature of the living organism. These interests brought him into close relations with Harald Høffding, the philosopher, who wrote in his "memoirs":

> The regular meetings from which I gained much pleasure . . . started when I used to join Christian Bohr the physiologist after the meetings at the Academy of Sciences and Letters, and we would then carry on the discussions in a café. As a physiologist and a disciple of the Leipzig scientist Ludwig

43. This is quoted from a much longer passage in S. Rozental, ed., *Niels Bohr: His Life and Work* (New York: Interscience Publishers, 1967), p. 11. The description of Christian Bohr on this and the four following pages of Rozental's book gives the best portrayal I have ever seen of his character and personality, to provide a background for the development of his son Niels.

he followed the line that requires the strict application of physical and chemical methods to physiology. Outside the laboratory he was a keen worshiper of Goethe. When he spoke of practical situations, of views of life, he liked to do so in terms of paradoxes and these were as a rule improvised. A conversation was given new life when he joined in. Our gatherings at the café after the meetings at the Academy soon included a third member, the physicist Christiansen. He and Bohr had many interests in common, as Bohr's physiological method led him to detailed studies in physics.

This trio which had been formed soon tired of café life, and it was therefore arranged that we should in turn go to each other's home on those Friday evenings when the Society was meeting. A fourth man now joined us, the famous philologist, Vilhelm Thomsen.[44]

Many years thereafter, in his memorial lectures on Høffding at the Danish Academy, Christian Bohr's famous son Niels described the profound influence he and his brother Harald had received, beginning in early childhood, by listening to these conversations that went on in their home. Clearly they served as an important stimulus to the development of his own later thinking on the philosophical aspects of science, including biology.

One short quotation may serve to illustrate Christian Bohr's outlook on the nature of the living organism:

Notwithstanding the differences in external factors, and despite the fact that the internal processes of vital functions are continually subject to great variation of intensity, the organism remains essentially an unaltered unity through a relatively considerable period; it forms then, in an empirical manner, a self-regulating whole.

The chief task of physiology—in the sense of its characteristic as a special branch of natural sciences—is to investigate the phenomena peculiar to the organism *qua* empirical object in order, if possible, thus to find out how the single factors in the self regulation are acting, how they are mutually adjusted and attuned to variations in external conditions and internal processes. Naturally this implies that the organism be looked upon as the aim and that the means of regulation contributory to its maintenance be regarded as expedient. It is in this

44. This passage, in its English translation, is quoted from Rozental, *Niels Bohr*, p. 13, and the information in the next paragraph is taken from the same source.

sense the term "adequate" will be applied to organic functions.[45]

From 1883 on, Bohr devoted himself to the physiology of blood and respiration. He determined the solubility of oxygen, nitrogen, hydrogen, and carbon dioxide in water, salt solutions, and other liquids. He made repeated studies of oxygen uptake by hemoglobin that led him to the conclusion (already mentioned above) that the blood of a single individual contains several distinct hemoglobins, differing from one another both in chemical composition and in oxygen combining capacity. His attempts to separate these by fractionation, however, led to unsatisfactory and inconclusive results. Hüfner in 1894 challenged Bohr's results, reporting again a constant ratio of bound O_2 to hemoglobin in a series of measurements on different bloods;[46] but Bohr apparently never retreated from the view that there were multiple hemoglobins, even in a single individual. He also drew a distinction between the genuine blood pigment in the red cells, which he called hemochrome, and the hemoglobin that can be prepared from it in free solution. Bohr upheld both these conceptions, apparently to the end of his career, although neither one has won acceptance since.[47]

The great achievement, with which his name is always associated, was published in 1904. Bohr, with his two gifted younger associates, K. A. Hasselbalch and August Krogh, set out

45. This is an English translation, taken from Fridericia (n. 42, pp. 173–174). The Danish original by Bohr was published in *Universitets Festskrift* (Copenhagen, 1910). For a longer quotation from Christian Bohr, developing similar thoughts in more detail, see Niels Bohr, *Atomic Physics and Human Knowledge* (New York: John Wiley, 1958), p. 96.

46. See n. 22 above.

47. For references to the papers involved, see Tigerstedt's obituary on Bohr (above, n. 42), and Bohr's own comprehensive summary of his views: C. Bohr, "Blutgase und respiratorischer Gaswechsel" in *Handbuch der Physiologie des Menschen,* W. Nagel, ed. (Braunschweig, F. Wieweg und Sohn, 1905), I, 54–222. The date of this *Handbuch* is often given as 1909 but the first half of Vol. I, in which Bohr's chapter appeared, was published in 1905.

In connection with Bohr's views we may note that biochemists in very recent years have shown that the blood of normal human individuals does indeed contain several different hemoglobins, which can be separated and purified. However, one of them (hemoglobin A) is present in far larger amount than any of the others. All of them have essentially identical oxygen combining capacity. Thus the modern view, although it bears some superficial resemblance to Bohr's concept of multiple hemoglobins, is really very different. In any case the presence of multiple hemoglobins could not in itself explain the sigmoid form of the oxygen-binding curve of hemoglobin, which is discussed below; indeed the presence of several distinct components, with different oxygen affinities, would tend to make the curve flatter, not steeper.

to examine critically the problem of the binding of oxygen by hemoglobin. As we have seen, Hüfner[48] in 1890 had reported a dissociation curve for oxyhemoglobin, in which he had assumed the validity of equations (2′) and (4) above; that is, he had assumed that the curve *must* be a rectangular hyperbola, and that the binding of oxygen must be described by a single constant k. What happened is best described in the words of Barcroft:

> Hüfner assumed the correctness of the equation and set out to find the value of k. This can be done from one point. He used a number of samples of hemoglobin prepared in different ways, determined a point for each, found the value of k, averaged these values and produced a curve. But a nemesis

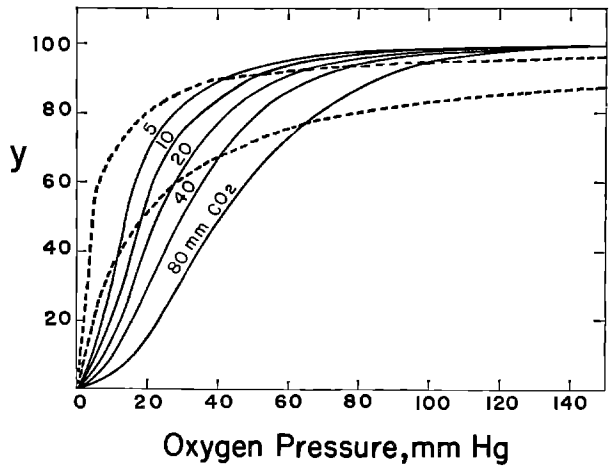

FIG. 1. The solid lines represent the data of Bohr, Hasselbalch, and Krogh, for binding of oxygen by hemoglobin in dog blood, at various partial pressures of CO_2. The two dashed lines are rectangular hyperbolas, showing binding curves that would correspond to equation (4); the hyperbolic curve on the left is for half saturation with oxygen at 4 mm Hg oxygen pressure K= 0.25 mm^{-1} the curve on the right is for half saturation at 20 mm. (K = .05 mm^{-1}). The ordinate, y, denotes *percentage* saturation with oxygen, i.e., the numerical values are 100 times as great as for y defined in equation (4) and other equations in the text.

> awaited Hüfner. His speculations fell into the hands of a physiologist of a diametrically opposite school. Bohr had inherited a tradition from the great laboratory of Ludwig

48. See n. 27.

> which, though it may carry its holders to excessive lengths, at least forms a useful corrective to unjustifiable generalisations. Bohr's motto was that every experiment had a value, nothing which was obtained as the result of a test in the laboratory was set aside on the ground of its inherent unlikelihood, of its failure to fit general principles. Bohr therefore determined to map out the curve relating the pressure of oxygen to the relative quantities of oxy- and reduced hemoglobin point by point, irrespective of laws, and to find out experimentally what the curve was like. . . The actual curve determined point by point differs fundamentally from Hüfner's hyperbola.[49]

Just how it differs can be seen by examining any one of the curves (solid lines) in Fig. 1; these are the actual curves derived from the data of Bohr, Hasselbalch, and Krogh[50] and published in their paper. We note, first of all, that their measurements were made on whole dog's blood, not on hemoglobin solutions as Hüfner's measurements were. This choice reflected Bohr's view that the native "hemochrome" within the red cells differed significantly from the hemoglobin that was released on laking the cells. For comparison with these curves I have inserted two rectangular hyperbolas, one corresponding to half saturation of the hemoglobin at 4 mm Hg, close to Hüfner's original curve, the other corresponding to half saturation at 25 mm Hg. These hyperbolic curves rise steeply from the origin at $p = 0$, with a steadily decreasing slope as p rises and the hemoglobin approaches saturation with oxygen. The experimental curves of Bohr et al., by contrast, start at $p = 0$ with a very small slope; as p increases, the curves become much steeper, until they flatten out once more as the hemoglobin approaches saturation with oxygen. In brief they are S-shaped or sigmoid curves. All the experimental curves converge, and approach 100% saturation, at oxygen pressures of 100 mm Hg or above, which is in the range of oxygen pressure found in the alveolar air of the lungs. Thus the arterial blood should normally be close to saturation with oxygen, which is then unloaded as the blood flows through the tissue capillaries. The sigmoid curves, which are

49. Joseph Barcroft, *"The Respiratory Function of the Blood,* 1st ed. (Cambridge: [Eng.] University Press, 1914), p. 21.

50. C. Bohr, K. A. Hasselbalch, and A. Krogh, "Uber einen in biologischen Beziehung wichtigen Einfluss, den die Kohlensäurespannung des Blutes auf dessen Sauerstoff bindung übt," *Skand. Arch. Physiol., 16* (1904), 401–412. There was an earlier brief report by the same authors in *Zentbl. Physiol., 17* (1904), 661–664, and an article, by Bohr alone, on the theoretical treatment of the oxygen uptake of hemoglobin *Zentbl. Physiol., 17* (1904), 682–688.

steep in the mid region, near half-saturation ($y = 0.5$) correspond to a far more efficient release of oxygen, as the oxygen pressure falls, than any hyperbolic curve would provide. The left-hand hyperbolic curve in Fig. 1 does indeed fall steeply over a narrow range of oxygen pressure, but this pressure is so low that a hemoglobin functioning in this manner would be of little physiological use. The sigmoid curve, therefore, however one might explain its chemical basis, clearly represented a biological adaptation.

Bohr, Hasselbalch, and Krogh, however, gave no explicit discussion of the biological significance of the sigmoid form of the curve. What they did stress was the obviously profound significance of the family of curves shown in Fig. 1. Each curve corresponds to a constant partial pressure of CO_2. As the CO_2 pressure increases, the sigmoid form of the oxygen dissociation curve is maintained, but the midpoint of the curve shifts to higher oxygen pressures. Hence, as they pointed out, the influx of CO_2 as the blood passes through the tissue capillaries must markedly decrease the affinity of hemoglobin (or "hemochrome") for oxygen, and release large amounts of oxygen into the respiring tissues, that would not have been released in the absence of CO_2. This coupling of oxygen affinity with CO_2 pressure, which had apparently been totally unsuspected, until this research revealed it, was obviously a biological adaptation of fundamental importance.

