ADVANCES IN PARASITOLOGY

Reflections on a Century of Malaria Biochemistry

67

IRWIN W. SHERMAN

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CHAPTER 1

Introduction

This volume examines the contributions of biochemistry—concepts and techniques—to our understanding of the intimate and integrated interrelationships between malaria parasites and their hosts. It is a status report on investigations carried out over the last 100 years describing the torturous path that has enabled us to know the molecular 'secrets' of *Plasmodium* better. Knowledge of these 'secrets' has helped us to develop better diagnostics and novel chemotherapies, provided a biochemical basis for drug resistance and contributed to a description of the molecular mechanisms of pathogenesis.

Reflections of a Century of Malaria Biochemistry is somewhat autobiographical: I have investigated various aspects of the field for more than half a century and contributed, I hope, to uncovering what it takes to make malaria parasites parasitic, and how with this understanding we may be better able to limit the disease they cause. The coverage is selective, based largely on personal experiences, and favors the work of investigators who are continuing well-respected contributors, as well as those I have known personally. Aside from chronicling biochemical successes (and failures), I want to provide those who have studied malaria biochemistry with an opportunity to speak for themselves, that is, reflections. These short biographical sketches, interspersed within the scientific text, tell of their background, how malaria parasites came to be a source of fascination and enthusiasm for them, and allows for their description of future goals.

It is my hope that this overview of the past century will inspire others to contribute their talents and expertise to achieve conquest of one of the world's most deadly diseases.

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CHAPTER 2

An Introduction to Malaria Parasites

By the middle of the 1800s, Pasteur, Koch and co-workers had provided convincing evidence that microbes can cause disease (the Germ Theory) and Koch had delineated a scheme (called Koch's Postulates) for the unequivocal identification of such disease causing agents, that is, parasites. It was into this environment that the military physician and microbe hunter Charles Alphonse Laveran (1845–1922) entered the picture. Laveran was born in Paris and studied medicine at the Imperial School of the Military Medical Service at Strasbourg and the Military Medical School at Val-de-Grace (Paris). In 1875, as an Associate Professor at Val-de-Grace, he wrote a treatise on military epidemiology (Foster, 1965). Laveran observed that although swamps and low humid plains were the most favorable environments for malaria, he decided that swamps themselves did not cause the fever as even in hot countries not all swamps gave rise to fever (Jarcho, 1984). He concluded, 'Swamp fevers are due to a germ.' After several military assignments in France, Laveran was transferred in 1878 to Bone, Algeria. On 20 October 1880, he examined a drop of blood from a soldier suffering from an intermittent fever. Under the light microscope, Laveran noticed some crescent-shaped bodies among the red blood cells that were almost entirely transparent, save for some pigmented inclusions. On 6 November 1880, while examining a drop of blood from a feverish artilleryman, he saw several transparent mobile filaments emerging from a clear spherical body. He recognized that these bodies were alive, and that he was looking at an animal parasite, not a bacterium or a fungus. Subsequently, he examined blood samples from 192 malaria patients: in 148 of these, he found the tell-tale crescents (Kean, Mott and Russell, 1977). Where there were no crescents, there were no symptoms of malaria. He named the parasite *Oscillaria malariae* and communicated his

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findings to the Société Médicale des Hôpitaux on 24 December 1880. The drawings in his paper provide convincing evidence that, without use of stains or a microscope fitted with an oil immersion lens, Laveran had seen the development of the malaria parasite. At first, Laveran's announcement was received with scepticism. Indeed, in 1882, when he visited Rome and showed his slides to Italian parasitologists they scoffed and told him the spherical bodies were nothing more than degenerating red blood cells (Harrison, 1978; Manson-Bahr, 1963). Initially, the Italians examined only preparations that had been heat-fixed and stained with methylene blue so they did not see any movement of the parasite that had caused Laveran to give it the name *Oscillaria*. However, 2 years later when, like Laveran, they began to examine fresh preparations of blood, they were able to observe the ameboid movements of the parasite within the red blood cell as well as emerging whip-like filaments from the clear spherical bodies within the red blood cell (Bruce-Chwatt, 1988; de Kruif, 1926; Sherman, 1998a; 2005).

In 1886, using thin smears of fresh blood, Camillo Golgi (1843–1926) discovered that the parasite reproduced asexually by multiple fission and showed that fever coincided with red blood cell lysis and parasite release. In 1891, Dimitri Romanowsky prepared heat-fixed thin blood films and used a combination of methylene blue and eosin to stain the nucleus and cytoplasm of the parasite differentially (Kean, 1977). The method was difficult to reproduce since, to obtain this differential staining, the methylene blue solution had to be 'aged' or 'turned mouldy'. As a result, Wright and Leishman made modifications to the Romanowsky method but none was easy to handle or reproduce. In 1899, Bernard Nocht, the chief medical officer of Hamburg harbor in Germany, developed an interest in diagnosing malaria among returning sailors who had acquired the disease in the tropics by staining blood samples. However, he was stymied by the lack of reproducibility of the Romanowsky method. When he became director of the Institute for Maritime and Tropical Disease in Hamburg, he enlisted the help of Gustav Giemsa (1867–1948), a pharmacist with extensive training in chemistry and bacteriology to solve the problem (Fleischer, 2004). Giemsa succeeded in identifying the compound in 'aged' methylene blue as azure B (trimethylthionine) and found that a stable stock solution with reproducible staining properties could be obtained by mixing eosin with azure B in a glycerol-methanol mixture (Barcia, 2007). Now using clinical patterns supplemented by the staining of blood films the four types of human malaria (*Plasmodium malariae*, *P. falciparum*, *P. vivax* and *P. ovale*) could be clearly distinguished from one another. But, it was only after 'the staining act was recognized to be of a chemical nature' that systematic studies of the biochemical properties of malaria parasites began (Wilson, 1907).

The significance of Laveran's observation of the release of motile filaments went unappreciated until 1896–1897 when William MacCallum (1874–1944) and Eugene Opie (1873–1971), medical students at Johns Hopkins University (Baltimore, Maryland), found that the blood of sparrows and crows infected with *Haemoproteus* (a bird parasite closely related to malaria) contained two kinds of sex cells, crescent-shaped gametocytes, and that filament extrusion (called exflagellation) reflected the release of microgametes from the male gametocyte (MacCallum, 1898; Opie, 1898). They also correctly interpreted their observations: gametocytes occur in the blood and when ingested by a biting fly, the gametes are released in the insect's stomach, where fertilization takes place, producing a worm-like zygote, the ookinete.

