Cellular and Molecular Biology of Erythrocytes

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CELLULAR AND MOLECULAR BIOLOGY OF ERYTHROCYTES

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PREFACE

The red cell has been an object of research since the beginning of modern science. There has been no waning of interest in this simple biological system ; on the contrary, the red cell continues to be in the focus of multidisciplinary research on a steadily expanding scale. In the study of this system the interests of the molecular biologists who are concerned with differentiation and maturation, protein synthesis or membrane processes with those of the clinical hematologists, pathologists and specialists in blood preservation meet. Corresponding to the diversity of disciplines, research results are published in a variety of journals with different groups of readers. Thus pertinent information is widely scattered and an accumulation of it is not easily available to any single group or individual. This circumstance, combined with the rapidity of progress, constitute the main reasons for the recurring pressing need for authoritative reviews by leaders in the field, in which scientific advances are summarized and digested for the benefit of the scientific community as a whole. While there have been in the last years a multitude of international symposia on various aspects of the red cell, no comprehensive collection of reviews has appeared in which the molecular and cellular biology of the red cell has been the main subject. For this reason the editors have approached a number of international experts with the request to contribute to such an enterprise. The response to the proposal was most gratifying, and those who responded spent much time and effort preparing their contributions. It is sincerely hoped that the monograph will fulfill its purpose to present an up-to-date account of the most significant areas of research on red cells, and will find wide readership.

From the beginning to the end of the preparation of the monograph the editors have been helped greatly by their colleagues Makoto Nakao and Shigeki Minakami, whom they wish to express their sincere thanks. They are also very grateful to the publishers, the University of Tokyo Press, for their understanding and patience and their willingness to permit additions and alterations even at a very late stage of publication in order to make the monograph as useful as possible.

The fact that this monograph appears in Japan and includes a large share of contributions by Japanese researchers reflects the im-

portance of the work of Prof. H. Yoshikawa, his pupils and his colleagues. It is a fitting tribute to the life's work of a dedicated resourceful and highly original scientist of great modesty and generosity.

January 1974

S. M. Rapoport

ABBREVIATIONS

Enzymes

HK	hexokinase
PHI	hexosephosphate isomerase
PFK	phosphofructokinase
TIM	triosephosphate isomerase
GAPD	glyceraldehyde-3-phosphate dehydrogenase
PGK	phosphoglycerate kinase
PGM	phosphoglyceromutase
PK	pyruvate kinase
LDH	lactate dehydrogenase
DPGM	diphosphoglycerate mutase
2,3-DPGase	2,3-diphosphoglyceratephosphatase
G-6-PD	glucose-6-phosphate dehydrogenase
TA	transaldolase
TK	transketolase
PGI	phosphoglucoisomerase
RNase	ribonuclease
DNase	deoxyribonuclease
GK	glucokinase
PPase	pyrophosphatase
ATPase	adenosinetriphosphatase
AK	adenylate kinase
SDH	succinade hydrogenase
ICDH	isocitrate dehydrogenase

Substrates and Others

DHAP	dihydroxyacetone phosphate
GAP	glyceraldehyde-3-phosphate
1,3-DPG	1,3-diphosphoglycerate
2,3-DPG	2,3-diphosphoglycerate
2-PG	2-phosphoglycerate
3-PG	3-phosphoglycerate
PEP	phosphoenolpyruvate
Pi	inorganic phosphate
ATP	adenosine triphosphate
ADP	adenosine diphosphate
GlcNAc	N-acetylglucosamine
NANA	N-acetylneuraminic acid
NGNA	N-glycolylneuraminic acid
CMH	ceramide monohexoside
CDH	ceramide dihexoside
CTH	ceramide trihexoside
F antigen	Forssman antigen
GNT	globo-N-tetraose
UTP	uridine triphosphate
XTP	xanthosine triphosphate
CTP	cytosine triphosphate
PRPP	phosphoribosyl pyrophosphate
GTP	guanosine triphosphate
IHP	inositol hexaphosphate
2,4-DNP	2,4-dinitrophenol
GSH	reduced glutathione
GSSG	oxidized glutathione
3-AT	3-amino-1,2,4-triazole
ALA	aminolevulinic acid
dsRNA	double-stranded ribonucleic acid