This effect of CO_2 is so large, as Fig. 1 shows, that one is surprised that all previous investigators had missed it. Bohr, Hasselbalch, and Krogh pointed out, however, that almost all their predecessors had worked at such high oxygen pressures that the blood (or hemoglobin) remained fully saturated with oxygen, even in the presence of substantial CO_2 pressures. As Fig. 1 shows, all the curves converge at high oxygen pressures, above 100 mm Hg, regardless of the CO_2 pressure; one had to work at lower oxygen pressures, where the hemoglobin was only partly combined with oxygen, in order to perceive this biologically important relation. The form of the curves in Fig. 1 also shows clearly that CO_2 does not displace oxygen by direct competition in binding to the iron atom—as carbon monoxide for instance does—for in that case the family of curves would not have reached a common saturation level at the higher oxygen pressures. If CO_2 was indeed bound to hemoglobin, and that was a highly doubtful point, it must bind somewhere else, not at the oxygen-binding iron atom.

Thus in this single paper, Bohr, Hasselbalch, and Krogh

reported two previously unrecognized effects of fundamental importance—the sigmoid form of the oxygen dissociation curve and the profound influence of CO_2 on the curve. In considering this great contribution we should be fully aware of the part played by Bohr's two younger collaborators, especially by Krogh. Both had been working with him for several years. In 1899 Bohr and Krogh had published a study of respiration in the skin and lungs of the frog, and Bohr and Hasselbalch had published two papers, in 1901 and 1903, on the CO_2 production and heat metabolism of the chicken embryo, and one on mammalian embryos. Hasselbalch later did important biochemical work on acid-base equilibria. To Krogh, however, I believe we must attribute a particularly important role in the discovery of the form of the oxygen dissociation curve and the influence of CO_2 upon it. Immediately preceding the paper of Bohr et al. was a paper by Krogh, describing the new tonometer he had developed for equilibrating blood and hemoglobin with gas mixtures, and measuring the uptake of oxygen and CO_2.[51] It was this improved tonometer that was used in all the measurements in the epoch-making paper that immediately followed. Krogh's great technical skill, combined with his penetrating and original mind, and his immense capacity for work, later made him one of the world's supremely eminent physiologists and the author of revolutionary studies on the blood capillaries.[52]

Indeed, Professor F. J. W. Roughton has told me of a conversation with Krogh at the time of Barcroft Memorial Symposium in Cambridge, England (June, 1948). Krogh stated unequivocally that it was he himself, and not Bohr, who had demonstrated the effect of CO_2 on the oxygen dissociation curve; and Roughton later learned that all of Krogh's Danish colleagues in physiology held the same view. Bohr, however, had already reported on the sigmoid character of the oxygen dissociation curve in a previous paper, so that this was apparently his discovery.[53] Logically it

51. A. Krogh, "Apparat und Methoden zur Bestimmung der Aufnahme von Gasen im Blute bei verschiedenen Spannungen der Gase," *Skand. Arch. Physiol.*, *16* (1904), 390–401.

52. A. Krogh, *The Anatomy and Physiology of Capillaries*, Silliman Lectures (New Haven, Yale University Press, 1922); revised and enlarged edition (1929).

53. See Bohr's paper in *Zentbl. Physiol.*, cited in n. 50 above. It is worth noting that later, in 1910, Krogh challenged one of Bohr's most firmly held views: namely, that the alveolar cells of the lungs can actively secrete oxygen, against a pressure gradient, from the alveolar air into the lung capillaries, and that they could similarly secrete CO_2 in the opposite direction (see Bohr's review in Nagel's *Handbuch*, pp. 142–160). Krogh, in a series of studies on rabbits reported in *Skand. Arch. Physiol.*, 23 (1910),

would appear that the effect of CO_2 on oxygen binding, now universally termed the Bohr effect, should have been called the Krogh effect; but any change of the name today would be confusing and undesirable. It did in any case emerge from the laboratory that Bohr headed and inspired.

The striking effect of CO_2 on the binding of oxygen by hemoglobin naturally raised the question whether the reciprocal effect existed; did oxygenation alter the amount of CO_2 held in the blood? The idea that such an effect existed was indeed an old story, as we have seen; it was just what Holmgren, in Ludwig's laboratory believed he had observed nearly forty years before,[54] although Ludwig himself had failed to confirm the effect; and it was considered a live possibility by Zuntz[55] in 1882. Naturally Bohr, Hasselbalch, and Krogh sought for this reciprocal effect, but by the techniques available to them they failed to find it. They concluded that the effect was absent, or at least negligibly small. Bohr reiterated this negative conclusion in his comprehensive review of 1905 in Nagel's *Handbuch*[56] and this apparently remained his considered opinion to the end of his life. Apart from Holmgren's early observations, there were other indications that the effect might really exist. In 1892 Werigo[57] had shown that, when one lung of an experimental animal was kept distended with oxygen and the other with hydrogen, the CO_2 content was always higher in the oxygen lung. From this

---

179–260, concluded that Bohr was wrong, and that all respiratory exchange in the lung could be accounted for by simple diffusion. He paid a warm tribute to Bohr as his great teacher, to whom he was profoundly indebted, but unequivocally rejected Bohr's conclusions on this question.

The controversy over secretion in the lungs continued, however, for J. S. Haldane upheld the view that secretion does occur after adaptation to high altitudes and to other conditions of stress, whereas Barcroft and others concluded that diffusion was adequate to explain all the facts. See J. S. Haldane, *Respiration* (New Haven: Yale University Press, 1922); 2nd ed. by Haldane and J. G. Priestley (1935); and J. Barcroft, "Lessons from High Altitudes," in *The Respiratory Function of the Blood*, 2nd ed. (Cambridge [Eng.] University Press, 1925), Vol. I. Haldane never gave up his belief in the existence and physiological importance of oxygen secretion in the lungs (see, for instance, *Respiration*, 2nd ed., p. 293), but few, if any physiologists today share his belief. The question, however is not necessarily closed. The whole controversy deserves an article to itself; we cannot pursue the matter further here.

54. See n. 39.

55. See Zuntz, n. 19, p. 81.

56. See n. 47, esp. pp. 106–107 of this review.

57. B. Werigo, "Zur Frage über die Wirkung des Sauerstoffs auf die Kohlensäureausscheidung in den Lungen," *Pflügers Arch. ges. Physiol., 51* (1892), 321–361.

Werigo had concluded that the presence of oxygen helped to expel CO_2 from the blood; but Bohr explained these results by the assumption that some additional oxidation was taking place in the oxygenated lung itself, and he offered the same explanation for similar results obtained in his own laboratory by Halberstadt.[58]

Bohr's resolute empiricism, as characterized by Barcroft above, may have prevented him seeing what later seemed an obvious inference, namely, that the reciprocal effect, of oxygen on CO_2 uptake by blood, *must* exist. If oxygen binding was a function of CO_2 pressure, and hence of the CO_2 content of the blood, then CO_2 content must be a function of the oxygen content. Failure to observe the effect must then simply be a reflection of the inadequacy of the experimental technique. However, Bohr was not alone in his failure to draw this necessary inference from his work. None of the English investigators, to whose work we turn next—Haldane, Barcroft, A. V. Hill, and the others—saw this point either; nor did L. J. Henderson, who had by 1910 begun to work on these problems in the United States.

As Henderson wrote later:

> To those who have not themselves experienced that state of bewilderment which is the usual condition of the investigator, it must seem strange that the physiologists who were studying the respiratory function of the blood should not have drawn from the discovery of the variation of oxygen saturation with carbon dioxide pressure the conclusion that, since carbonic acid influences the oxygen equilibrium in blood, oxygen must influence the carbonic acid equilibrium. . . Yet, so little are physiologists accustomed to mathematics, and such is the natural inertia of the mind, that this conclusion escaped us all.[59, 60]

We shall see how the problem was later resolved experimentally in the laboratory of J. S. Haldane.

58. Halberstadt's results were not published separately but were discussed by Bohr in his review (n. 47, p. 207).

59. Henderson, *Blood*, pp. 80–81.

60. See also J. Parascandola, "Organismic and Holistic Concepts in the Thought of L. J. Henderson," *J. Hist. Biol.*, 4 (1971), 63–113. A discussion of this point, with a quotation from Henderson's unpublished "Memories," occurs on pp. 88–90.

INTERACTIONS IN THE HEMOGLOBIN MOLECULE: THE BINDING OF OXYGEN AND THE SIZE OF THE HEMOGLOBIN MOLECULE

The work of Bohr, Hasselbalch, and Krogh had brought to light two fundamental effects—the sigmoid form of the oxygen dissociation curve and the effect of CO_2 on oxygen binding. The further study of these effects, in the decade 1904–1914, centered in two laboratories in England—that of John Scott Haldane in Oxford and that of Joseph Barcroft in Cambridge. I will turn first to the significance of the sigmoid curve for oxygen binding, a problem soon seen to be inextricably interwoven with the question of the true size of the hemoglobin molecule. Did this indeed correspond to the minimum molecular weight of 16,700, as given by Zinoffsky's[61] and Jaquet's[62] analyses, or was it in fact some multiple of this? We shall pursue this problem beyond the year 1914, into the period following the first world war, before turning in the next section to the interactions between oxygen and carbon dioxide in the blood and hemoglobin solutions. Eventually the study of both sets of phenomena became merged in a larger conceptual scheme, but for the moment it is easier to follow these complex developments by considering them separately.

Haldane[63] was twelve years older than Barcroft, and by 1900 his work was already widely known. His contributions to fundamental science, in the physiology of blood and respiration, were combined with intense activity devoted to the practical problems of men exposed to foul air and hazardous gases, and to high and low atmospheric pressures—miners, deep sea divers, factory workers, and later aviators and others. His work with miners, in particular, led to great improvements in the conditions of work in the mines; indeed, his great services were recognized by his election to the Presidency of the British Institution of

61. See n. 8.

62. See n. 9.

63. For a biographical study of Haldane, see C. G. Douglas, "John Scott Haldane, 1860–1936," *Obituary Notices of Fellows of the Royal Society*, 2 (1936), 115–139; also J. B. S. Haldane, "The Scientific work of J. S. Haldane," *Nature*, *187* (1960), 102–105. The latter article gives references to a number of Haldane's most important papers. See also Garland E. Allen, "J. S. Haldane: The Development of the Idea of Control Mechanisms in Respiration," *J. Hist. Med.*, 22 (1967), 392–412. Allen deals with what was probably Haldane's most important single contribution to physiology; the discussion in the present paper treats of other, though closely related, aspects of this work.

Mining Engineers (1924–28), although he was a physiologist, not an engineer. These practical concerns bore intimate relations to his fundamental research; the hazards of carbon monoxide poisoning in mines, for instance, certainly played a part in his studies of the combination of carbon monoxide with hemoglobin, the competition of carbon monoxide with oxygen, and their relative affinities for the hemoglobin molecule. Characteristically, after first showing that mice would die when exposed to 0.2 per cent carbon monoxide in air, he experimented on himself, and reached 49 per cent saturation of his hemoglobin with carbon monoxide, at which point he stopped the experiment, recording "vision dim, limbs weak. Had some difficulty in getting up or walking without assistance, movements being very uncertain." [64] These studies led him to recommend the use of small birds in mines, to detect the presence of carbon monoxide, since their very high metabolism made them much more rapidly sensitive than men to the effects of this gas.

