# MODERN CONCEPTS IN HEMATOLOGY

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Symposia of the International Committee for Standardization in Hematology

*Edited by*

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# PREFACE

Standardization and quality control have been rather slow in penetrating medical science. The recognition of the hazards to patient care, brought about by the inaccuracy of laboratory procedures, and the need to ensure reliable comparison of data between laboratories have, however, made the introduction of well defined standard references and procedures into laboratory medicine of primary importance.

The establishment of the International Committee for Standardization in Hematology (ICSH) was a major step forward. The Committee, after having selected items for standardization, brings together eminent scientists to form expert panels for scrutinizing the chosen problems. Once conclusions have been reached, the panels offer recommendations for methods and standard preparations. These recommendations are published, critically evaluated by the laboratories involved and, after further review by the panel, offered for international adoption.

The work presented in this book describes the results of the efforts of four expert panels on as many different subjects. These are linked by a common concern for the needs for quality control and standardization in hematology. It is hoped that further volumes will be published containing the results of the work of other panels, and that these will be put at the disposal of both clinicians and investigators.

This work was presented at four symposia at the XIII Congress of the International Society of Haematology. The sections were initially edited by the individual chairmen of the scientific sessions at which they were presented. Dr. O. W. van Assendelft (Groningen, The Netherlands) carried out this work for Part One; Drs. M. E. Conrad (Washington, D. C.), J. Fielding (London) and W. N. M. Ramsay (Edinburgh) for Part Two, Dr. S. M. Lewis (London) for Part Three and Dr. R. J. Eilers (Kansas City, Kansas) for Part Four.

Throughout the book we have used the international standard nomenclature for expression of units, but in view of the uncertainty prevailing *at present* we have not extended it to the use of molar concentrations for hemoglobin and related parameters.

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Although we have accepted editorial responsibility for the publication of this book, it stems essentially from the activities of ICSH, and we are grateful for the support and cooperation of our colleagues of the ICSH Secretariat, G. Astaldi, Ch. G. de Boroviczeny, J. Coster, R. J. Eilers and J. Spaander (Chairman of the Secretariat and President of ICSH).

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# PART ONE

# HEMOGLOBIN STRUCTURE AND MEASUREMENT

# INTRODUCTION

The standardization of hemoglobinometry began with the work of Sahli and Haldane during the first years of this century and was followed by that of Heilmeyer and King and their co-workers in the 1930s and 1940s. More recent developments stem from a proposal made in 1958 by the United States National Academy of Sciences-National Research Council (NAS-NRC), to establish a certified hemoglobin standard and to recommend that hemoglobin should be measured as hemiglobincyanide (HiCN) (1).

By 1962 further investigation had indicated that there were some discrepancies in the original specification for the standard. Several investigators, notably Zijlstra and van Kampen, had performed accurate optical density measurements and iron analysis on purified hemoglobin solutions, by means of which they had determined the extinction coefficient with greater precision, while Braunitzer and co-workers had been able to calculate that the exact value for the molecular weight of $\alpha_2\beta_2$ hemoglobin was 64,458.

A standardizing committee was established by the European Society of Haematology in 1963 in order to study the problems of hemoglobinometry and other aspects of hematological standardization. One of its first recommendations was that the newer values of 11.0 for $\frac{1}{4}\varepsilon$ and 64,458 for the molecular weight of hemoglobin should be used for the establishment of a HiCN reference preparation (2). In 1964 when the committee was expanded to form the International Committee for Standardization in Hematology (ICSH), an international expert panel on hemoglobinometry was established. Its recommendations were studied and reviewed by the national hematology societies which form the ICSH, by NAS-NRC and by other national and international organizations. As a result of these deliberations NAS-NRC agreed to revise their specification, as did the International Union of Pure and Applied Chemistry (IUPAC). In 1966 these recommendations were unanimously adopted by the ICSH Assembly and published under the title "Recommendations for hemoglobinometry in human blood" (3,4). These recommendations led to the establishment by ICSH of an international HiCN reference preparation, to be manufactured by the Netherlands National Institute of Public Health (RIV) and to be controlled by a group of inter-

national laboratories appointed by ICSH. In 1968 the World Health Organization gave the name "the WHO international HiCN reference preparation" (5) to the solution. The reference preparation is now widely distributed in most countries and the ICSH recommendations on hemoglobinometry have been universally adopted.

Control of the reference preparation remains a cornerstone of the work of the Expert Panel. The Panel is, however, aware of the need for continuing research and of the fact that the standard and methods of hemoglobinometry must be critically reevaluated continually, and if necessary, amended or replaced as new techniques and further research bring new data to light. It is, thus, fundamental to the work of the Panel that it should have regular symposia for the exchange of information and ideas. The latest symposium on the subject of hemoglobinometry was organized by ICSH in collaboration with the International Society of Haematology at the XIII Congress of the Society in Munich in August 1970. Part One contains the papers which were presented at this symposium.

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# THE MEASUREMENT OF HEMOGLOBIN

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## INTRODUCTION

Hemoglobin is a chromoproteid consisting of four heme groups and two $\alpha$ and two $\beta$ globin chains; it has been the object of intensive studies since the mid-19th century. However, even as late as 1941 Campbell (1) rightly stated, "Although the determination of the amount of hemoglobin is one of the most important of all chemical tests of the blood, yet as a rule it is one which is determined with less care and by methods more inaccurate than those in use for any other constituent of the body."