DCIP	2,6-dichlorophenol-indophenol
ACD	acid-citrate-dextrose solution
CPD	citrate-phosphate-dextrose solution
mRNA	messenger ribonucleic acid
tRNA	transfer ribonucleic acid
DOPA	dihydroxyphenylalanine
FAD	flavin adenine dinucleotide
FH_4	tetrahydrofolic acid
IU	international unit(s)
HADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
PCMB	*p*-mercuribenzoic acid
deoxy-Hb	deoxyhemoglobin
Met-Hb	methemoglobin
DNA	deoxyribonucleic acid
cyt c	cytochrome c
Co A	coenzyme A
OPP	oxidative pentose phosphate pathway
EM	Embden-Meyerhof pathway
REF	renal erythropoietic factor
EDTA	ethylenediaminetetraacetic acid

CONTENTS

CELLULAR AND MOLECULAR BIOLOGY OF ERYTHROCYTES

Introduction

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INTRODUCTION

While blood has been regarded as the seat of "vital spirit" since ancient times, there were no serious scientific inquiries on circulation and on the red cell until the sixteenth century. The publication of William Harvey's monograph, "Motion of the Heart and Blood" in 1628 was an epoch-making event in the progress of medical knowledge. In 1658, one year after Harvey's death, Jan Swammerdam noted an immense number of "ruddy globules" in the blood of frogs. Shortly thereafter, in 1665, Malpighi, the discoverer of capillary circulation, also observed corpuscles "looking like a rosary of red coral" in a blood vessel of a hedgehog, but he mistook them for fat globules.

It was in 1674 that the first accurate description of the erythrocyte was given by Leeuwenhoek. He showed that the corpuscles were circular in mammals, but oval in birds and fishes, and that the red color of the blood was due to the corpuscles and not to the fluid. He established the size of erythrocytes at about 8 microns in diameter by comparing them with the known size of a grain of sand. A century later, William Hewson showed that these red particles were not globules, as they were called, but biconcave discs, and that if they were put in water, they swelled and ruptured, whereas they shrank in hypertonic saline.

The recognition of the principal physiological function of the red cell, the transport of oxygen and carbon dioxide, had to wait for nearly two centuries after the discovery of the blood circulation. In 1777 Antoine Laurant Lavoisier identified respiration with the process of oxidation, and showed that venous blood became bright by absorbing "Oxygine", as he called it, from the air and that bright blood turned dark on absorbing "Aeriform calcic acid" which was carbon dioxide.

It was Menghini who first showed the presence of iron in the ash of red blood cells in 1747. Justus von Liebig proposed in 1852 the idea

that red cells contained a compound of iron which could combine with oxygen or carbon dioxide in a reversible process, but he did not clarify the real nature of the iron compound. Hemoglobin was isolated in a crystalline form from the blood of guinea pig by Reichert in 1849, and Hoppe-Seyler demonstrated the unique property of this pigment in absorbing and discharging oxygen in 1862. It was then that the functional significance of hemoglobin and of the red cells in which it is carried became clear.

Another problem was the origin of the red cells. The question remained unresolved until 1868, when Ernst Neumann demonstrated that mammalian red corpuscles were derived, throughout life, from colorless nucleated cells in the bone marrow. With the introduction of the aniline dyes by Ehrlich, morphological studies of the blood and bone marrow received much attention and remarkable progress in the understanding of hematopoiesis was attained. On morphological grounds, a number of stages in the development of the erythrocyte have been identified. The stem cells give rise to proerythroblasts which divide to form basophilic erythroblasts. Successive cell divisions yield polychromatophilic erythroblasts and orthochromatophilic erythroblasts, which mature into normoblasts and reticulocytes without further cell division. The reticulocyte has been so named because these cells can be stained with supravital dyes such as brilliant cresyl blue, revealing a reticulum. Final maturation takes place both in the bone marrow and the peripheral circulation.

Recent studies on the structure and metabolism of the red blood cell reflect the development of cellular biochemistry in many directions, such as the molecular structure and function of proteins, the biosynthesis of cell constituents and its general control, the mechanism of metabolic regulation, the active transport of cations across cell membranes, and the process of differentiation and aging in individual cells.