In New College, Oxford, there stands a Memorial Tablet to Haldane; it is worth recording here:

In Memory of
John Scott Haldane
C.H., F.R.S., M.D., LL.D., D.Sc.

Fellow of New College 1901–1936
Honorary Professor and Director of the Mining Research
Laboratory in the University of Birmingham,
Physiologist and Philosopher
whose researches on respiration on air and in kindred
subjects were applied by him to the signal benefit of
miners, divers, the crews of submarines and all who work
in crowded factories or fly at high altitudes.

a deep thinker
unselfish and single-minded

Born in Edinburgh
2nd May 1860

Died in Oxford
14th March 1936

Haldane's interest in the study of blood gases led him, with his collaborator J. Lorrain Smith, to visit Bohr's laboratory in Copenhagen in 1894, during which as noted by Douglas "he learned much from Bohr's experience of methods of gas analysis." [65] Haldane later made great improvements in such methods, and in 1898 he made a new observation that was of central

64. J. S. Haldane, "The Action of Carbonic Oxide on Man," *J. Physiol., 18* (1895), 430–462.

65. See Douglas, n. 63, p. 119.

importance in the study of hemoglobin for many years to come. He found that the addition of potassium ferricyanide to a solution of oxyhemoglobin led to the evolution of gas, and the gas, on analysis, proved to be pure oxygen. The ferricyanide in fact, oxidized the hemoglobin to methemoglobin with release of any bound oxygen. The volume of oxygen released furnished a direct measure of the amount of oxyhemoglobin present, and this provided a much simpler method of oxyhemoglobin determination than the more cumbrous gas pump methods previously used. A few years later Haldane, with Barcroft, adapted the method for accurate determination of oxygen content on small volumes of blood, and also determined the bound CO_2 in blood by releasing it with added tartaric acid.[66]

It was Joseph Barcroft (1872–1947) and his co-workers who most actively took up the study of the oxygen dissociation curves of hemoglobin, at the point where Bohr had left off. Barcroft himself told the story up to 1914, not only in a series of papers in the *Journal of Physiology*, but in a coherent and vivid narrative in the first edition of his book, *The Respiratory Function of the Blood.*[67] The reader can learn here, not only of the scientific results achieved, but also of the errors, the confusions, the false starts later corrected, the promising ideas that failed to work out. Lawrence J. Henderson used to say that this book, more than any other that he knew, conveyed an impression of what scientific research was really like, unlike the usual scientific paper, where the final results are presented in tidy form, with the errors and gropings omitted. Here I can touch only briefly on the story that Barcroft set forth in detail.[68]

66. J. S. Haldane, "A Contribution to the Chemistry of Haemoglobin and its Immediate Derivatives," *J. Physiol.*, 22 (1898), 298–306; "The Ferricyanide Method of Determining the Oxygen Capacity of Blood," ibid., 25 (1900), 295–302; J. Barcroft and J. S. Haldane, "A Method of Estimating Oxygen and Carbonic Acid in Small Quantities of Blood," ibid., 28 (1902), 232–240.

67. Published by Cambridge University Press, 1914. The second edition appeared in two volumes from the same publisher: vol. I, *Lessons from High Altitudes* (1925), and vol. II, *Haemoglobin* (1928). This edition, of course, contains material of great importance that was not in the first edition and is written with Barcroft's characteristic vividness and charm, but it does not have quite the zest and vitality of the first edition.

68. Barcroft's personal influence was certainly greater than the record of his distinguished scientific achievement alone would convey. One may appreciate this by reading the personal recollections and tributes of his colleagues, E. D. Adrian, Sir Henry Dale, A. S. Krogh, C. G. Douglas, A. V. Hill, R. A. Peters, G. S. Adair, and F. J. W. Roughton, in *Hemoglobin: A Symposium based on a Conference held at Cambridge in June 1948 in Memory of Sir Joseph Barcroft,"* ed. F. J. W. Roughton and J. C. Kendrew

In his early experiments with Camis, Barcroft was unable to get reproducible oxygen dissociation curves for hemoglobin solutions; even the form of the curves differed, for samples from different animals or even for different samples of the same hemoglobin solution. Barcroft's description in his book of this long series of frustrating and inconclusive experiments conveys vividly the kind of struggle that all experimental scientists know from their own experience. Eventually, one day, in making up the hemoglobin solution, they omitted the ammonium carbonate which they had previously included (it "had been added somewhat casually" to use Barcroft's own words). To their surprise the resulting measurements fitted Bohr's sigmoid curve for blood remarkably well.[69] Barcroft and Camis then proceeded to study the effects of various electrolytes and found in time that they could regularly make up solutions that would match the dissociation curves of whole blood. Roberts, who had recently come to work with Barcroft, then studied the effect of removing nearly all the electrolytes from the solution by dialysis. The oxygen dissociation curve of the resulting solution was nearly a rectangular hyperbola, and they inferred that this was the limiting state that the curve would assume in the complete absence of salts. Barcroft laid much stress on this finding in writing "The Respiratory Function of the Blood"; for a number of years it was the generally accepted view. Yet later workers, beginning with G. S. Adair in 1925, were unable to confirm it, and found a sigmoid curve even at extremely low salt concentrations.[70] The reason for Barcroft's early findings on this point remained an enduring puzzle. F. J. W. Roughton has told how Barcroft, a few months before his death in 1947, said to him, "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."[71]

(London, 1949). The biography by K. J. Franklin, *Joseph Barcroft, 1872–1947* (Oxford, 1953), portrays the man and his career in great detail and gives an essentially complete bibliography of his published work. For one aspect of Barcroft's work and thought, which has some close relations with our discussion here, see F. L. Holmes, "Joseph Barcroft and the Fixity of the Internal Environment," *J. Hist. Biol.*, 2 (1969), 89–122.

69. Barcroft, *Respiratory Function of the Blood*, 1st ed., pp. 42–47.

70. G. S. Adair, "The Hemoglobin System," a series of six papers, some written in collaboration with A. V. Bock and H. Field Jr., *J. Biol. Chem.*, 63 (1925), 493–546.

71. See *Hemoglobin*, p. 30 (above, n. 68). For the original papers, see J. Barcroft and M. Camis, "The Dissociation Curve of Blood," *J. Physiol.*, 39 (1909), 118–142; J. Barcroft and Ff. Roberts, "The Dissociation Curve of Hemoglobin," ibid., 39 (1909), 143–148.

In any case it was now apparent that hemoglobin in salt solutions, under physiological conditions, would give just the sort of sigmoid curve that Bohr had found for whole blood. There was no need for Bohr's conception that "Hemochrome" inside the red cell differed from the hemoglobin that was released from the cell. Barcroft and Orbeli[72] in 1910 and 1911 showed that the influence of CO_2 on the oxygen dissociation curve was probably due to the acid properties of carbonic acid, since the addition of lactic or other acids shifted the curve to the right, i.e. decreased the oxygen affinity. Addition of alkali had the opposite effect; the curve remained sigmoid but shifted to the left. This observation served to generalize Bohr's fundamental observation; the role of CO_2 was not unique; it was the change in the acidity or alkalinity of the medium that was fundamental. We must remember that the quantitative formulation of the latter concepts was still in its infancy; it was only in 1908 that L. J. Henderson had formulated the theory of buffer action, and in 1909 that S. P. L. Sørensen had formulated the concept of pH and shown how to measure it.[73] Until these concepts were more closely woven into the thinking of physiologists, the full implications of the work of Barcroft and Orbeli could not be appreciated; but an important start had been made.

There remained, however, the idea often expressed by Bohr and others, that CO_2 had a rather special character in its relation to hemoglobin, and could react with it directly, in ways not possible for other acids. Although this idea remained dormant for almost twenty years after this period of Barcroft's work, it underwent an important revival thereafter, as we shall see later, in Part II.

One elementary fact remained to be established, or rather reestablished. Was there a constant chemical relation between the mass of the hemoglobin molecule, or the iron contained in it, and the amount of oxygen taken up at saturation? As we have

72. J. Barcroft and L. Orbeli, "The Influence of Lactic Acid upon the Dissociation Curve of Blood," *J. Physiol.*, *41* (1910), 355–367; J. Barcroft, "The Effect of Altitude on the Dissociation Curve of Blood," ibid., *42* (1911), 44–63.

73. L. J. Henderson, "Concerning the Relation between the Strength of Acids and their Capacity to Preserve Neutrality," *Am. J. Physiol.*, *21* (1908), 173–179; "The Theory of Neutrality Regulation in the Animal Organism," ibid., *21* (1908), 427–448; S. P. L. Sørensen "Enzymstudien: II Mitteilung, Ueber die Messung und die Bedeutung der Wasserstoffionen Konzentration, bei Enzymatischen Prozessen," *Biochem. Z.*, *21* (1909), 131–304. See also J. Parascandola, "L. J. Henderson and the Theory of Buffer Action," *Medizinhistorisches Journal*, 7 (1972), 9–21.

seen, Hüfner[74] believed that he had established such a relation firmly, with one O_2 molecule combining with one atom of hemoglobin iron, and his spectroscopic studies convinced him that the hemoglobins of various species were essentially identical. Bohr however, had found wide variations in the "specific oxygen capacity" of hemoglobin from different species, from different animals of the same species, and even in the same individual at different times. Moreover the rise of colloid chemistry in the early twentieth century had led to great emphasis on the importance of nonspecific adsorption, in contrast to specific chemical combination. Wolfgang Ostwald, a leader of the colloidal school, had proposed that hemoglobin could take up additional oxygen by adsorption, beyond what it could bind by chemical reaction involving the iron atom, and one chemist, Manchot,[75] had claimed that he had experimental evidence for the binding of substantially more oxygen than the amount equivalent to the iron content.

It was vital to settle the matter, and Barcroft set one of his students, R. A. Peters (now Sir Rudolph Peters), to reinvestigate the problem. Peters determined the oxygen combined with the hemoglobin by releasing it by oxidation with ferricyanide (Haldane's method, as further developed in Barcroft's laboratory). To determine the iron he used a method that was then new, converting it to ferric chloride, which was then reduced to ferrous chloride by titration with titanous chloride. The results were decisive; the data fitted the expectations for a 1:1 ratio of Fe to O_2 well within the experimental error.[76] After that, there were few who seriously questioned this fundamental fact. One of these, however, was Sir William Bayliss, the eminent physiologist in London. He still held, in the third edition of his famous textbook, that "the subject would repay more investigation from the adsorption point of view than it has yet received," [77]

74. See n. 22.

75. Wolfgang Ostwald, "Ueber die Natur der Bindung der Gase im Blut und in seinen Bestandteilen," *Kolloidzeitschrift*, 2 (1907–08), 264–272, 294–301; W. Manchot, "Untersuchungen über die Sauerstoffbindung im Blute," *Liebigs Ann. Chem.*, *370* (1909), 241–285. Wolfgang Ostwald should not be confused with his father, Wilhelm Ostwald, the well-known physical chemist and founder of the *Zeitschrift für Physikalische Chemie*. I have discussed elsewhere (above, n. 3) the often confusing influence of the "colloidal" school on the development of protein chemistry.