Neither Laveran nor MacCallum solved the problem of malaria transmission. It was Ronald Ross, a surgeon-major in the Indian Medical Service, who showed how 'bad air' could cause malaria (Harrison, 1978; Ross, 1923). After many years of frustrating efforts, Ross decided to see whether mosquitoes could become infected with malaria. Ross fed them on a patient who had crescents in his blood and then dissected the mosquitoes. Four or five days after feeding on infected blood, the mosquito had wart-like oocysts on its stomach. Ross reported his findings to the *British Medical Journal* in a paper entitled, *On some peculiar pigmented cells found in two mosquitoes fed on malarial blood*. It appeared in print on 18 December 1897. But before he could complete this work on human malaria, Ross was posted to Calcutta. However, there was not a large number of malaria cases in the Calcutta hospitals, so he turned to something that his mentor, Patrick Manson, had suggested earlier: the study of mosquitoes and malaria in birds. Soon Ross found that crows and pigeons had malaria parasites in their blood; he also observed malaria-pigmented cells in the stomachs of mosquitoes that had fed on infected birds. The parasites grew to their maximum size within about six days. Then, he noticed that some of the oocysts seemed to have stripes or ridges in them; this happened on the seventh or eighth day after the mosquito had been fed on infected blood. On 4 July 1898, near the mosquito's head, Ross found a large branching salivary gland. He suspected that this salivary gland might pour parasites into the blood of a healthy creature during mosquito feeding. During 21 and 22 July of that year, he took some uninfected sparrows, allowed malaria-infected mosquitoes to bite them and was able to show that the healthy sparrows became infected in a few days. This was the proof that showed malaria was transmitted by the bite of an infected female mosquito and not 'bad air'. Ross communicated his findings on 28 July 1898.

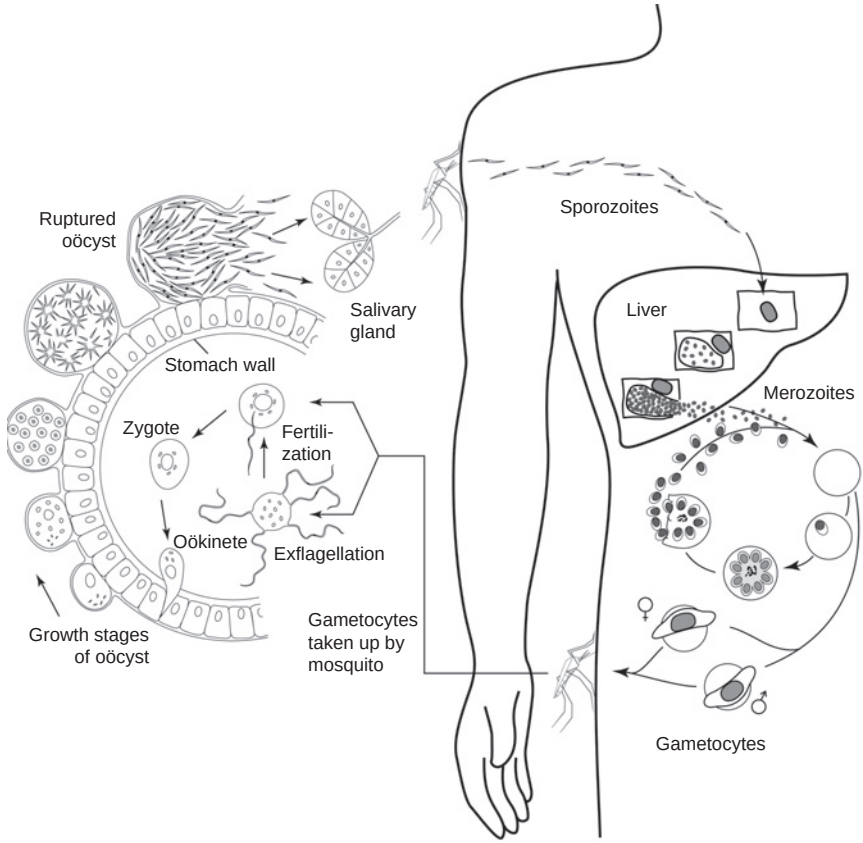
Ross wanted the glory of discovery for himself and for England, but he was not alone in his quest (de Kruif, 1926; Harrison, 1978; Sherman, 1998a). The German government had dispatched a team of scientists under the leadership of Robert Koch to work in the Roman Campagna,

an area notorious for endemic malaria. They isolated a bacillus from the air and mud of the marshes, and, rejecting the claims of Laveran, named the causative agent for malaria, *Bacillus malariae*. However, when the bacillus could not be grown in the laboratory, Koch discarded it as the cause of the disease; undeterred he continued to look further. Koch then visited the laboratory of Professor Giovanni Batistta Grassi at the University of Rome, Italy and told him of his failure with the 'germ' of malaria and mentioned Ross's communication. At that moment Grassi had what is today called an 'ah-ha' moment.

Where Ross was patient, perseverant and willing to carry out a seemingly endless series of trial-and-error experiments, Grassi was methodical and analytical—he was also able to distinguish the different kinds of mosquitoes. Grassi observed 'there was not a single place where there is malaria—where there aren't mosquitoes too, and either malaria is carried by one particular blood-sucking mosquito out of the forty different kinds of mosquitoes in Italy—or it isn't carried by mosquitoes at all' (de Kruif, 1926). He recognized there were still two tasks left: identify the specific kind of mosquito that transmitted human malaria and then demonstrate the mosquito cycle for human malaria. Working with Amico Bignami, Giovanni Bastianelli, Angelo Celli and Antonio Dionisi he went into the highly malarious Roman Campagna, and the area surrounding it, collecting mosquitoes, and at the same time recording information on the incidence of malaria among the people (Sherman, 2005; Shortt *et al*., 1951). It soon became apparent that most kinds of mosquitoes could be eliminated as carriers of the disease because they occurred where there was no malaria. But, there was an exception. Where there were '*zanzarone*', as the Italians called the large brown spotted-winged mosquitoes, there was always malaria. Grassi recognized that the *zanzarone* were *Anopheles* and he wrote, 'It is the *Anopheles* mosquito that carries malaria . . .' Grassi and his team were able to infect clean *Anopheles* mosquitoes by having them feed on patients with crescents in their blood and he was able to trace the development of the parasite from the mosquito stomach to the salivary glands. The life cycle in the human was, as Ross had correctly surmised, similar to that of the bird malaria with which he had worked.

Although malaria can be induced in a host by the introduction of parasites (called sporozoites) through the bite of a suitable infectious female mosquito, the parasites do not immediately appear in the blood. In 1948, Shortt, Garnham and their colleagues in England inoculated rhesus monkeys with a syringe of sporozoites that they had obtained from the salivary glands of mosquitoes infected with *P. cynomolgi* (a parasite similar to the benign tertian malaria, *P. vivax* of humans) and in 1 week parasites, called exo-erythrocytic (EE) stages, were found in the livers of the monkeys (Shortt and Garnham, 1948). Later, they

FIGURE 2.1 The life cycle of *Plasmodium.* Source: I.W. Sherman.



demonstrated similar stages in biopsy material taken from the livers of human volunteers who had been infected by the bite of mosquitoes carrying *P. vivax* (Shortt, 1948). After infected mosquitoes had fed on other volunteers, this stage was found at the same site in malignant tertian malaria, *P. falciparum* (Shortt *et al.*, 1951) and *P. ovale* (Garnham *et al.*, 1955). It was now clear that when an infected female anopheline mosquito feeds it injects sporozoites that go first to the liver, where they live and multiply for several weeks, and subsequently they move on to the blood stream where they reproduce asexually in the red blood cells or they differentiate into male or female gametocytes (Fig. 2.1).