Hemoglobin, as readily available protein, has been the subject of intensive investigations. It is widely distributed in nature and possesses heme as the prosthetic group, which is attached to the colorless basic protein, globin. Hemoglobins of different species differ in their physical, chemical and biological properties. Further, different hemoglobins have been found within a single species and even in the same individual. Thus, human hemoglobin formed in fetal red cells differs from that formed after birth as regards the oxygen-dissociation curves,[1] the resistance to denaturation by alkali,[2] and electrophoretic mobility.[3] It is now established that all these differences are entirely due to the

different primary structures of the globin moieties since the heme structure is the same.

Teichmann, in 1853, was the first to observe the formation of hemin crystals, known as Teichmann's crystals, by reacting blood with glacial acetic acid and sodium chloride. The task of elucidating the chemical structure of hemin, which is the chloride of the prosthetic group, and related porphyrins engaged some of the most brilliant minds in organic chemistry: Willstätter, Nenck, Piloty and Küster. Finally in 1929, Hans Fischer[4] achieved the triumph of synthesizing hemin identical in every respect with that obtained from hemoglobin.

Information about the molecular structure of hemoglobin remained scanty until the advent of modern techniques of protein chemistry. It is known that the smallest possible molecular weight of hemoglobin, which contains 1 gram atom of iron, is around 17,000. In 1925, Adair[5] estimated the molecular weight to be about 700,000 by measurements of the osmotic pressure, and later, in 1926, Svedberg[6] obtained the same figure by employing an ultracentrifuge devised by himself. This corresponds to the tetramer.

Further investigations have shown that all mammalian hemoglobins consist of 4 polypeptide chains, usually in pairs. Two kinds of chains in hemoglobin A have been termed α and β, and those in hemoglobin F, α and γ. The complete amino acid sequences of these 3 chains have been established by the concurrent efforts of 3 groups, Braunitzer *et al.*,[7] Konigsberg and Hill,[8] and Schroeder *et al.*[9] The secondary, tertiary and quaternary structures of horse hemoglobin was determined X-ray crystallographically by Perutz and his collaborators.[10] In a hemoglobin molecule, 4 subunits are arranged in a tetrahedral array, the 4 heme groups lying in separate crevices on the surface. Of particular importance is the finding that tertiary structure of the chains of hemoglobin is very similar to that of myoglobin, as demonstrated by Kendrew *et al.*,[11] and that oxygenation of hemoglobin results in a decrease in the distance between the β chains.[12]

In 1910 Herrik discovered sickle-cell anemia in a Negro, and suggested that the abnormality in shape might be related to some unrecognized change in the corpuscle itself. In 1949, Pauling, Itano, Singer and Wells[13] showed that the red cells from sickle-cell anemia contained an unusual form of hemoglobin. This discovery introduced into medicine the concept of "molecular disease", which implies that disease may result from a genetically determined fault in protein synthesis. They found that this form was different from normal hemoglo-

bin not only in its lower solubility of the reduced form but also in its reduced electrophoretic mobility. Later, in 1956, Ingram[14] demonstrated that in sickle-cell hemoglobin, the glutamic acid residue at position 6 in the normal β chain is replaced by a valine residue. To date, a large number of abnormal haemoglobins have been discovered, and characterized by abnormalities in the amino acid sequence of one of the polypeptide chains. The occurrence of such a change in the protein is explained as due to a mutation in the gene that controls the synthesis of the polypeptide chains.

Much investigative work on hemoglobin biosynthesis has employed the reticulocyte, largely because this denucleated cell, which can be obtained in high concentrations in the peripheral blood, still possesses the mitochondria, endoplasmic reticulum and ribosomes required for the biosynthesis. The nucleated nonmammalian erythrocyte is also suitable for this purpose, as indicated by its active metabolic processes.