76. R. A. Peters, "Chemical Nature of Specific Oxygen Capacity of Hemoglobin," *J. Physiol.*, *44* (1912), 131–149. Barcroft, in his *Respiratory Function of the Blood* (1914), gives an interesting description of the progress of Peters's work, as seen by himself and by others in the laboratory.

77. W. M. Bayliss, *Principles of General Physiology*, 3rd ed. (London: Longmans Greene, 1920), pp. 618–625. The remark quoted is on p. 625.

and he maintained that view again in 1923, only a year before his death. A. V. Hill, in his Bayliss-Starling Memorial Lecture,[78] has affectionately recalled the debate on this question between Bayliss, on one side, and Barcroft, N. K. Adam, and himself on the other, with a series of letters published in *Nature* in 1923. Bayliss still remained unconvinced by the arguments against the adsorption hypothesis.

In 1926 Conant and Scott, in a paper on adsorption of nitrogen by hemoglobin,[79] again suggested that hemoglobin might bind oxygen by a combination of adsorption and chemical reaction. Barcroft, in the second edition of his book, devoted several pages of discussion to the adsorption hypothesis.[80] He ended, as might be expected, by concluding that it was unnecessary to postulate any adsorption in order to explain the facts. No one since that time, to my knowledge, has wished to challenge that conclusion, and all later workers have assumed that the combination of oxygen and hemoglobin occurs by chemical reaction at the iron atoms of the heme groups.

THE TRUE MOLECULAR WEIGHT OF HEMOGLOBIN AND THE CHEMICAL BASIS OF THE OXYGEN DISSOCIATION CURVE

It was now clearly apparent that the sigmoid oxygen dissociation curve, as first described by Bohr, Hasselbalch and Krogh, was characteristic not only of blood but of hemoglobin solutions under physiological conditions. Barcroft, I believe, also appreciated its biological value, for the steepness of the curve in the middle range of y values promoted the efficient unloading of oxygen in the tissues, as compared with the hyperbolic curve that Hüfner had supposed to represent the facts. The most forceful statement on the subject came later from Haldane: "A man would die on the spot of asphyxia if the oxygen dissociation

78. A. V. Hill, "Bayliss and Starling and the Happy Fellowship of Physiologists: The Third Bayliss-Starling Memorial Lecture," *J. Physiol.*, 240 (1969), 1–13. See esp. p. 3. This article includes an interesting set of photographs of eminent British physiologists and biochemists.

79. J. B. Conant and N. D. Scott, "The Adsorption of Nitrogen by Hemoglobin," *J. Biol. Chem.*, *68* (1926), 107–121.

80. J. Barcroft, *The Respiratory Function of the Blood* 2nd ed., pt. II: "Haemoglobin" (1928), chaps. VI and XII. On pp. 120–122 he quotes in full a very interesting letter from N. K. Adam in *Nature, 101* (1923), 496, on the criteria of adsorption and the combination of oxygen and hemoglobin. This was one item in the controversy with Bayliss, referred to by A. V. Hill; see the text above, and n. 78.

curve of his blood were suddenly altered so as to assume the form which Hüfner supposed it to have in the living body." [81]

However, the question remained: what was the chemical basis for the fact that hemoglobin bound oxygen in this fashion? If hemoglobin had a molecular weight near 16,700, with a single iron atom—the minimum value compatible with the data of Zinoffsky and others—would it not necessarily react as Hüfner had supposed, according to the equation: $Hb + O_2 \rightleftharpoons HbO_2$? If this was indeed the case, how could it give any kind of dissociation curve except a rectangular hyperbola? Yet, during the period of Barcroft's early work, it was generally believed that the molecular weight of hemoglobin actually did correspond to the minimum value.

How clearly this dilemma may have been perceived by the early physiologists is uncertain. The first who did perceive it, we may be quite sure, was the young A. V. Hill (1886–), who, as an undergraduate at Cambridge, had devoted himself to mathematics, but with the encouragement of his tutor, Sir Walter Fletcher, had made a radical shift into physiology, and had begun to work in Barcroft's laboratory.[82]

Before considering how Hill dealt with the problem, however, we must consider the little that was known, in the first decade of the twentieth century, concerning the true molecular weight of hemoglobin. The usual methods employed by physical chemists to determine the size of small molecules in solution—freezing point depression, or vapor pressure measurements on the solvent were hopelessly insensitive for big molecules of molecular weight 10,000 or more. The one hope appeared to lie in osmotic pressure measurements. The work of van't Hoff had shown that, in very dilute solution, the osmotic pressure of any solute that did not dissociate should be equal to RT times the molar concentration (c) of solute, R being the gas constant and T the absolute temperature. Since $c = g/M$, where g is the weight concentration (grams per cc) and M the molecular weight, van't Hoff's equation states that π, the osmotic pressure, is related to M by the relation

$$\pi/RTg = 1/M \qquad (5)$$

as a limiting equation at small values of g. The osmotic pressure of a protein solution is measured as the pressure difference re-

81. J. S. Haldane, *Respiration*, 1st ed. (1922), p. 72.

82. A. V. Hill, "Autobiographical Sketch," *Perspectives in Biology and Medicine, 14* (1970), 27–42.

quired to maintain equilibrium between two solutions, with protein present in only one of them, separated by a membrane impermeable to the protein but permeable to all other components. Small molecules that can cross the membrane freely distribute themselves equally on both sides and do not contribute to the measured pressure. The same was supposed to be true for the ions of simple salts, but here there was in fact a complication, unappreciated by the early workers, if the protein molecule carried a net electric charge and therefore required the presence of an excess of diffusible ions of opposite charge in the protein solution. The problems arising from this complication will call for close attention in Part II of this account. Here we note only that their inevitable neglect in the early work led to uncertainties and probably to errors.

As early as 1896 C. J. Martin[83] in London was working on "a rapid method of separating colloids from crystalloids in solutions containing both." He used membranes as "filters," and to calibrate them used the method of Starling[84] to determine whether hemoglobin or albumin exerted any osmotic pressure. He found a small constant pressure, varying linearly with absolute temperature, for each of these proteins, and concluded that they must be large molecules. He remarked incidentally that "the molecular weight of hemoglobin is at least 16,669, and is probably some multiple of this quantity" (p. 370).

Nearly ten years later, E. Waymouth Reid[85] undertook a more systematic study. Having found the ratio of osmotic pressure to concentration to be fairly constant for hemoglobin solutions, he concluded that hemoglobin was in a state of true solution, not merely in a colloidal suspension, as some of the colloid chemists believed. He also noted that the osmotic pressure he had determined was about one third of what was to be expected if the molecular weight were 16,669. At 15°C the osmotic pressure, for a 1 per cent hemoglobin solution, ranged from 3.51 to 3.85 mm Hg. From equation (5), this would correspond to a molecular weight near 48,000. Reid stated this conclusion somewhat diffidently.

Looking back, twenty years later, with the perspective derived from his own far more reliable work, G. S. Adair concluded that Reid's work was by all odds the best of the early osmotic

83. C. J. Martin, *J. Physiol.*, *20* (1896), 364-371.

84. E. H. Starling, "On the Absorption of Fluids from the Connective Tissue Spaces," *J. Physiol.*, *19* (1896), 312–326; "The Glomerular Functions of the Kidney," ibid., *24* (1899), 317–330.

85. E. W. Reid, "Osmotic Pressure of Solutions of Hemoglobin," *J. Physiol.*, *33* (1905), 12–19.

studies of hemoglobin. Yet it failed to convince his contemporaries, who largely disregarded it. Reid had published an earlier paper on serum and egg white from which he had concluded that the osmotic pressures found in their solutions were probably produced by substances other than the proteins present, perhaps by protein breakdown products. There must have appeared, in the eyes of other workers, a discrepancy between these negative findings and the positive results on hemoglobin solutions which would have weakened confidence in the significance of Reid's work.

Moreover, in 1907 Hüfner and Gansser[86] reported an osmotic pressure study from which they concluded that the molecular weight was "doubtless" 16,700, and not a fraction or a multiple of this value. Their evidence was in fact no more convincing than that of Reid, indeed probably less so. However, Hüfner had the prestige of a senior distinguished contributor to the study of hemoglobin over many years. Moreover, his conclusion undoubtedly accorded better with the intellectual prejudices of the time. It was difficult enough in 1907 for most chemists to believe in definite molecules with molecular weights as large as 16,700. To think of molecules several times as large as this was a strain on one's credulity.[87] The work of Roaf[88] in Liverpool, which had started before that of Hüfner and Gansser appeared, showed great variations in apparent molecular weight under various conditions, and led to no firm conclusions.

At this stage, indeed, we can now see that no firm conclusions were likely to emerge. The influence of salt concentration and of the electric charge carried by the protein molecule at varying degrees of acidity or alkalinity—the concept of pH had not yet been formulated by Sörensen—was still unknown. Inconsistency of results and inconsistency between the findings of different investigators were practically inevitable.

This was the situation that confronted Barcroft and Hill in their attempt to interpret the oxygen dissociation curve of hemoglobin. The rising slope of the sigmoid curve, as the oxygen pressure increases from zero, clearly implies that the binding of

86. G. Hüfner and E. Gansser, "Ueber das Molekulargewicht des Oxyhämoglobins," *Arch Physiol., Anat.* (1907), 209–216.

87. In another article (n. 3) I have discussed in more detail the intellectual resistance to the concept of definite macromolecules and the gradual acceptance of this concept as the evidence for it accumulated, especially from about 1925 on.

88. H. E. Roaf, "The Relation of Proteins to Crystalloids. I.: The Osmotic Pressure of Hemoglobin and the Laking of Red Blood Corpuscles," Q. *J. exp. Physiol.*, 3 (1910), 75–96.

a little oxygen increases the tendency of the hemoglobin to bind more oxygen. There must therefore be a transmitted influence between the oxygen-binding centers (the hemoglobin iron atoms) such that oxygen binding at one center increases the oxygen affinity at one or more other centers. But, if each molecule contains only one such center, how can such mutual interaction arise? It could hardly be an interaction between different molecules, separated by solvent from one another. In that case the interaction would decrease rapidly as the solution was made more dilute, and the facts did not correspond to this.

Hill, in 1910, came up with a proposed solution to the paradox.[89] He assumed that hemoglobin existed in solution, not only as single molecules of molecular weight 16,700 but also largely in the form of aggregates of variable size. The single molecules would bind oxygen according to the equation: $Hb + O_2 \rightleftharpoons HbO_2$. Molecules n times as large could bind n molecules of oxygen: $Hb_n + nO_2 \rightleftharpoons Hb_n(O_2)_n$. It was implicit in the whole scheme that the n molecules of oxygen were bound more or less simultaneously, i.e., that such forms as $Hb_n(O_2)_x$, where x is less than n, but greater than zero, were present in very small or negligible amounts. To this vital assumption we return later.