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CHAPTER 3

The Early Years

The past century has witnessed spectacular advances in our understanding of the biochemistry (and molecular biology) of malaria parasites. This work has had three objectives:

- Establishing the *raison d'être* for obligate parasitism,
- Amelioration of the disease through an understanding of the pathogenic mechanisms,
- Rational design of chemotherapeutic agents.

During World War II as American and Allied forces engaged in battles in the malarious areas of North Africa, Asia and the Pacific, the troop losses due to malaria were sometimes as great as those due to bullets and bombs. Further, with the fall of Java (today Indonesia) to the Japanese the sources of quinine, the only effective anti-malarial, became unavailable and so the military began to use atabrine (also named quinacrine or mepacrine). Atabrine was not actually a product of the wartime effort but had been developed by the Germans in the 1930s and the Allies resurrected the drug for military use. Atabrine, an acridine, was a marginally effective anti-malarial that turned the skin a bright yellow, caused gastroenteritis and most disturbingly occasionally caused temporary insanity. Atebrine was hardly the ideal anti-malarial but there were no other drugs available and it did prevent death among the troops.

It was the search for the mode of action of atabrine (and to a lesser degree quinine) that stimulated the earliest biochemical studies on malaria parasites. The first such study was conducted at the London School of Hygiene and Tropical Medicine (LSHTM, London, United Kingdom) by Christophers and Fulton (1938) using *Plasmodium knowlesi*, discovered in 1932 in a Malayan kra monkey that had been sent to the

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Calcutta School of Tropical Medicine *via* Singapore. Benign in the kra monkey, the infection was virulent in rhesus monkeys available in the United Kingdom.

In 1898, Sir Rickard Christophers (1873–1978) was appointed to study the relationship of quinine therapy to blackwater fever; his long career in malaria continued with Colonel H. E. Shortt in India (1904–1932). Returning to the United Kingdom, Shortt became a reader in medical parasitology and Christophers became a professor of malarial studies at the LSHTM. James D. Fulton (1899–1974) trained in chemistry and medicine (bachelor of medicine (MB), 1934), began his work on anti-protozoan chemotherapy with Warrington Yorke at the Liverpool School of Tropical Medicine (LSTM, Liverpool, United Kingdom) and under the aegis of the Medical Research Council was appointed to the staff of the London School of Tropical Medicine and Hygiene (London, United Kingdom) where Sir Rickard introduced him to the malaria parasite. From 1939 until his retirement in 1963, Fulton worked at the National Institute for Medical Research (NIMR, London, United Kingdom) on chemotherapy, metabolic physiology and the immunology of all the major protozoan diseases of humans.

From 1938 to 1945, Fulton and his associates, using the available analytical tools of Barcroft-Haldane manometry and colorimetry, found: the parasites did not store glycogen, and *P. knowlesi*-infected red cells rapidly depleted the medium of glucose; respiration stimulated by the presence of glucose was inhibited by 0.001-M cyanide. Erythrocyte-'free' parasites prepared by saponin lysis behaved similarly. Surprisingly, uptake of oxygen also occurred with glycerol, fructose and mannose (Fulton, 1939).

During this same period, work on the biochemistry of malaria (*P. gallinaceum, P. lophurae* and *P. knowlesi*) was initiated at several universities in the United States, including Harvard, Johns Hopkins and the University of Chicago (Chicago, Illinois) under contracts with the Board for the Coordination of Malaria Studies. *P. gallinaceum*, described by Emile Brumpt in 1935, naturally infects jungle fowl, but it can be maintained in chickens, and in this host, it was established in laboratories across the globe. *P. lophurae*, isolated in 1938 by L. T. Coggeshall from a fireback pheasant, *Lophura igniti*, living in the New York Zoo, was maintained by blood passage in chicks and ducklings (Coggeshall, 1938b). The studies in the United States with bird and monkey malarias also used the then current techniques of manometry, photometry supplemented by spectrophotometry.

Manometric measures of intact infected red cells, parasites removed from infected cells by saponin or hemolytic serum or cell-free extracts showed that malaria parasites contained cytochromes and increased flavin adenine dinucleotide (FAD) levels (Ball *et al*., 1948). Ball *et al*. (1947)

found that several 2-hydroxy-3-alkylnaphthoquinones, which were active against both *P. lophurae* and *P. knowlesi*, strongly inhibited the respiration of the parasites and suggested that they acted by inhibiting some step in the parasite electron transport system at the level of the cytochromes. However, because the effect of nine other drugs (several being sulphas as well as quinine and atabrine) on the respiration of malaria parasites *in vitro* did not correlate with the *in vivo* responses Coggeshall *et al*. (1941) wisely cautioned, 'inhibition of respiration ... alone should not be depended upon to furnish an index of chemotherapeutic efficiency but should be used as an adjunct to *in vivo* experiments.'

Glucose, very rapidly consumed by parasitized red cells, was found to be indispensable for *in vitro* growth and its breakdown was by classical glycolysis yielding lactic acid in both monkey (*P. knowlesi*) and bird (*P. gallinaceum*) malarias (reviewed by Fulton, 1951; McKee, 1951; Moulder, 1948). However, with *P. gallinaceum*, Speck claimed pyruvate was oxidized to CO_2 and H_2O by infected red cells and free parasites 'by a cycle like that proposed by Krebs' (Speck *et al*., 1946). In addition, free parasites oxidized the Krebs-cycle intermediates succinate, fumarate, oxaolacetate and α-ketoglutarate at rates equal to pyruvate. These findings led Moulder (1948) to conclude, 'Almost every type of enzymic reaction discovered in higher animals has been found in malaria parasites, and the known growth factors required by plasmodia are similar to those needed by many extracellular parasites ... Therefore, we cannot explain why the rapidly metabolizing blood stages ... live and grow only within erythrocytes. These metabolic similarities must be considered in attempting to inhibit selectively the metabolism of malarial parasites with chemotherapeutic agents.'

Moulder's contention of 'metabolic similarities' would eventually be shown to be incorrect; however, examination of the basis for his conclusion allows an appreciation of how concepts and techniques can influence interpretation. In 1944, the United States Office of Scientific Research and Development was created with a Board for the Coordination of Malaria Studies 'to better understand the mode of action of anti-malarials in order to protect United States troops in Southeast Asia, North America and the Pacific.' A member of that Board was W. Mansfield Clark (1884–1964), Professor of Physiological Chemistry (1927–1952) at the Johns Hopkins School of Medicine. His colleague in the department was Leslie Hellerman (1896–1981) whose work focused on metallo-enzyme functions. Hellerman and his student, Marianna Bovarnick (1911–1995), involved themselves in the testing of the biochemical effects of atabrine. Another malaria project was sponsored at Harvard under A. Baird Hastings, chair of the Department of Biological Chemistry, also a member of the Board. Eric Ball (1904–1979) was a graduate student and then a faculty member in Mansfield Clark's department at Johns Hopkins (1930–1939) and spent 1

year (1937–1938) in Berlin with Otto Warburg (1883–1970) where microdetermination of oxygen had been developed using the manometric technique of Barcroft and Haldane. In Berlin, as a colleague of Otto Meyerhof (1884–1951), Ball demonstrated that xanthine oxidase contained flavin as a co-factor, and measured the oxidation–reduction potentials of the cytochromes. Ball was recruited by Hastings to join the Harvard faculty and after arriving at Harvard in the autumn of 1940 became acting head of the department (1943) when Hastings was heavily involved in the Committee on Medical Research. Shortly thereafter, he became secretary to the Panel on the Biochemistry of Anti-malarials and embarked on a project to cultivate the malaria parasite, *P. knowlesi, in vitro* and to study its metabolism. Ball, in turn, recruited to the malaria project another faculty member, Christian Anfinsen (1916–1995), who had received his doctor of philosophy (PhD) under Hastings in 1943. The group was joined by Ralph W. McKee (1912–1992) who had joined the Harvard faculty in 1940 after receiving a PhD in Biochemistry from St. Louis University (St. Louis, Missouri) where he had isolated vitamin K from fish meal, and Quentin M. Geiman (1904–1986), an assistant professor in the Department of Comparative Pathology; Geiman, trained as a parasitologist, received his PhD from the University of Pennsylvania in 1934 working on amebiasis.