Application of isotope tracer techniques demonstrated that the complicated protoporphyrin molecule is synthesized from 2 simple precursors, glycine and acetate. Deuterioacetic acid was fed to rats, and the isolated hemin was found to contain excess deuterium (Bloch and Rittenberg, 1945).[15] The next step was the demonstration that the feeding of glycine labeled with ^{15}N resulted in the presence of excess isotope in hemin.[16] Most of the later experiments have been carried out by means of *in vitro* techniques, using nucleated red cells of the duck[17] as well as mammalian reticulocytes.[18] The pathway of heme synthesis has been elucidated by the work of Shemin, Rittenberg, London, Granick, Neuberger, Rimington and others, as outlined below.[19] Acetate is utilized as succinyl coenzyme A, which combines with glycine to form δ-aminolevulinic acid. Two molecules of δ-aminolevulinic acid condense under the influence of a specific enzyme to yield porphobilinogen, identical with the chromogen excreted in acute porphyria. Four molecules of porphobilinogen undergo enzymatic condensation to form uroporphyrinogen, type III plus some type I. Uroporphyrinogen III is converted to coproporphyrinogen by decarboxylation, and this is oxidized and decarboxylated to yield protoporphyrin IX. Mitochondria are required for the formation of succinyl coenzyme A via the tricarboxylic acid cycle. δ-Aminolevulinic acid synthetase and coproporphyrinogen III oxidase are bound to mitochondria, while the enzymes responsible for the pathway from δ-aminolevulinic acid to coproporphyrinogen are contained in the cytosol.

The final step in heme synthesis involves the incorporation of ferrous iron into protoporphyrin by heme synthetase or ferrochelatase bound to mitochondria. This reaction was investigated initially by studying the incorporation of radioactive iron *in vitro* into mammalian reticulocytes[20] and avian erythrocytes.[21] The enzyme was solubilized by treating cellular particles with surface-active agents,[22,23] and the properties of the partially purified enzyme were investigated.[24,25]

The formation of peptide bonds in red-cell protein *in vitro* was first observed by Shemin and co-workers in 1950[26] with intact duck erythrocytes. Subsequent studies on the biosynthesis of globin have been conducted effectively with mammalian reticulocytes. Incorporation of radioactive amino acids into the protein was followed both in the intact cell[27] and in cell-free systems.[28] Schweet and his associates[29] and Dintzis[30] demonstrated that the formation of peptide chains starts at the N-terminal amino acid, valine, and continues by steady sequential addition of amino acids until elongation of the chain terminates at the C-terminal end. Electron microscopic observations of reticulocyte microsomes indicated that protein synthesis occurs in aggregations of ribosomes, the so-called polysomes, which are strung along a messenger RNA strand.[31]

Newly synthesized heme is accepted by globin, apparently by a nonenzymatic process, to yield hemoglobin.[32] Earlier studies on the synthesis of heme and globin have indicated a parallelism in the rates of the 2 synthetic processes.[27,33,34] London and his associates[35] reported that on the addition of hemin to rabbit reticulocytes *in vitro*, there was marked inhibition of heme synthesis at the step of δ-aminolevulinic acid synthetase, together with stimulation of incorporation of valine into newly formed hemoglobin.[36] The claim by Schwartz and others[37] that the insertion of iron into protoporphyrin was enhanced in the presence of globin could not be confirmed by subsequent investigations.[32] Details of the mechanism by which the syntheses of heme and of globin are normally coordinated must await further investigation.

The reticulocyte possesses functioning glycolytic and hexose monophosphate shunt pathways, tricarboxylic acid cycle, and an intact electron transfer and oxidative phosphorylation systems. Besides the biosynthesis of hemoglobin, the reticulocyte also has the capability to carry out *de novo* synthesis of purine nucleotides from low molecular precursors to some extent.[38]

In contrast to the reticulocyte, the mature mammalian erythrocyte

lacks mitochondria and ribosomes. Hence the electron transfer and oxidative phosphorylation systems are absent, the tricarboxylic cycle is not functioning although some of its enzymes are present, and synthesis of hemoglobin does not occur. It was natural that, for many years, the nonnucleated mature erythrocyte was thought to be an inert membranaceous bag containing a concentrated solution of hemoglobin, and was therefore called a red corpuscle. But by now it is clear that the erythrocyte is metabolically quite active: in fact, pure human red cells utilize about 2 μmoles glucose per ml per hour, producing nearly an equivalent amount of lactic acid.