Given this assumption, and with a single value of n—that is, a uniform degree of aggregation of all molecules in the solution —one would obtain the equation for the oxygen saturation of hemoglobin (y) as a function of the oxygen pressure (p):

$$y = kp^n / (1 + kp^n) \qquad (6)$$

Here k is the association constant. The reasoning is exactly the same as that involved in deriving equation (4) from equation (1), and equation (6) of course reduces to (4) if $n = 1$. If n is an integer greater than one, equation (6) describes a sigmoid curve. The assumptions of uniform aggregation and absence of intermediates would indeed require n to be an integer. Hill found, however, that he could obtain a good fit to the existing sigmoid curves for hemoglobin only by taking for n a non-integral value, not far from 2.5. Hill offered a ready explanation for this, however, by assuming that hemoglobin in solution existed as a mixture of aggregates, with $n = 1, 2, 3, 4$, etc., and that the observed value of n in equation (6) was a statistical average over all these varying forms.

Hill's first report[90] on his aggregation theory was only a

89. A. V. Hill, "The Possible Effects of the Aggregation of the Molecules of Hemoglobin on its Dissociation Curve," *J. Physiol., 40* (1910), iv–vii.
90. Ibid.

preliminary note, but it attracted much attention. In Haldane's laboratory it stimulated a different approach.[91] Douglas and Haldane had been investigating the combination of hemoglobin with oxygen and carbon monoxide, both separately and in combination; the resulting study is—only incidentally for our present purposes—one of the most important contributions ever made to the understanding of carbon monoxide poisoning. They found the binding of CO to hemoglobin to follow essentially the same sigmoid curve as that for oxygen, except that the partial pressure of CO, for a given degree of saturation, was only a very small fraction—of the order of 1/250, but varying somewhat between individuals—of that required for oxygen. They also made the striking observation that the binding of CO, at very low and constant partial pressures, was actually increased by adding small amounts of oxygen to the gas mixture. They wrote: "In presence of low partial pressures of CO the hemoglobin of blood may take up more than twice as much CO with a low partial pressure of oxygen as when oxygen is entirely absent. There can be no doubt that the converse is also true, namely, that in presence of a low partial pressure of oxygen the hemoglobin will take up more than twice as much oxygen when a low partial pressure of CO is present as when no CO is present." [92]

This "promoter" action of the one gas on the binding of the other naturally could exist only at relatively low degrees of saturation of the hemoglobin with the two gases; as the partial pressure of oxygen rose further, in the presence of a small fixed concentration of CO, the amount of CO bound by the hemoglobin passed through a maximum, and fell steadily toward zero as the oxygen pressure increased. Because of the sigmoid nature of the binding curve, the binding of either oxygen or CO, at low values of y, raises the value of y into a region where the curve is rising more steeply, and therefore where both gases are bound more strongly. As the degree of total saturation of the binding sites on the hemoglobin rises still further, however, the competition between oxygen and CO for the available sites becomes the dominant effect.

J. B. S. Haldane, then a nineteen-year-old scholar of New College, Oxford, proposed to explain the sigmoid binding curve for either oxygen or CO by assuming that both Hb and HbO_2 (or HbCO) would form aggregates, but that the tendency to

91. C. G. Douglas, J. S. Haldane, and J. B. S. Haldane, "The Laws of Combination of Haemoglobin with Carbon Monoxide and Oxygen," *J. Physiol.*, *44* (1912), 275–304.

92. Ibid., 291–292.

aggregation was much stronger for the unbound Hb than for either HbO_2 or HbCO. Therefore, as more oxygen (or CO) was bound, the aggregates tend to break up. With this general assumption, and by suitable choice of constants, it was possible to obtain a good fit to the experimental curves.

In the following year Hill[93] gave a full statement of the thinking underlying his equation. The central point was "the idea that the aggregated hemoglobin molecule, taken e.g., to be Hb_2, is oxidised into the form $Hb_2(O_2)_2$, by combination with two oxygen molecules simultaneously, and that the unsaturated molecule Hb_2O_2 either does not exist at all or exists in negligibly small quantities," or, "in other words, that the partially saturated molecule is very unstable, is difficult to form and easy to combine further with oxygen."[94] In modern biochemical terminology, Hill was saying that the aggregated hemoglobin molecule exhibited very strong cooperative interactions in the binding of oxygen (or CO) molecules. Barcroft,[95] in an immediately following paper, considered the fit of Hill's equation to the available experimental data, and concluded that the fit was excellent, and well within the experimental error, the value of n in equation (6) being close to 2.5. This clearly required that the average degree of aggregation must be greater than 2; obviously, if there were no aggregates higher than Hb_2, the value of n could not be higher than 2. Barcroft also concluded that the effect of acids (as shown for example by the effect of CO_2 on oxygen binding in Fig. 1) was only to change the value of k in equation (6), the degree of aggregation, as indicated by n, being unchanged.

Barcroft and Hill both concluded that Hill's equation fitted the experimental facts better than did the Haldane hypothesis, which assumed that the binding of O_2 would tend to break up the aggregates. It was certainly a simpler hypothesis. It contained one important implication, stressed by Hill, concerning the slope of the curve at very low p values. The limiting slope, dy/dp, as p approaches zero, is nkp^{n-1} from equation (6). If $n = 1$, the limiting slope is finite and equal to k; but if $n>1$, it goes to zero. In other words, at very low p, Hill's equation for $n>1$ requires that the curve be tangent to the abscissa at the origin. Measurements of oxygen saturation, at very low values of y, are exceedingly difficult and subject to large experimental error.

93. A. V. Hill, "The Combinations of Hemoglobin with Oxygen and with Carbon Monoxide. I," *Biochem. J.*, 7 (1913), 471–480.

94. Ibid., 472, 474.

95. J. Barcroft, "The Combinations of Hemoglobin with Oxygen and with Carbon Monoxide. II," *Biochem. J.*, (1913), 481–491.

Barcroft and Hill, in testing Hill's equation, were probably misled by the data they obtained in this portion of the curve. As Professor Roughton has pointed out to me in discussion, the ferricyanide method for determining oxyhemoglobin, which Barcroft and his associates used, is subject to small errors because the ferricyanide not only releases the oxygen bound to hemoglobin but also, to a very small extent, oxidizes other constituents that may be present in the system. These secondary oxidations consume a fraction of the oxygen released from the hemoglobin, thus making the measured value of y too small. At very low y values, this error was appreciable in Barcroft's work, so that the measured values of y at very low p gave a curve that appeared to be horizontal at $p = 0$. It was only much later that very careful measurements showed that the limiting slope of the curve, at $p = 0$, was actually finite. If this fact had been known to Hill, he might never have proposed his equation. However, at intermediate y values, say between 0.2 and 0.8, the Hill equation gives an excellent fit to the best modern data, and it is still widely used as an empirical equation.

The coming of the First World War brought a halt to all progress in this field. When research resumed after the war, a young investigator, G. S. Adair in Cambridge, undertook further study of the osmotic pressure and molecular weight of hemoglobin. Both in his theoretical background and his experimental technique, Adair was far ahead of the earlier workers. He pointed out that Hüfner and Gansser had used relatively impermeable parchment membranes, within which salts, even without proteins present, can give rise to temporary pressures lasting 10 days or more; and they had measured what they supposed to be the osmotic pressure of the protein after only a day or two. With special care Adair prepared, by a technique he described in detail, collodion membranes that were readily permeable to water and salts, but did not permit hemoglobin to leak out. As he reported: "After a few years' practice, the proportion of failures was below 10 per cent."[96] He imposed rigorous criteria for the validity of the measurements. They must be steady over a long period; the pressure must return to the same level if the osmometer is reset at a different pressure; and

96. G. S. Adair, "A Critical Study of the Direct Method of Measuring the Osmotic Pressure of Hemoglobin," *Proc. Roy. Soc.* [108A] (1925), 627–637. The quoted sentence is on p. 630. This paper was actually communicated in April 1924, although not published until nearly a year later. We may suspect that the referees were disturbed by Adair's then highly unorthodox conclusions concerning the size of the hemoglobin molecule, and therefore delayed the publication of the paper.

they must be reproducible if the technique is varied. Moreover, the influence of the electric charge on the protein molecule, and of consequent uneven distribution of salts across the osmometer membrane, had not been well understood by the earlier workers. It was later clarified, thanks primarily to the work of Donnan.[97] Adair was well aware of all the problems involved; he was indeed almost unique among biochemists in having studied deeply the great thermodynamic treatise of Willard Gibbs,[98] and was the first to recognize that Gibbs had in fact anticipated the analysis given independently by Donnan more than thirty years later.[99]

Adair had begun to get significant osmotic pressure measurements on hemoglobin as early as 1921, although his first paper on the subject[100] was not published until 1925. His conclusions were unequivocal; the osmotic pressure in dilute solutions, divided by the hemoglobin concentration, was only about one quarter of that reported by Hüfner, and the molecule was therefore 4 times as large as Hüfner and others had supposed, the true molecular weight being 67,000. The same value applied even in the absence of salts[101] under the conditions where Barcroft and Roberts[102] had reported the dissociation curve to be a rectangular hyperbola.

Thus, if Adair was correct, the hemoglobin molecule contained 4 atoms of iron and 4 heme groups. Therefore, a hemoglobin solution was not composed of a system of aggregates, of various sizes, in equilibrium with one another, as Hill had supposed. It was instead a definite molecule, of molecular weight close to 67,000.

These conclusions, startling to many workers at the time, soon received powerful and completely independent support from another source. In Uppsala, the eminent colloid chemist The Svedberg had, since about 1920, been developing the ultracentrifuge as a tool for the study of the sizes of colloidal

97. F. G. Donnan, "Theorie der Membrangleichgewichte und Membranpotentiale bei Vorhandensein von nicht dialysierenden Elektrolyten. Ein Beitrag zur Physikalisch-chemischen Physiologie," *Z. Elektrochem., 17* (1911), 572–581.

98. J. W. Gibbs, "On the Equilibrium of Heterogenous Substances," *Trans. Connecticut Acad. Sci., 3,* (1875–78), 108–248 and 343–524; also in *The Collected Works of J. Willard Gibbs* (New York: Longmans Green, 1931), I, 54–353.

99. See G. S. Adair, "On the Donnan Equilibrium and the Equation of Gibbs," *Science, 58* (1923), 13.

100. See n. 96.

101. G. S. Adair, "The Osmotic Pressure of Hemoglobin in the Absence of Salts," *Proc. Roy. Soc.* [109A] (1925), 292–300.