At the University of Chicago School of Medicine another malaria research group was formed under the leadership of Earl A. Evans Jr. (1910–1999) who had worked as an undergraduate in Mansfield Clark's department at Johns Hopkins, received his PhD from Columbia University (New York, New York) in Hans Clarke's laboratory, one of the earliest centers for radioisotope techniques, and joined the Chicago faculty in 1937. In 1939, he worked with Hans Krebs (1900–1981) on CO_2 fixation and the newly formulated Krebs tricarboxylic acid (TCA) cycle. James W. Moulder (1921–), who received his PhD in 1946, was a graduate student under Evans, as was Joseph Ceithaml (1906–), who received his PhD in 1941, and John F. Speck. Moulder joined the faculty in the Department of Microbiology at the University of Chicago and remained there until his retirement in 1986. Thus, it was the 'connected' background and training of these biochemists that inspired and influenced their work on the metabolism of malaria parasites (and as might be expected it also affected their interpretations). In short, their findings did not deviate from the views on energy metabolism that had been described for muscle, liver and yeast and which were prevalent in the 1940s and early 1950s.

What did the future hold for these United States workers on malaria biochemistry? When World War II ended so too did the contracts for malaria research. None of these pioneers of malaria biochemistry ever worked with malaria parasites again. Ball remained on the faculty at Harvard working on oxidation–reduction mechanisms under hormonal influence, Anfinsen moved to the National Institutes of Health (NIH,

Bethesda, Maryland) where he worked on protein folding ultimately receiving the Nobel Prize in 1972. Hellerman continued to work on the structure of enzymes and enzymatic processes. Bovarnick moved to the Public Health laboratories in New York City where she studied rickettsial metabolism. Neal Groman (1922–2001), Moulder's graduate student, who received his PhD in 1950 working on nitrogen metabolism of *P. gallinaceum*, subsequently became a faculty member at the University of Washington (Seattle, Washington) (retiring in 1989) and Earl Evans redirected their research to phage studies, and Moulder's subsequent research and that of his graduate students was on rickettsias. Speck studied glutamine synthesis in pigeon liver and died prematurely in a mountaineering accident, and Geiman who came to Stanford in 1955 maintained a research program on pathogenic amoebas until retirement in 1970, but between 1962 and 1967 he once again worked with *P. knowlesi* and was able to adapt two strains of *P. falciparum* (FUP, Falciparum Uganda Palo Alto and FVO, Falciparum Vietnam Oak Noll) to *Aotus* monkeys; McKee left Harvard and joined the University of California, Los Angeles (UCLA, Los Angeles, California) faculty in 1953 where he studied the glycolytic pathway enzymes and their regulation in mouse ascites cells, eventually becoming Dean of Students. Ceithaml remained at Chicago carrying out research on *Escherichia coli* and CO_2 fixation in barley roots; in 1951, he became dean of students and retired in 1986.

The inescapable conclusion to be drawn from this brief historical review is that although the kind of biochemical research on malaria parasites between 1938 and 1955 was restricted to the animal models then in vogue it was principally influenced by the availability and sources of funding.

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CHAPTER 4

Show Me the Money

Funding can be critical to drug design, drug testing, uncovering the mode of action of an anti-malarial, and for the development and deployment of vaccines. Therefore, as evidenced by past experiences, research on malaria parasites—both type and focus—can be affected by a particular funding agency, the level of support and its duration. In 1938, with World War II looming, the United Kingdom Medical Research Council (MRC), an organization dedicated to 'promoting a balanced development of medical and related biological research' and with a prime role to distribute medical research funds, established a Malaria Committee. This committee was composed of MRC members specialized in specific medical areas, as well as representatives from the London School of Hygiene and Tropical Medicine (LSHTM, London, United Kingdom) and from pharmaceutical companies (May and Baker, Imperial Chemical Industries and Glaxo), thus enabling the exchange of information about promising compounds designed to replace quinine, which had become unavailable to the Allies (due to the Japanese occupation of Indonesia). In particular, paludrine and some diaminopyridines, which had been screened and evaluated by Len Goodwin at the Wellcome Research Laboratories (Beckenham, United Kingdom) using the convenient and inexpensive bird malaria, *Plasmodium gallinaceum*, aroused interest in the possibility of their capacity to inhibit metabolic processes in human malarias. Some of these were even sent to biochemists for determination of mode of action. However, after World War II (1945) most of the pharmaceutical companies dropped out of conducting research on anti-malarials. Despite this, the life of the Malaria Committee was extended and became the Tropical Medicine Research Board of the MRC; although the MRC would continue to fund research at thirty-five units nationwide and at three main institutes (Cambridge, Mill Hill and Hammersmith,

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United Kingdom) as well as in The Gambia and Uganda, only a small portion of the budget was allocated to the biochemistry of malaria parasites and the mechanisms of action of anti-malarials.

When the United States entered World War II (1941) and was faced with limited supplies of quinine, there developed a need for a better understanding of the malarial parasite and to discover new anti-malarials in order to protect United States troops in Southeast Asia, North America and the Pacific. The Office of Scientific Research and Development, created a Board for the Coordination of Malaria Studies to develop a large-scale pharmacological research program. Of the thousands of compounds synthesized and screened only a few emerged, the most important of these was chloroquine, a 4-aminoquinoline. However, despite this success, at the end of World War II the Board was dissolved and with it funding for malaria biochemistry disappeared.

The pitfalls in the discovery of chloroquine, the successor to atabrine, have been recounted by Coatney (1963) and even today the story provides enlightenment for us. In the 1930s, the German company I. G. Farben synthesized a compound called resochin that cured malaria; however, when it was found to be too toxic, the I. G. Farben chemists altered it to make it less toxic, producing sontochin. Farben informed its United States counterpart Winthrop Stearns about sontochin's anti-malarial activity and how it could be synthesized, but this was forgotten and for more than a decade, it languished. In 1940, Winthrop gave a sample of sontochin to malaria researchers at the Rockefeller Institute where it was shown to be effective against bird malaria, but because the chairperson of an expert panel (who was not a chemist) misidentified sontochin as a toxic 8-aminoquinoline it was dismissed for further consideration. In 1943, the chemical structure of sontochin, which had been tested by French physicians in North Africa, was determined; and with a slight chemical change, the compound was re-named chloroquine. Ironically, when the structure of resochin was re-examined it was found to be identical to chloroquine and non-toxic. By the 1950s, chloroquine was being hailed as the 'magic bullet' for eradicating malaria and it spread throughout the tropics like a therapeutic ripple.