The gradual disappearance of sugar from the blood *in vitro* was first demonstrated by Bernard in 1876. This phenomenon was later extensively investigated, and Evans[39] showed that the breakdown of glucose was paralleled by the formation of lactic acid. It occurred only in the presence of blood cells, and especially of white blood cells, and some investigators believed that the glycolytic activity of normal blood resided predominantly, or even exclusively, in leucocytes. However, quantitative studies[40] revealed that the glycolysis of blood was attributable mostly to the red blood cells. By 1930, acid-soluble phosphorus compounds such as hexose phosphate, adenosine triphosphate (ATP), and 2,3-diphosphoglycerate (2,3-DPG) had been found in whole blood, and the significance of these compounds in the glycolytic process was recognized. The existence of hexose phosphate was reported by Lawaczek[41] and by Goodwin and Robison.[42] "Adenylic acid" separated from whole human blood by Jackson[43] appears to be identical with ATP which was isolated from muscle by Embden and Zimmermann[44] and given the correct chemical formula by Lohmann[45] later. 2,3-DPG was discovered in red blood cells by Greenwald[46] in large amounts. The importance of phosphorylation in blood glycolysis was suggested by Rona and Iwasaki[47] and hexose phosphate was regarded as an intermediate of glycolysis by Roche and Roche.[48] Subsequent studies yielded evidence that the maintenance of a normal level of phosphoric esters in the blood was a consequence of their continuous synthesis and decomposition during glycolysis.[49,50]

The breakdown of sugars is carried out almost exclusively via the well-known Embden-Meyerhof pathway, which comprises 11 sequential reactions catalyzed by specific enzymes. For each molecule of glucose metabolized, 2 molecules of ATP are consumed and 4 molecules of ATP are produced, thus generating 2 molecules of ATP per molecule of glucose. One function of the glycolytic system of the erythrocyte is to

provide an easily available supply of energy in the form of ATP. ATP is decomposed to adenosine diphosphate (ADP) and inorganic phosphate by adenosinetriphosphatase. The energy released by this hydrolysing process is utilized for the active transport of cations across the erythrocyte membrane. It has been demonstrated that ATP is also essential for the maintenance of the normal biconcave discoidal shape of the cell. While in the circulation, the energy-rich terminal phosphate groups of ATP undergo rapid turnover and each of the glycolytic intermediates, as well as the nucleotides, is in a dynamic equilibrium, generally kept at a constant level under continuous supply of glucose and removal of lactic acid.

In addition to ATP formation, glycolysis plays an important role in preventing hemoglobin from autoxidation to methemoglobin. The existence of an enzyme which catalyses the reduction of methemoglobin by NADH in human red blood cells was suggested by Gibson.[51]

The hexose monophosphate shunt, or pentose phosphate cycle, has been detected in erythrocytes.[52] This pathway diverges from glycolysis at the level of glucose-6-phosphate and produces pentose phosphates after dehydrogenation and decarboxylation.[53,54] The pentose phosphates are finally converted to fructose-6-phosphate and glyceraldehyde phosphate, and so return to the glycolytic pathway. The metabolic flow through this pathway is estimated at only 10% of the total glucose metabolism.[55] When inosine is added to erythrocytes, a nucleoside phosphorylase splits the nucleoside to give hypoxanthine and ribose-1-phosphate and the latter is converted to ribose-5-phosphate and disposed of by the same cycle.[56] More recently, it has been demonstrated that there is another pathway which metabolizes xylitol via xylulose and its phosphate, leading to the pentose phosphate cycle.[57] In a normal state, however, this enzyme system seems to be dormant because of a lack of substrate in the erythrocyte.

Characteristic of the human erythrocyte is the existence of a bypass, called the Rapoport-Luebering cycle, via a large pool of 2,3-DPG. 1,3-DPG, which is produced by a phosphorylating dehydrogenase from triose phosphate, is converted to a low-energy 2,3-diester by diphosphoglycerate mutase[58] and this in turn is hydrolysed to give 3-phosphoglycerate (3-PG) by a specific phosphatase.[59] 2,3-DPG combines with hemoglobin, causing a decrease in its affinity for oxygen and a displacement of the oxyhemoglobin dissociation curve to the right.[60,61] Thus, its presence in the erythrocyte assists oxyhemoglobin to unload oxygen.