102. See n. 71.

particles, by measuring their distribution in an intense centrifugal field. The development of this machine, which in later models could produce centrifugal accelerations several hundred thousand times as great as the acceleration of gravity, represented a major triumph of instrument design; and Svedberg applied it eagerly to the study of proteins. The first protein he chose for study was hemoglobin, because of its great physiological interest, its ease of preparation, and its well-defined iron content. Its color also made it easy to observe the migration of the hemoglobin molecules in the optical cell that held the solution and was inserted in the rotor of the centrifuge. In the first study, Svedberg and Fåhreus[103] used the method of sedimentation equilibrium, which required only a relatively low centrifugal field (in this case about 5000 times gravity). In this method the centrifuge had to run long enough, in this case several days, to permit a distribution of protein in the cell of the rotor that is independent of time; the outward motion of the protein, due to the centrifugal field, being balanced by its diffusion from regions of higher to regions of lower concentration.[104] Svedberg and Fåhreus were unaware of Adair's work, but their conclusions were the same; the hemoglobin molecule was 4 times as large as the molecular weight calculated from the iron content. They also noted that the molecular weight, as calculated from the distribution of hemoglobin in different parts of the cell at equilibrium, was independent of the distance from the center of rotation. This indicated that all the molecules were probably of the same size, a conclusion that could not be established from osmotic pressure measurements alone.

A year later, Svedberg and Nichols[105] reported on the use of the sedimentation velocity method in studying hemoglobin. This required a much more powerful centrifugal field, in this case about 100,000 times gravity. The measurement required determining the rate at which the boundary between protein and solvent moved outward as the run proceeded; this rate of motion, divided by the centrifugal acceleration, gave the sedimentation coefficient s:

$$s = (dr/dt) \,/\, \omega^2 r$$

103. T. Svedberg and R. Fahreus, "A New Method for the Determination of the Molecular Weight of the Proteins," *J. Amer. Chem. Soc.*, *48* (1926), 430–438.

104. For the details of the method see the comprehensive book by T. Svedberg and K. O. Pedersen, *The Ultracentrifuge* (Oxford: Clarendon Press, 1940).

105. T. Svedberg and J. B. Nichols, "The Application of the Oil Turbine Type of Ultracentrifuge to the Study of the Stability Region of Carbon Monoxide Hemoglobin," *J. Amer. Chem. Soc.*, *49* (1927), 2920–2934.

Here r is the radial distance from the center of rotation, t is time, and ω is the angular velocity of the rotor in radians per second. To know the molecular weight one must also know the diffusion coefficient D, which they obtained by measuring the spreading of the boundary as sedimentation proceeded. Finally, it was essential to know the partial specific volume (v) of the protein in solution—that is, the increment in volume per gram of dry protein added to the solution (this quantity was also essential for the sedimentation equilibrium determination). Then the molecular weight is obtained from the equation

$$M = RTs/\mathrm{D}(1-v\rho),$$

where ρ is the density of the solvent. Svedberg and Nichols were now aware of Adair's work, and noted the excellent agreement between Adair's findings and their own. The sedimentation velocity runs could be done rapidly, requiring only a few hours instead of several days. The form of the moving protein boundary was evidence that all the protein molecules had essentially the same molecular weight, and the resulting value was 68,000 within the limits of error, in complete agreement with Adair and with the sedimentation equilibrium studies. They also showed that this value was independent of pH between 6 and 9, but that at lower or higher pH values there appeared to be a partial breakdown to lower molecular weights.

The work of Adair and of Svedberg was now rapidly accepted, and a drastic change in outlook naturally ensued. The aggregation theory was now discredited, and the remarkable cooperative properties of hemoglobin in the binding of oxygen had to be explained on another basis. Here Adair led the way, by formulating the interaction of hemoglobin with oxygen in terms of a series of intermediate compounds. Denoting the molecule of hemoglobin, with its 4 iron atoms, Hb_4, it could obviously form a series of compounds with oxygen: Hb_4O_2, $Hb_4(O_2)_2$, $Hb_4(O_2)_3$, and $Hb_4(O_2)_4$. The relations between all of these could be described by a series of equilibrium constants.

$$Hb_4 + O_2 \rightleftharpoons Hb_4O_2;\ Hb_4O_2 + O_2 \rightleftharpoons Hb_4(O_2)_2, \text{ etc.}$$

Thus there would be four equilibrium constants, as follows:

$$\frac{[Hb_4O_2]}{[Hb_4]p} = K_1 \qquad \frac{[Hb_4(O_2)_2]}{[Hb_4O_2]p} = K_2$$

$$\frac{[Hb_4(O_2)_3]}{[Hb_4(O_2)_2]p} = K_3; \qquad \frac{[Hb_4(O_2)_4]}{[Hb_4(O_2)_3]p} = K_4$$

The fractional saturation (y) of the hemoglobin with oxygen

is given by counting all the oxygen-binding sites that are occupied by oxygen molecules—one for each Hb_4O_2 molecule, two for each $Hb_4(O_2)_2$ molecule etc.—and dividing by the total number of such sites, 4 per molecule.

$$y = \frac{[Hb_4O_2] + 2\,[Hb_4(O_2)_2] + 3\,[Hb_4(O_2)_3] + 4\,[Hb_4(O_2)_4]}{4\,([Hb_4] + Hb_4O_2] + [Hb_4(O_2)_2] + [Hb_4(O_2)_3] + [Hb_4(O_2)_4])} \quad (7)$$

Then y can be expressed in terms of the 4 binding constants, K_1 ..... K_4, which gives:

$$y = \frac{K_1p + 2K_1K_2p^2 + 3K_1K_2K_3p^3 + 4K_1K_2K_3K_4p^4}{4\,[1 + K_1p + K_1K_2p^2 + K_1K_2K_3p^3 + K_1K_2\,K_3K_4p^4]} \quad (8)$$

This is Adair's equation. [106] It is obviously more complex than the Hill equation, which involves only two adjustable constants, whereas Adair's equation involves four. This corresponds to the fact that the Hill equation assumes only two classes of hemoglobin molecules—those with no bound oxygen at all and those that are completely oxygenated. Hill assumed variable degrees of aggregation of Hb to form Hb_2, Hb_3, Hb_4, etc., whereas Adair concluded that only Hb_4 exists in appreciable quantities, but that all possible intermediate states of oxygenation can occur.

Certain differences between the oxygen dissociation curves predicted by the Hill and by the Adair equation were manifest. The Hill equation, as we have seen, predicts that the initial slope, dy/dp, at $p = 0$, should be zero if n is greater than unity in equation (6). In the Adair equation (8) on the other hand, as p approaches zero, all terms but the first must vanish in numerator and denominator. Thus the initial slope of $dy/dp = K_1/4$ as $p \to 0$. This indeed furnishes a direct method of determining K_1, though experimentally it is extremely difficult to make accurate measurements of y in the range below 0.01–0.02, which is what is needed here. The most accurate measurements of y are probably those of Roughton, Otis, and Lyster,[107] made thirty years after Adair proposed his equation; they clearly confirm the prediction of Adair's equation that the initial slope of the curve y versus p is finite, not zero.

Likewise, it can easily be shown that, as y approaches unity, at very large values of p, the concentration of all intermediates

106. G. S. Adair, "The Hemoglobin System," A series of six papers, some in collaboration with A. V. Bock and H. Field, Jr., *J. Biol. Chem.*, 63 (1925), 493–546. Adair's equation for oxygen binding is presented in the last paper.

107. F. J. W. Roughton, A. B. Otis, and R. L. J. Lyster, "The Determination of the Individual Equilibrium Constants of the Four Intermediate Reactions between Oxygen and Sheep Hemoglobin," *Proc. Roy. Soc. Lond.* [144B], (1955), 29–54.

except $Hb_4(O_2)_3$ and the fully saturated $Hb_4(O_2)_4$ must vanish. Thus measurements of y as a function of p, in the range of $y = 0.98–1.00$, depend essentially only on K_4, and give a means of determining this constant. Here also it is technically very difficult to make such measurements with the required degree of accuracy; again the measurements of Roughton et al. are the most accurate available.

The Adair equation is entirely capable of accounting for the sigmoid form of the oxygen dissociation curves (and therefore also for the carbon monoxide dissociation curves, which are also sigmoid, but with far larger values of K_1, ... K_4). However, this imposes certain restrictions on the relative values of the different K's. In brief, at least one of the higher K values must be larger, in fact a good deal larger, than K_1; all of them—K_2, K_3, and K_4—may be larger than would be expected on a basis of independent binding by four equivalent sites.[108] Both Adair and later workers such as Roughton[109] had evidence that K_4 was particularly large. In any case, to account for the experimental facts it was certainly necessary to accept the hypothesis of cooperative binding, as originally stated by Hill. That is, the binding of oxygen (or carbon monoxide) at one of the 4 sites in the molecule must, by some mechanism, strongly promote the binding at the other sites. This, of course, does not mean that intermediates are absent—by Adair's hypothesis they must be present—but it does mean that Hb_4 and $Hb_4(O_2)_4$ will be present, at y values near 0.5, in much larger relative quantities than would be expected on the basis of chance. In its subsequent history this fundamental concept has had far-reaching ramifications, not only in the further study of hemoglobin, but more widely in the study of many enzymes and in the understanding of adaptation in biochemical systems.

THE MUTUAL INTERACTION OF OXYGEN AND CARBON DIOXIDE IN THE HEMOGLOBIN MOLECULE: COUPLING OF ACIDITY CHANGES AND OXYGEN AFFINITY

We must now return to another aspect of the hemoglobin story, to the phenomenon that Christian Bohr had sought and

108. If all 4 sites were equivalent, and reacted independently of each other with an intrinsic binding constant K_0, then the observed K values would be $K_1 = 4K_0$, $K_2 = 3K_0/2$, $K_3 = 2K_0/3$, $K_4 = K_0/4$. The factors 4, 3/2 etc., are statistical factors determined by the number of binding sites in each molecule at which the "on" and "off" reactions can occur. For further discussion, see, for instance, J. T. Edsall and J. Wyman, *Biophysical Chemistry*, (New York: Academic Press, 1958), vol. I, chap. 9.