Experiences during the Korean War (1950–1953) led the United States Armed Forces Epidemiologic Board (AFEB) to establish a permanent Commission of Parasitic Diseases that would 'anticipate or prevent an outbreak of disease that might affect military operations.' By 1957, when the World Health Organization's (WHO, Geneva, Switzerland) Global Eradication Program for Malaria appeared to be failing, a special session of the AFEB on the status of malaria research was convened in Washington, DC. The Commission concluded (http://history.amedd.army.mil/booksdocs/itsfirst50yrs/default.htm; last accessed 16 July 2008) that basic research was needed to close the gaps in our knowledge about vectors, insecticides and the parasite. Funding for such research would

come from the United States Army through the Office of the Surgeon General. During the 1960s, Professor Harry Most (who had worked on the development of chloroquine while in the United States Army during World War II and upon return to civilian life was chairman of the Department of Preventive Medicine at the New York University (NYU, New York) Medical Center served as chair of the AFEB and director of the Commission on Malaria. He initiated a project at NYU on the biology of two rodent malaria parasites, *P. yoelii* and *P. berghei*, and, in subsequent years, the Commission funded several additional malaria projects at other universities. The principal focus at NYU has been, and continues to be, on malaria vaccines (sporozoite and EE stage) and has involved, to name but a few, Meier Yoeli, Jerome Vanderberg, Ruth Nussenzweig, Victor Nussenzweig, Elizabeth Nardin, Fidel Zavala, John Barnwell, Alan Cochrane, Ute Frevert, Photini Sinnis and Mary Galinski.

Because malaria had exacted a heavy toll on United States troops during both World Wars as well as the Korean conflict, the Department of Defense, and in particular the Naval Medical Research Institute (NMRI, Bethesda, Maryland) and the Walter Reed Army Institute of Research (WRAIR, Washington, DC), established and maintained active malaria research programs dedicated to protecting the health of United States military personnel. In 1959, the United States military became involved in a war in Vietnam. As the numbers of American troops in Vietnam increased and more and more soldiers were being killed by chloroquine-resistant malaria, a separate Commission on Malaria was formed (1964). It encouraged further expansion of research, training and the development of new anti-malarials. Thus, after a decade-long hiatus, malaria once again became a priority of the military and monetary investments in malaria research began to increase. Elvio Sadun (1918–1974), chief of Medical Zoology at WRAIR, and also an AFEB Commission Member from 1965 to 1972, recognized the need for basic research on malaria and he helped to establish a program to develop new prophylactic and therapeutic drugs for malaria. In 1965, the funding for the WRAIR malaria drug program amounted to $3.3 million and, in 1967, it peaked at $13 million, with most funding going into extramural research and drug synthesis (Arrow *et al*., 2004). David Jacobus and Craig Canfield led the drug program effort that culminated in the organization of the world's first malaria private-public partnership that involved WRAIR, World Health Organization/Special Program for Research and Training in Tropical Diseases (WHO/TDR, Geneva, Switzerland) and Hoffman LaRoche for the development of Lariam (mefloquine). During this time period, Sadun organized, directed and edited several malaria research symposia (1966, 1969 and 1972). Although funding at WRAIR dropped after the Vietnam conflict ended, by 2004 funding at WRAIR had rebounded to $30 million with 46% for drug development, 38% for vaccine development and 9% for basic research (Arrow *et al*., 2004).

The Office of Naval Research (ONR, Arlington, Virginia) was established in 1946 and during the 1960s when its funds were at a peak had a small extramural malaria grants program. With the advent of the Vietnam War and a decline in funding (that reached its lowest point in 1975), the few extramural grants for malaria research largely disappeared. In 1942, the NMRI was commissioned (http://www.nmrc.navy.mil/history.htm; last accessed 16 July 2008). Since then it has had a strong intramural program, initially under Clay G. Huff (1947–1969), and focused on the biology of the exo-erythrocytic (EE) stages of malaria, first with the avian malarias, *P. gallinaceum, P. lophurae* and *P. fallax*, and later under Richard Beaudoin (1970–1990) with the rodent malarias, *P. berghei* and *P. yoelii* and the human malarias, *P. falciparum* and *P. vivax*. In the early 1970s, in collaboration with the Rush Medical Center in Chicago, NMRI pioneered immunization of humans by the bite of irradiated mosquitoes carrying *P. falciparum* sporozoites, still the gold standard for malaria vaccine development. Subsequently under Stephen Hoffman, NMRI became a leader in the effort to develop subunit malaria vaccines and to sequence the genome of *P. falciparum* with an annual budget reaching $12 million, and including more than 100 staff working at NMRI and overseas Navy laboratories in Jakarta, Lima, Cairo and for several years in western Kenya. In 1998, NMRI was re-commissioned as the Naval Medical Research Center (NMRC, Silver Spring, Maryland). The NMRC has an Infectious Disease Directorate with three research departments and a budget exceeding $10 million per year. One of three departments, Malaria, now under the direction of Thomas Richie, has, as its primary objective, the development of a combined EE- and erythrocytic-stage vaccine using recombinant deoxyribonucleic acid (DNA) techniques, viral vectors and prime-boost approaches. The Malaria department is also developing an attenuated sporozoite vaccine in collaboration with biotechnology partners and pursuing an active antigen discovery program based on genomic and proteomic approaches.

From the mid-1960s onwards the WHO had a small (around 40) grants program ($100,000–$150,000 per annum) with the purpose of encouraging research activities in the fields of parasite biology, chemotherapy and immunology; it was hoped that by recognizing the efforts of individual research groups, more substantial funding would be obtained from other agencies.In 1976, the WHO established TDR, co-sponsored by the United Nations Children's Fund (UNICEF), the United Nations Development Program (UNDP), the World Bank and the WHO with a mission to promote scientific collaboration and to help co-ordinate, support and influence global efforts to combat a portfolio of major diseases of the poor and disadvantaged, including malaria (Arrow *et al*., 2004). Once the TDR was established, the small grants program of WHO was phased out. In 2004, the TDR budget for malaria was $13.4 million with 49%

allocated to drug discovery and development, 32% to implementation research and 8% to basic research.

By the 1970s, the pharmaceutical industry had once again largely abandoned research and development of new drugs for malaria since such drugs offered little potential return on investment. Indeed, between 1975 and 1998 of all the new drugs developed worldwide only six were anti-malarials, that is, fansidar, amodiaquine, mefloquine, atovaquone, artemisinin and halofantrine. Recognizing this need, a number of interested parties from the public and not-for-profit private sectors and the pharmaceutical industry initiated in 1999, after much discussion and debate, the Medicines for Malaria Venture (MMV, Geneva, Switzerland), a public–private partnership designed to combine the expertise of the pharmaceutical industry in drug discovery and development and the public sector's expertise in parasite biology, clinical medicine and field trials. Financial support for the MMV comes principally from philanthropic donors: Bill & Melinda Gates Foundation, Rockefeller Foundation, The Wellcome Trust, the Swiss, United Kingdom and Dutch governments, The World Bank and Exxon Mobil. In 2007, the needs of the MMV are projected to be greater than $45 million.