This paper reviews the advances in hemoglobinometry made during the 1950s and 1960s. A survey of the methods used for the determination of hemoglobin will be given, as well as the standardized method which was accepted in 1966 and the work accomplished by the Expert Panel on Hemoglobinometry of the International Committee for Standardization in Hematology will be briefly reviewed. An extensive review of the literature has not been included as this may be found in the work of, among others, Sunderman (2), Henry (3) and van Assendelft (4).

The (total) hemoglobin concentration ($c_t$) may be determined by certain physical properties of blood, such as specific gravity and refractivity, the chemical composition of the hemoglobin molecule, the ability of hemoglobin to unite reversibly with $O_2$ or CO and the spectral characteristics of hemoglobin derivatives.

## MEASUREMENT OF $c_t$ BASED ON PHYSICAL PROPERTIES OF BLOOD

*Measurement of specific gravity.* The specific gravity of whole blood is related to hemoglobin concentration and may be measured by the falling drop technique, in which drops of blood are allowed to fall into a series of fluids of known specific gravity. The specific gravity of the sample corresponds to the specific gravity of the solution in which the drop floats at the center. Errors are said to be small (5).

*Measurement of refractive index.* The refractive index of an aqueous solution of hemoglobin is dependent on $c_t$ (6). The measurement of the refractive index of a sample of hemolyzed blood has been used to calculate $c_t$ after correcting for the refractive index of the plasma.

### MEASUREMENT OF $c_t$ BASED ON THE COMPOSITION OF THE HEMOGLOBIN MOLECULE

*Iron analysis.* Because the non-hemoglobin iron content of whole blood is negligible in comparison with hemoglobin iron, only hemoglobin and its iron-containing derivatives are determined. Although an accuracy of $\pm 2\%$ is often claimed, the methods are too tedious and time-consuming to be used for routine analysis in the clinical laboratory. They can, however, be of value as a reference method to check other, more rapid techniques.

Hemoglobin iron cannot react with iron reagents until it is liberated. This may be accomplished by either dry or wet ashing. Dry ashing involves heating the samples to a high temperature with the danger of losing iron by volatilization and the formation of compounds difficult to dissolve for further analysis. This time-consuming process does, however, assure destruction of all organic matter, leaving a neutral ash. The simpler and more rapid wet ashing techniques involve the addition of concentrated oxidizing agents. The iron content of the resulting solutions is finally determined either titrimetrically or photometrically after making certain that all the iron is in either the ferrous or the ferric state, depending on the reagent used.

During the development of atomic absorption spectrophotometry this technique has, of course, also been applied to the measurement of hemoglobin iron. However, it has been shown (7) that this technique is prone to errors, and both the instrument used and the method of sample preparation greatly influence the results.

### MEASUREMENT OF $c_t$ BASED ON THE ABILITY OF HEMOGLOBIN TO UNITE REVERSIBLY WITH $O_2$ OR CO

*Measurement of $O_2$ capacity.* To determine the oxygen capacity, the blood sample is first saturated with $O_2$, which is then liberated and measured. Knowing that one molecule of hemoglobin contains four atoms of iron and that one atom of hemoglobin iron can bind reversibly to one molecule of $O_2$, $c_t$ can be calculated in (m)mole from the amount of $O_2$ liberated from the sample. Knowing, also, either the Fe content of hemoglobin or its

molecular weight, $c_t$ can be calculated in g. Only hemoglobin actually capable of binding $O_2$, i.e. active hemoglobin, is measured and the amount of $O_2$ liberated from the sample must be corrected for $O_2$ dissolved in blood.

The measurement of $c_t$ by the determination of the $O_2$ capacity is usually performed using the van Slyke manometric method. This method is tedious and difficult and requires highly trained laboratory technicians. Also, there are problems in determining when the blood samples are fully oxygenated and in correcting for the amount of $O_2$ dissolved.

*Measurement of CO capacity.* Determination of $c_t$ by measuring the CO capacity of a blood sample is based on the assumption that the CO and $O_2$ capacity are identical for normal human blood. A blood sample is saturated with CO, the $O_2$ liberated is ejected from the apparatus, CO is then set free from the HbCO through the action of acid $K_3Fe(CN)_6$ and measured manometrically. $c_t$ is calculated after correcting for the CO dissolved in the blood sample. It has been found that, at times, the CO capacity of normal human blood determined after reduction of the sample with $Na_2SO_4$ was greater than prior to reduction. This difference is due to the inactive hemoglobin and is said to be composed of hemiglobin (methemoglobin) (Hi) — major component — hematin, sulfhemoglobin and methemalbumin.

## MEASUREMENT OF $c_t$ BASED ON THE SPECTRAL CHARACTERISTICS OF HEMOGLOBIN DERIVATIVES

*Determination as Hb.* The determination of $c_t$ colorimetrically after reduction of a diluted blood sample with $Na_2SO_4$ was originally based on the observation that deoxygenated hemoglobin, diluted and stored anaerobically, did not show any change of color over a number of years (8). In the Sicca hemoglobinometer (Testa-lab. A/S, Copenhagen, Denmark), developed later, a few drops of blood are collected in a small glass vessel and a powdered reducing and hemolyzing reagent is added. The sample is stirred and then allowed to flow into a shallow wedge-shaped chamber. The color of the reduced hemolyzed sample is finally compared visually to a colored glass reference. Even in the hands of well trained technicians this instrument has been found to give errors in $c_t$ of up to 10% (9) when compared with the results obtained by iron analysis and by a hemiglobincyanide method.

*Determination as $HbO_2$.* The simplest technique ever devised for the determination of $c_t$ is undoubtedly that described by Tallqvist in 1900