109. See n. 107.

failed to find—the effect of oxygenation on the capacity of blood to take up carbon dioxide. Bohr's failure, in repeated attempts, to observe any such effect, had clearly discouraged other physiologists from looking for it. Haldane, however, was puzzled by Bohr's curve for dog's blood,[110] showing uptake of CO_2 as a function of the partial pressure of the gas. His own great earlier researches with Priestley[111] on the regulation of respiration had shown the extraordinary sensitivity of the respiratory center in the medulla to the exact partial pressure of CO_2 in the alveolar air and hence in the arterial blood. Bohr's curve suggested that, if about a third of the oxygen in arterial blood is consumed as it passes through the tissues, the corresponding rise in the CO_2 pressure in venous blood (involving an increase of about 5 volumes of transported CO_2 per 100 volumes of blood) would raise the CO_2 pressure in the venous blood by 16–17 mm Hg. Haldane and Priestly had found the arterial CO_2 pressure to be regulated within less than 1 mm Hg; indeed a rise or fall of 1 mm Hg could increase or decrease the breathing by fully as much as 60 percent. Haldane found it hard to believe that such changes between arterial and venous blood could normally occur in a subject at rest, and he suspected that some compensating factor must act to diminish the actual variation of the partial pressure of CO_2 between arterial and venous blood, so as to keep it well below the variation indicated by Bohr's curve. This led him to reinvestigate the effect of oxygen on the CO_2 dissociation curve, in collaboration with C. G. Douglas, who had worked with him for years, and with a young Danish woman, Johanne Christiansen, who had recently taken her M.D. in Copenhagen before coming to work with Haldane in Oxford.[112]

They used, of course, Haldane's methods of blood gas analysis,

110. See Bohr's review in Nagel's *Handbuch* (n. 47).

111. J. S. Haldane and J. G. Priestley, "The Regulation of the Lung Ventilation," *J. Physiol.*, 32 (1905), 255–266.

112. J. Christiansen, C. G. Douglas, and J. S. Haldane, "The Absorption and Dissociation of Carbon Dioxide by Human Blood," *J. Physiol.*, *48* (1914), 244–271. Johanne Christiansen (1882–) returned to Copenhagen in 1913, where she taught at the Institute of General Pathology and later practiced medicine, and wrote books and articles on nutrition, many of them addressed to the general public (see *Kraks Blabog*, Copenhagen, 1966 ed.). For a brief account of her with a photograph, see P. Astrup "Early Danish Contributions to Oxygen and Acid-Base Research, in "Oxygen Affinity of Hemoglobin and Red Cell Acid-Base Status," M. Rørth and P. Astrup, eds. (Munksgaard, Copenhagen, and Academic Press, New York, 1972), pp. 809–822. This article includes photographs of Bohr, Hasselbalch, Krogh, and other notable Danish investigators, with brief discussions of the work of each.

which were much more accurate than those that Bohr had used, and the effect that Bohr had failed to detect emerged with unmistakable clarity. As shown in their Fig. 2, reproduced here,

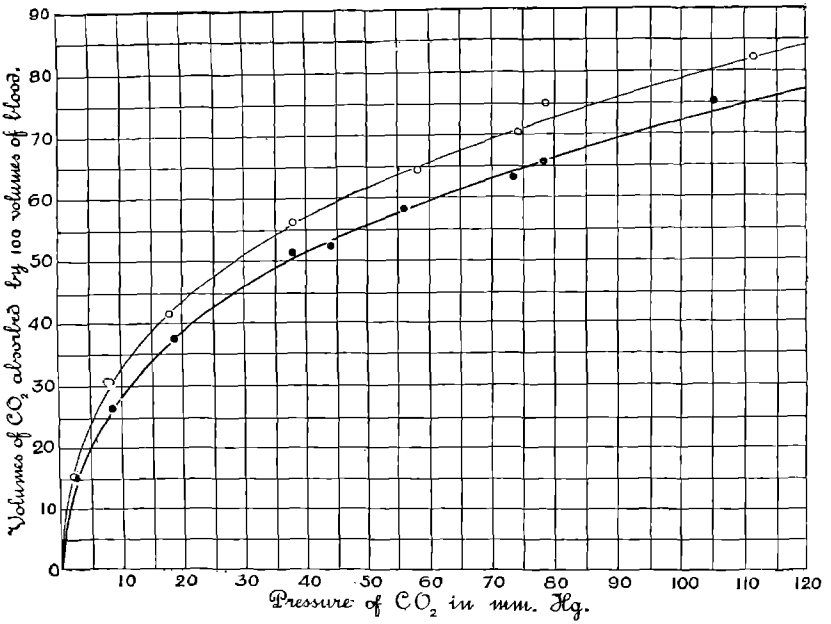

FIG. 2. The total carbon dioxide content of the blood of J. S. H. in presence of air and CO_2 (lower curve) and in presence of hydrogen and CO_2 (upper curve). From Christiansen, Douglas, and Haldane.

the lower curve shows the CO_2 uptake for Haldane's blood as a function of p CO_2 in the presence of enough oxygen to saturate the iron-binding sites almost completely. The upper curve shows the CO_2 uptake in the presence of an inert gas that would not bind to hemoglobin (in this case, hydrogen). Both curves, for a given individual, proved to be highly reproducible, day after day and month after month.

Thus this effect, which Holmgren[113] believed he had discovered, with far less accurate techniques in 1865, was now demonstrated by very accurate measurements. The physiological implications, as Christiansen, Douglas, and Haldane pointed out, were far-reaching. As the arterial blood flowed through the

113. See n. 39.

capillaries, releasing oxygen and taking on CO_2, the discharge of oxygen would increase the capacity to take on CO_2, even at a fixed CO_2 pressure. "At the physiologically important part of the curve, between 35 and 60 mm pressure of CO_2, the extra amount of CO_2 taken up is about 5 to 6 vols. per 100 vols. of blood." [114]

For a given shift in $p\ CO_2$, between the arterial blood and the mixed venous blood that returns to the heart, the amount of CO_2 the blood will take up in the tissues is nearly twice as great as if the oxygen effect, shown in Fig. 2, did not exist. Likewise, in the lungs, the influx of oxygen causes the release of far more CO_2 into the alveolar air than would occur if there were no change in the oxygen pressure. Moreover, the fall in CO_2 pressure and concentration, as the blood flows through the lungs, increases the oxygen affinity of the hemoglobin according to the reciprocal relation discovered earlier by Bohr, Hasselbalch, and Krogh (Fig. 1). The two reciprocal effects together enormously enhance the efficiency of blood as a transporting agent for both oxygen and CO_2.

This discovery had a profound impact on the thinking of L. J. Henderson. As he wrote later: "The discovery by Christiansen, Douglas and Haldane. . . finally led me to the conclusion, which should have been drawn a decade earlier, that every one of the variables involved in the repiratory exchanges of blood must be a mathematical function of all the others." [115] It thus marked a turning point in Henderson's thought, which led him in the course of the next decade to his formulation of blood as a physicochemical system of multiple interdependent variables. Haldane had arrived experimentally at the fact that the reciprocal relations existed; it was probably Henderson who first perceived that their reciprocal character was a mathematical necessity. However, as he himself emphasized, he had failed like everyone else involved to perceive this necessity before the experimental evidence was in.[116]

How was the action of oxygen, in driving off CO_2 from the blood, to be explained? Christiansen, Douglas, and Haldane proposed two alternative explanations: (1) oxyhemoglobin is a stronger acid than hemoglobin, or (2) if reduced hemoglobin is more strongly aggregated than oxy, as J. B. S. Haldane had

114. See n. 112, p. 256.

115. L. J. Henderson, *Blood*, pp. 95–96. See also the discussion by Parascandola, n. 60, pp. 88–89.

116. In his unpublished "Memories," however, Henderson claimed that he had finally realized the necessary character of such a relation, shortly before the paper of Christiansen, Douglas, and Haldane appeared; so this statement need some qualification (see Parascandola, n. 60, p. 81).

proposed, then "the aggregated molecules would have less influence in expelling CO_2." [117] The reason for this latter statement is not immediately clear. Nevertheless, J. S. Haldane, eight years later, in the first edition of his great treatise on *Respiration* (p. 90), still preferred the latter explanation, but he was virtually alone in doing so. All those who later dealt with the problem adopted the former explanation, that oxygenation of hemoglobin made it a stronger acid; and this hypothesis proved adequate to account for all the facts.

The next workers to take up the problem were T. R. Parsons, a young investigator in Barcroft's laboratory, and L. J. Henderson at Harvard. Both made the same basic assumptions, that oxyhemoglobin was a stronger acid than reduced hemoglobin, and that the total carbon dioxide carried in the blood could be accounted for as carbonic acid (including both CO_2 and H_2CO_3) and bicarbonate.[118] Parsons,[119] whose work was the first to appear, assumed the available "base"—or in modern terminology, the cations, especially sodium and potassium—to be distributed between bicarbonate and the blood proteins, especially hemoglobin, acting as acids, according to the equation:

$$\text{H Protein} + \text{NaHCO}_3 \rightleftharpoons \text{Na Protein} + \text{H}_2\text{CO}_3$$

or, if we treat the salts as fully ionized, we can omit the sodium from the equations; and write:

$$\text{H Protein} + \text{HCO}_3 \rightleftharpoons \text{Protein} + \text{H}_2\text{CO}_3$$

This leads to the equation:

$$K = \frac{[\text{Protein}^-]\,[\text{H}_2\text{CO}_3]}{[\text{H Protein}]\,[\text{HCO}_3]} = \frac{K_{\text{Protein}}}{K_{\text{H}_2\text{CO}_3}} \qquad (9)$$

117. See n. 112, p. 258.

118. At this time there was no way of determining the ratio of CO_2 to H_2CO_3, in aqueous solutions, but since the two molecules must be present in a constant ratio, according to the law of mass action, they could be lumped together as a single component for analytical purposes. Within a few years it became possible, and important, to discriminate between CO_2 and H_2CO_3, and to determine the rates of transformation of one into the other. We return to these questions in Part II.

The fundamental assumption, that carbonic acid and bicarbonate accounted for all the CO_2 in blood, had been denied by Bohr and several later workers—Parsons gives references to the controversy—but it seemed to most workers the best hypothesis available at the time. The later developments, which showed that this assumption was inadequate to account for all the facts, are also to be taken up in Part II.

119. T. R. Parsons, "The Reaction and Carbon Dioxide Carrying Power to Blood—a Mathematical Treatment. Part I," *J. Physiol.*, 53 (1920), 42–59. Part II is in the same volume, pp. 340–360, but is less important for the present purposes.

Here $K_{H_2CO_3}$ is the ionization constant of carbonic acid,

$$K_{H_2CO_3} = \frac{[H^+]\,[HCO^-_3]}{[(CO_2) + (H_2CO_3)]} \qquad (10)$$

and $K_{Protein}$ is the acidic ionization constant of the protein. If the protein is hemoglobin, and if K for oxyhemoglobin is greater than for reduced hemoglobin, then oxygenation will cause the ratio [Protein$^-$]/[H Protein] to increase. The result is to transfer acidic hydrogen (protons) from hemoglobin to bicarbonate, and reduce the amount of carbon dioxide carried by the blood. Parsons showed that, by assuming suitable values for the acid strength of the proteins of the blood, with and without oxygenation, it was possible to fit the two curves of Fig. 2, and other data from Haldane's work, very satisfactorily. Henderson's analysis[120] of the problems was along very similar lines, but he took account explicitly of the fact that "every protein molecule contains a considerable number of acid and basic radicals" and therefore, as the pH varies, the system will behave, not like a single weak acid, but like a mixture of several weak acids. Only a few of these, perhaps only one per hemoglobin molecule, and none in the blood plasma proteins, will be affected by oxygenation. Taking account of these facts, and using Haldane's data, Henderson calculated acid ionization constants for oxygenated (K_0) and reduced hemoglobin (K_R), assuming that one acidic group per iron atom changed its acid strength on oxygenation.

$$K_R = [H^+]\,[Hb^-]/[HHb = 2.3 \times 10^{-8} m \qquad (11)$$

$$K_0 = [H^+]\,[HbO^-_2]/[HHbO_2] = 2.0 \times 10^{-7} \mathrm{m} \qquad (12)$$

Expressed in terms of negative logarithms, according to the usual convention of chemists and biochemists subsequently, these values correspond to

$$pK_R = -\log K_R = 7.64$$

$$pK_0 = -\log K_0 = 6.70$$

In view of the inherent uncertainties of Haldane's data for Henderson's purposes, the calculated values are astonishingly close to the far more accurate ones obtained later, by D. D. Van Slyke and his collaborators, and by Jeffries Wyman. Henderson's calculations were in fact something of a *tour de force;* he himself later rather deprecated the significance of this paper.[121] It was,

120. L. J. Henderson, "The Equilibrium between Oxygen and Carbonic Acid in Blood" *J. Biol. Chem., 41* (1920), 401–430.

121. For Henderson's attitude toward this piece of work, see Parascandola, n. 60, pp. 89–90.

however, a preliminary step to his comprehensive study of blood as a physicochemical system, and is therefore notable in any case for the later work to which it led.