In addition to WRAIR and the NMRC, three other United States government agencies currently fund malaria research: the United States Agency for International Development (USAID), the Centers for Disease Control and Prevention (CDC) and the National Institute of Allergy and Infectious Diseases (NIAID, Bethesda, Maryland), an institute within the National Institutes of Health (NIH, Bethesda, Maryland). Since its establishment in 1961, USAID has been involved in numerous health projects around the globe. Prior to the 1990s, the bulk of USAID malaria research funding was devoted to malaria vaccine development. During the 1990s, USAID was the leading donor for research that led to insecticide-treated bed nets, artemisinin-based combination therapies and intermittent preventative therapy for pregnant women as well as continuing the Malaria Vaccine Development Program. During 2004–2006, USAID contributed more than $6 million to malaria vaccine development annually and, in 2006, a total of $10 million was allocated for malaria research (including malaria vaccine development), $60 million for ongoing malaria prevention and treatment programs and $30 million for the Presidential Malaria Initiative (http://www.usaid.gov/policy/budget/cbj2007/si/malaria.html; last accessed 16 July 2008) that will provide leadership and means to halve malaria-related mortality in fifteen African countries.

The CDC, created as a new component of the United States Public Health Service in 1946, was a successor to the Office of Malaria Control in War Areas, an agency established in 1942 to limit the impact of malaria and other vector borne diseases in the south-eastern United States (CDC,

1996). By 1951, when malaria was considered eradicated from the United States, the CDC had 400 employees. In 2008, the CDC includes 12 centers, institutes and offices and employs approximately 8500 people who are stationed in all 50 states and 45 countries with a mission to protect and monitor the health of United States citizens. From 1985 to 1989, approximately $6 million was spent annually by the CDC for malaria surveillance and laboratory investigations, including vaccine and immunity research and some of the latter was supported by USAID funding. In 2004, the $1.2 million spent by the CDC for malaria research was broad-ranging and included: studies of host–parasite relationships, immune responses and immunity; host genetic factors; parasite diversity and drug resistance; methods of malaria control; vaccine development and evaluation (using non-human primate models); evaluation of long-lasting bed nets; larval ecology; and the adaptive characteristics and molecular variation in the immune responses of *Anopheles gambiae*, and is a WHO collaborating center for evaluation of new insecticides, identification and typing of insect vectors, production and distribution of sporozoite ELISAs.

With funding appropriated by the United States Congress, the NIH, comprised of 27 institutes and centers, invests more than $27 billion annually in medical research (Oaks *et al*., 1991; Western, 1992). Five NIH entities fund malaria research: the NIAID, the Fogarty International Center, the National Center for Research Resources (NCRR, Bethesda, Maryland), the National Institute of Child Health & Human Development (NICHD, Rockville, Maryland) and the National Heart, Lung, and Blood Institute (NHLBI, Bethesda, Maryland). Malaria investment by these five agencies totaled $87.7 million in 2004 and in 2006 was $98 million, with NIAID accounting for 92% of the funding. The NIAID's mission is to conduct and support 'basic and applied research to better understand, treat, and ultimately prevent infectious, immunological, and allergic diseases.' In 2006, NIAID allocated approximately 25% of its budget for intramural malaria research and the reminder was used to fund more than 150 principal investigators. Vaccine development and vaccine trials received the largest share of total NIAID investment, followed by antimalarial drug discovery and development and basic research (at 36%, 26% and 23% of total NIAID funding, respectively).

The commercial interests of the United Kingdom—much of it in the tropical and sub-tropical regions of the world—provided the driving force for research on malaria and other diseases of humans and domestic cattle. Beginning at the turn of the nineteenth century, malaria research in the United Kingdom was carried out at several academic institutions and one commercial laboratory. In 1899, the Liverpool School of Tropical Medicine (LSTM, Liverpool, United Kingdom) opened with Ronald Ross as its first staff member. After World War I, Warrington Yorke (1883–1943) developed a team (that included Frank Hawking, J. D. Fulton

and James Williamson) at the LSTM to work on malaria chemotherapy and this continued under the direction of Wallace Peters (1966–1979) who was later chair of medical protozoology (1979–1989) at the LSHTM. The LSHTM was formed in 1922 (after the death of Sir Patrick Manson) by an amalgamation of a School of Hygiene with the School of Tropical Medicine that had been founded in London by Manson in 1904. It has had several distinguished malariologists, including: H. E. Shortt (1887–1987), P. C. C. Garnham (1901–1994), R. S. Bray, R. Killick-Kendrick and Allistair Voller. In 1919, Clifford Dobell (1886–1949) resigned his lectureship at Imperial College and was appointed to the post of protistologist to the MRC at the new National Institute for Medical Research (NIMR, London, United Kingdom) where he spent 30 years; shortly before he died, the NIMR was relocated to Mill Hill where research on malaria (and other parasitic diseases) was conducted in the Division of Chemotherapy under Frank Hawking; in 1965, the section was re-named the Division of Parasitology.

In 1902, Frederick Quick, a prosperous coffee merchant, left the bulk of his estate to Cambridge University (Cambridge, United Kingdom) for the study of biochemistry, genetics and parasitology; the first holder of the Quick chair was G. F. Nuttall (1862–1937). In 1920, after he discovered trypan blue as a treatment for red water fever, Nuttall convinced the Molteno family of South Africa to provide funds for a new building, named the Molteno Institute for Parasitological Research. David Keilin (1931–1952) succeeded Nuttall, followed by Parr Tate (1952–1966), Nuttall's doctor of philosophy (PhD) student, who was co-discoverer with S. P. James of the EE stages of malaria in birds (see p. 7), tested antimalarial drugs using *P. gallinaceum*. This work was continued by Ann Bishop (1899–1990). Keith Vickerman and co-authors (2000) commented, 'Through the 1960s relatively few universities in the United Kingdom encouraged research on protozoan biochemistry or very few biochemists decided to commit themselves to protozoa!'

Sir Henry Wellcome founded the Wellcome Bureau of Scientific Research in London in 1913, to carry out research in tropical medicine. The job of trust director was offered to Andrew Balfour, who had been in charge of the Wellcome Tropical Research Laboratory in Khartoum since its creation in 1902. Although funding was derived from the commercial activities of the Wellcome Foundation Ltd. (later Wellcome PLC), including Burroughs Wellcome Inc., its wholly owned American subsidiary, the scientific staff were relatively free to establish their own lines of investigation. Henry Wellcome's tenet for his research laboratories had always been 'Freedom of research—Liberty to publish,' and this attracted some of the most talented scientists of the day to work there. After the bureau moved to its Euston Road site in London in 1934, it became known as the Wellcome Laboratories of Tropical Medicine. In 1965, these laboratories moved to the Beckenham site to be merged with the existing Wellcome Research

Laboratories, which had been there since 1922. Len Goodwin (see pp. 15, 135) who had been in charge of the Tropical Medicine Laboratories in its latter years at Euston Road, and had been involved in development of pyrimethamine as an anti-malarial, did not wish to leave central London, and became director of science of the Nuffield Institute of Comparative Medicine, based at the Zoological Society of London (Ginger, personal communication).

Research on tropical medicine at Beckenham was carried out in the Departments of Protozoology and Helminthology, which were later merged into a Department of Parasitology headed by Ralph Neal. In 1972, when Colin Ginger (1937–), who had completed his PhD in 1964 at NIMR with James Fulton and Frank Hawking, joined the Parasitology Department at Beckenham, there were no biochemists either in that department or any other. Wellcome wished to create in the United Kingdom a biochemical parasitology group based on the approaches pioneered in the United States by George Hitchings and Gertrude Elion (see p. 132–134) at Burroughs Wellcome Co. As in many pharmaceutical company laboratories, the research goal at Wellcome was to design inhibitors for a particular biological target. The underlying principle, based on the successful work of Hitchings and Elion, was that even if there was not an absolute difference in the metabolism of parasite and the host, there were probably enough differences at the active or binding site of iso-functional enzymes (isozymes) to allow for differential inhibition. The best early example of this concept was the work on dihydrofolate reductase (DHFR) that led to pyrimethamine (for malaria), diaveridine (for chicken coccidiosis) and trimethoprim (for human bacterial infections). Using the Hitchings-Elion model, Wellcome (Beckenham) decided on a team approach, employing the talents of biologists (for *in vitro* and *in vivo* work), biochemists (for analytical and metabolic work, including enzymology) as well as synthetic and physical chemists (who would identify and synthesize the potential inhibitor molecules). In the mid-1970s, two major projects were initiated to develop drugs for malaria and other related parasites: one project was to develop mechanism-based inhibitors of the enzymes of the pyrimidine biosynthetic pathway, while the other targeted the ubiquinone coenzyme Q site of this electron transport system. While the pyrimidine biosynthesis project had some general inhibitor molecules already available from the Elion laboratory, generally more novel compounds were being synthesized by Roger Wrigglesworth and his chemist colleagues at Beckenham. The electron transport project was further advanced as hydroxy-naphthoquinones were already known to have good anti-malarial properties (see p. 11), and menoctone had been identified by Nick McHardy at Wellcome as a good drug, both *in vitro* and *in vivo*, against *Theileria parva*. It was, therefore, decided to close down the pyrimidine project, as it had now been shown conclusively that inhibition of electron transport in malaria leads to inhibition of dihydroorotate dehydrogenase

(DHOD)—a key enzyme in pyrimidine biosynthesis! This allowed more resources to be allocated to the electron transport project headed by Alan Hudson, the senior chemist for the parasitology area. It was fortunate that during the 1970s good *in vitro* assays utilizing the target species *P. falciparum, Eimeria tenella* and *T. parva* had become available, together with more useful *in vivo* models, for example, *P. berghei* in mice, *P. falciparum* in *Aotus* monkeys, *E. tenella* in chickens, and *T. parva* in cattle. For *Plasmodium* and *Eimeria* spp., biochemical assays were also available for both parasite and host electron transport systems. Finally, the key to developing a successful drug from the naphthoquinone series was to understand metabolism of the compound by the host liver systems, and to prevent degradation to inactive metabolites. The availability of human liver microsomes allowed studies of lead compounds in an *in vitro* system. Additionally, problems of bioavailability and pharmacokinetics needed to be addressed (See: The Road to Atovaquone p. 101).

In 1982, Winston (Win) Gutteridge (see p. 103) joined Wellcome as head of the Biochemical Parasitology Department, and together with Alan Hudson and other colleagues, three compounds from the naphthoquinone series were successfully marketed by Wellcome, namely parvaquone and buparvaquone for *Theileria* spp. and atovaquone for malaria. Despite these successes, biochemical research on parasites at Wellcome (Beckenham) was largely abandoned by the end of the 1980s, when the Animal Health Division (the original Cooper, McDougall and Robertson Laboratories based in Berkhamsted and acquired by Wellcome in 1959) was sold to ICI. First Colin Ginger and later Win Gutteridge moved to the WHO Tropical Medicine Programs (where they re-joined Peter Trigg who had moved there in 1977), and their colleagues from the Wellcome Parasitology Group moved to other fields.

In 1936, the monies from Burroughs Wellcome Co. were used by Henry Wellcome to establish the Wellcome Trust; it was re-named in the United Kingdom as the Wellcome Foundation Ltd. In 1986, the Trust sold 25% of Wellcome stock to the public, beginning a process of separating itself from the pharmaceutical industry, and, in 1995, the Trust divested itself of any interest in pharmaceuticals by selling all remaining stock to Glaxo, creating GlaxoWellcome. In 2000, the Wellcome name disappeared from the drug business when GlaxoWellcome merged with yet another British firm, SmithKline Beecham, to form GlaxoSmithKline (GSK). In 2006, the Trust had net assets of over $27 billion. The Trust's mission is 'to foster and promote research with the aim of improving human and animal health' and most of its funds go to support basic research for malaria and other tropical diseases, development initiatives and some support for the public understanding of science. In 2006, the Wellcome Trust invested approximately $1 billion in support of its mission, with previous years' funding having supported projects such as the

sequencing of the human and *P. falciparum* genomes, the development of the anti-malarial drug artemisinin, and other malaria-related projects, including a change in the WHO guidelines on the treatment of malaria as a result of Nick White's Wellcome-funded work on artemisinin.

The Bill & Melinda Gates Foundation is the world's largest private philanthropic organization (http://www.gatesfoundation.org; last accessed 16 July 2008) with an endowment of approximately $28.8 billion. The mission of the Gates Foundation's Global Health Program 'is to ensure that people in the developing world have the same chance for good health as people in the developed world,' and it funds a variety of malaria research initiatives. The number of Gates Foundation grantees and its total annual giving have been rapidly increasing. By 2004, 11 organizations received a total of $77.6 million to support malaria research and development. More than 60% of 2004 investment went to public–private partnerships. In 2005, the Gates Foundation (through PATH, Program for Appropriate Technology in Health) provided $35 million to the government of Zambia and other partners to help cut malaria deaths in that country by 75%. The partnership will document the impact of the national malaria control campaign that will provide insecticide-treated bed nets, anti-malarials and other tools with the aim of developing a replicable model of malaria control for application in other African countries. In October 2005, $258.3 million was provided to spur innovation in three critical areas. A vaccine initiative will conduct clinical trials in collaboration with GSK, drug development will be in collaboration with the MMV and mosquito control will be through the Innovative Vector Control Consortium at the LSTM.

In 2004, the MRC funded $6.4 million for malaria research and the London School of Tropical Medicine and Hygiene (London, United Kingdom; where about 70 staff work partially or wholly on malaria) and funded approximately $7 million for drug discovery (35%), basic research (24%), implementation research (22%) and diagnostics (13%), with the Gates Foundation through the Gates Malaria Partnership providing 41% of the total.