During this period began that close informal cooperation between Henderson at Harvard and D. D. Van Slyke and his associates at the Rockefeller Institute in New York, which played such a major role in the studies of blood as a physicochemical system. Most of the story belongs in Part II of this history, but we must note the great advance in experimental technique, contributed by Van Slyke's laboratory, in determining the pK_0 and pK_R values for oxygenated and reduced hemoglobin.[122] They determined pH values indirectly, by measuring $p\ CO_2$ and total CO_2 in the hemoglobin solutions, and used the Henderson-Hasselbalch equation:

$$\text{pH} = pK' + log\ \frac{[\text{bicarbonate}]}{[CO_2]}$$

to determine the pH. Then, by a series of titrations, they determined the base bound by (or, in modern terminology, the net electric charge of) oxy and reduced hemoglobin as a function of pH, over a pH range from about 6.8 to 8.2. It is interesting to reread their paper, with its long series of detailed protocols of individual experiments, and contrast it with the terse reports of essential results, perhaps accompanied by some statistical analysis, that are required today by the pressure for space in journals and by present editorial policies.

Their analysis of the data laid the essential groundwork for all subsequent workers on this problem. If we take the acid dissociation constants K_R and K_0 as defined by (11) and (12), without assuming the specific values given there, then we can write, for reduced hemoglobin

$$\frac{[Hb^-]}{[Hb^-] + [HHb]} = \frac{K_R}{K_R + [H^+]} \quad (13)$$

Here the left-hand side of the equation represents the fraction of all the hemoglobin that is present in the negatively charged form, i.e. which has given up a proton; likewise for oxyhemoglobin:

$$\frac{[HbO^-_2]}{[HbO^-_2] + [HHbO_2]} = \frac{K_0}{K_0 + [H^+]} \quad (14)$$

122. A. B. Hastings, D. D. Van Slyke, J. M. Neill, M. Heidelberger, and C. R. Harington, "Studies of Gas and Electrolyte Equilibria in Blood VI. The Acid Properties of Reduced and Oxygenated Hemoglobin", *J. Biol. Chem.*, *60* (1924), 89–153.

If we completely oxygenate reduced hemoglobin, while keeping the hydrogen ion concentration, $[H^+]$, constant, by titrating with acid or alkali, then the number of protons released on oxygenation is given by the increase in negative charge on hemoglobin that accompanies the process. We note that in this case $[Hb^-] + [HHb]$ in (13) must be equal to $[HbO^-_2] + [HHbO_2]$ in (14), since in both cases the sum of these two terms equals the total hemoglobin concentration. Thus, subtracting (13) from (14):

$$\Delta = \frac{[HbO^-_2] - [Hb^-]}{\text{total hemoglobin}} = \frac{K_0}{K_0 + [H^+]} - \frac{K_R}{K_R + [H^+]} \quad (15)$$

This difference gives the number of moles of hydrogen ions released per mole of hemoglobin iron. By measuring this quantity at a series of $[H^+]$ values, but in each case keeping pH constant during oxygenation, one obtains Δ as a function of $[H^+]$, or pH. It is readily seen that Δ must go to zero if $[H^+]$ is either very much greater, or very much less, than K_0 and K_R. By differentiation it is also easy to prove that Δ is a maximum when $[H^+] = \sqrt{K_0 K_R}$, i.e., when $\text{pH} = [pK_0 + pK_R]/2$.

It is thus possible, by determining Δ as a function of pH, to calculate K_0 and K_R. Hastings, Van Slyke, Neill, Heidelberger, and Harington thus found for horse blood at 38°C:

$$pK_0 = 6.87 \text{ and } pK_R = 8.33$$

at a concentration of sodium ions equal to 50 millimolar. Both pK_0 and pK_R decreased as the ionic concentration increased, but their difference remained nearly constant. Thus these measurements indicated that pK was shifted downward by 1.46 units on oxygenation; or, in other words, that the ratio K_0/K_R was close to 27—a striking increase in acid strength, about three times as great as in Henderson's preliminary calculations. Moreover, they concluded that all their data could be fitted on the assumption that only a single acid group, per hemoglobin iron atom, changed its acid dissociation constant during oxygenation. They were careful to point out that their data did not prove this assumption; there might be several groups, the acidity of which could be changed by oxygenation, each contributing a fraction of the total effect. In that case the two terms on the right-hand side of equation (15) would be replaced by the sum of a series of pairs of terms of the same form. No such complexity, however, was required by the data.

Hastings et al.[123] naturally speculated as to the chemical

123. Ibid.

nature of the groups affected by oxygenation; for instance, the conversion of propionic acid to lactic acid, by inserting a hydroxyl oxygen on the carbon atom adjoining the carboxyl group, caused approximately a tenfold increase in the acid strength of the carboxyl. But the nature of the acidic group in the hemoglobin molecule—the heme-linked group, as Wyman later termed it—was still completely obscure. Indeed, the work of the subsequent period, of nearly half a century, is only now beginning to yield what looks like a decisive answer (see n. 129 below).

As we have seen, it became obvious after the work of Christiansen, Douglas, and Haldane that there was a reciprocal interaction between the uptake of oxygen and that of CO_2 by blood; if increase of CO_2 pressure diminishes oxygen uptake, at constant partial pressure of oxygen, then increase of oxygen pressure *must* cause CO_2 to be released at constant partial pressure of CO_2. Each effect implies the other. The fundamental equations first stated by Willard Gibbs[124] not only indicate that such reciprocal relations must exist, but also specify how their magnitudes must be related. Adair,[125] in a short note occupying less than a single page, showed by the use of Gibbs equation that the effect of CO_2 on oxygen binding, as determined by Barcroft, could be used to calculate the magnitude of the effect of oxygen on the CO_2 content of blood as determined by Christiansen, Douglas and Haldane; and because of the symmetry of the relations involved, the reverse calculation was of course equally direct. This calculation was the forerunner of the comprehensive treatment, developed in detail by Wyman,[126] of the theory of linked functions and reciprocal relations: a theory of general application in physicochemical systems, but particularly useful for such highly organized and adapted systems as hemoglobin.

It is interesting to note how ideas and procedures, after apparently being rejected and forgotten, may emerge again much later in a somewhat different guise. Thus A. V. Hill's equation (6), derived from his aggregation theory, went into eclipse when Adair demonstrated that hemoglobin normally contained 4 heme groups. However, in recent years the use of

124. See n. 98.

125. G. S. Adair, "Thermodynamical Proof of the Reciprocal Relationship of Oxygen and Carbon Dioxide in Blood," *J. Physiol.*, *58* (1923–24), iv–v.

126. J. Wyman, "Heme Proteins," *Advances in Protein Chem.*, *4* (1948), 407–531; "Linked Functions and Reciprocal Effects in Hemoglobin: a Second Look," ibid., *19* (1964), 223–286; "Regulation in Macromolecules as Illustrated by Haemoglobin," *Q. Rev. Biophys.*, *1* (1968), 35–80.

the equation has undergone a great revival, and Wyman,[127] in particular, has shown the power of its use in describing many of the molecular interactions.

Likewise, the proposal of J. B. S. Haldane[128] in 1912, that deoxygenated hemoglobin is more aggregated than oxyhemoglobin, appeared to be dead and buried after the work of Adair. Recent work, however, has shown that hemoglobin can dissociate in concentrated solutions of certain salts, or under other conditions, according to the equation:

$$Hb_4 \rightleftharpoons 2Hb_2$$

The evidence is now unequivocal that this dissociation proceeds much more readily if the hemoglobin is oxygenated than if it is in the deoxy form. The latter, indeed, hardly dissociates at all, and therefore has a stronger tendency to aggregate than the oxy form. This is exactly what Haldane's theory predicted, although we now think not in terms of the aggregation of Hb (or HbO_2) into larger units, but in terms of the dissociation of Hb_4 (or Hb_4 (O_8)) into subunits. Moreover, under physiological circumstances the dissociation is usually so small as to be negligible, so the phenomenon lacks the physiological significance that Haldane (and Hill) ascribed to it.

The interactions of hemoglobin with oxygen and CO_2 are only a part of a larger story. These interactions are accompanied by the flow of water, and of bicarbonate and chloride ions, between the red cells and the plasma, all these events being mutually interdependent. Part II of this study will explore these developments, in which L. J. Henderson and D. D. Van Slyke were the central figures. Closely related were the pioneer studies in the kinetics of oxygen uptake and release by Hartridge and Roughton, and the previously unsuspected role of the enzyme carbonic anhydrase in the kinetics of CO_2 uptake and release, as established by Meldrum and Roughton. Finally, the discovery of the role of carbamate formation by hemoglobin, for the transport of CO_2 in blood, enlarged the picture of the role of hemoglobin in serving the needs of the organism, and explained some previously baffling anomalies. Taken together they provide a remarkably coherent picture of chemical adaptation to biological function—a picture that was essentially complete, within its limits, by 1935.

The great further advances of recent years in our knowledge

127. Wyman, "Heme Proteins," especially the two latter references in n. 126.

128. See also n. 91.

of the structure and function of the hemoglobin molecule will provide a magnificent subject for the future historian, but they lie for the present, outside our scope.[129]

Acknowledgements

I am indebted to the late Professor F. J. W. Roughton and to Jeffries Wyman, and John Parascandola for helpful comments on an earlier draft of this paper; and particularly to Thomas F. Ziobrowski, who not only assembled and photocopied many of the references cited here but also made thoughtful notes and comments on the material he collected. These have proved of great value.

Most of the research for this paper was done at the National Library of Medicine while I was a Scholar of the Fogarty International Center of the National Institutes of Health, Bethesda, Maryland, I express my appreciation for the helpfulness of the staff, both at the National Library and at the Fogarty Center. I am indebted to the National Science Foundation for a research grant (GS2723X) for studies in the history of biochemistry.

129. *Note added in proof.* I must call attention to the recent paper of Charles A. Culotta "Respiration and the Lavoisier Tradition: Theory and Modification, 1777–1850" *Transactions of the American Philosophical Society, New Series* 62, *pt.* 3 (1972), 41 pp. This provides important information, which I have not seen elsewhere, concerning this early period, including a detailed critical discussion of the work of Gustav Magnus in relation to his contemporaries.

The fundamental paper by M. F. Perutz: "Stereochemistry of Cooperative Effects in Hemoglobin." *Nature* 228 (1970), 726–738, based on his x-ray diffraction studies from 1937 to the present, appears now to offer a satisfactory basis for interpreting cooperative interactions and the Bohr effect in hemoglobin. Several subsequent papers from Perutz's laboratory, on mutant or chemically modified forms of hemoglobin, provide data in general accord with his proposals of 1970.