The 2005 report *Malaria Research and Development, An Assessment of Global Investment* (Malaria R&D Alliance, Program for Appropriate Technology and Health) found that in 2004 anti-malarial drug discovery and development received the largest amount of investment: $120.2 million (37% of the total) and the largest sources of this were the pharmaceutical and biotechnology companies, followed by the Gates Foundation, NIAID and the Department of Defense (DoD). Vaccine development and vaccine trials were the next highest funded category (24% of the total). NIAID was the top investor in this category followed by the Gates Foundation and DoD. Investment in basic research totaled $50.8 million with three organizations contributing 67% of total basic research funding: NIAID, followed by the Wellcome Trust and the MRC. By 2004, some members of the pharmaceutical industry returned to malaria drug development funding a total of $38 million in intramural research and development.

CHAPTER 5

In Vivo and *In Vitro* Models

Contents

1. LIFE IN THE ERYTHROCYTE

The earliest biochemical research on malaria used *Plasmodium gallinaceum* and *P. lophurae* in chicks and ducklings and *P. knowlesi* in rhesus monkeys because infections in these unnatural hosts are virulent and the large numbers of asexual parasites found in the blood were necessary for the available analytical techniques (reviewed in Fulton, 1951; McKee, 1951; Moulder, 1948). By the 1950s, the rodent malarias, *P. berghei, P. chabaudi* and *P. vinckei* (discovered in Africa) were adapted to laboratory rodents such as mice and rats (reviewed in Cox, 1988; Landau, 1998). These hosts were cheap, easily maintained and, by displaying different pathologies, served as convenient surrogates for the human malarias and for drug testing. (Maintenance of the *P. berghei* life cycle is described in Sinden, 2002.) Then, in the late 1960s, Geiman and Meagher (1967) succeeded in infecting *Aotus* night owl monkeys with a strain of *P. falciparum* and infections of *P. falciparum* were induced in *Saimiri* squirrel monkeys (Young and Rossan, 1969). In 1980, the *P. falciparum* FUP (Falciparum Uganda Palo Alto) strain was adapted to splenectomized squirrel monkeys (Gysin *et al*., 1980). Strains of *P. vivax* and *P. malariae* have been adapted to *Aotus* and *Saimiri* monkeys (Collins, 2002a,b; Schmidt, 1973, 1978). Having *P. falciparum* and other human malarias in the laboratory provided suitable primate models for human infections. However, using these New World monkeys has limitations: they are

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expensive, they come from great distances and frequently arrive in a deplorable condition, they can be difficult to handle, rearing them can be a problem and sometimes after infection the parasite numbers in the blood are low. Studies of the biochemistry of *P. falciparum* and its relatives would require *in vitro* cultivation of the parasites so that adequate amounts of parasite material would be available for analysis and primate use minimized.

Paralleling the early metabolic studies on *Plasmodium* were those involving nutrition. At Harvard, Geiman *et al*. (1946) and Ball *et al*. (1945) described rocker dilution and perfusion methods for the short-term cultivation of rhesus red cells bearing *P. knowlesi*. The simplest medium—based on an analysis of normal rhesus serum—that supported parasite growth required additions of glucose and *p*-aminobenzoic acid (pABA) as well as a mixture of purines and pyrimidines to the diluted serum. Although parasite growth and multiplication was best at 0.39% oxygen, cultures were routinely gassed with 20% oxygen 'since this approaches the content of alveolar air and thus duplicates *in vivo* conditions.'

In 1965, **Peter Trigg (1941–)** who had completed his doctor of philosophy (PhD) at Imperial College was recruited to Frank Hawking's Division of Parasitology at the National Institute for Medical Research (NIMR, London, United Kingdom). Hawking (1905–1986) started his career by working on drug resistance in trypanosomes at the Liverpool School of Tropical Medicine (LSTM, Liverpool, United Kingdom) under Warrington Yorke, later he studied the uptake of acriflavine by resistant parasites, and during World War II determined the best method for treating gas gangrene with sulphonamides. Although he became an expert on sulphonamides, from 1954 onwards, his principal interest was in circadian rhythms of malaria (see p. 271) as well as filariasis, not in chemotherapy, so he did not direct Peter Trigg's research. At NIMR, Trigg began to cultivate malaria parasites with the objective of providing sufficient antigens to immunologists for the eventual production of a vaccine. (A similar project was underway at Guy's Hospital (London, United Kingdom) by Geoff Butcher under the leadership of Sydney Cohen and by William Trager at the Rockefeller University, New York.) Trigg's *in vitro* culture studies with *P. knowlesi*-infected red cells (Trigg, 1968a) used the Harvard culture system (Ball *et al.*, 1945) and he was able to show: a requirement for cholesterol (Trigg, 1968b), the favoring effect of low (i.e. <5%) oxygen tensions (Trigg, 1968b) and that some commercial media (i.e. NCTC 135, medium 199) were adequate substitutes for the Harvard medium (Trigg, 1969). Though these short-term *in vitro* studies by Trigg were significant, most of the advances in the cultivation of malaria parasites would come from the Rockefeller University laboratories of William Trager.

Short-term *in vitro* growth of *P. falciparum* and *P. vivax* was reported as early as 1912 by Bass and Johns, however, attempts to reproduce this system met with failure. From 1947 onwards, Trager maintained *P. lophurae*-infected red cells using the Harvard rocker-dilution method. The culture system consisted of red cells suspended in the nutrient 'Harvard' medium, gently rocked to simulate blood flow, and gassed with humidified 5% CO_2 and 95% air. Under these conditions parasite growth was less than optimal and re-invasion rates were low so that continuous culture of the parasites could not be achieved. In 1971, Trager decided to abandon the rocker-dilution method and to substitute a perfusion system in which the culture medium would flow gently over a settled layer of cells. His reasoning was this: since *P. falciparum*-infected red cells spend most of their 48 h developmental cycle attached to the walls of the post-capillary venules, agitation of the infected red cells might be detrimental to parasite growth and invasion. As a result, red cells were removed from an *Aotus* monkey infected with the FVO (Falciparum Vietnam Oak Noll) strain of *P. falciparum* (obtained from Trager's former post-doctoral Wasim Siddiqui who at the time was working at Stanford with Geiman), the cells were washed, diluted with human AB red cells suspended in 15% human serum and placed in 'flow vials' and a variety of tissue culture media were screened. The newly developed RPMI 1640 medium was found to be superior to all others tested. Trager also changed the gas mixture from 5% CO_2 plus 95% air to 7% CO_2 plus 5% O_2 plus 88% N_2. Under these conditions, and with a settled layer from a 2–8% red blood cell suspension, it was possible to maintain the parasites for 24 days by adding fresh uninfected red cells every 3–4 days (summarized in Jensen, 1983; Trager, 1997; Trager and Jensen, 1980). Although this flow vial system was a clear success in growing parasites, it was cumbersome and would have had limited use were it not for the contributions of James B. Jensen, who fortuitously had joined Trager's laboratory as a post-doctoral fellow in 1976.

In 1966, United States Agency for International Development (USAID) funded a project for the development of a malaria vaccine at the University of Illinois (under Paul Silverman) and later at the University of New Mexico (Albuquerque, New Mexico) (under Karl Rieckmann). By the mid-1970s, USAID decided to diversify and expand its vaccine effort and so it enlisted the support of William Trager to undertake the cultivation of *P. falciparum*—an enterprise he had abandoned some 5 years earlier. In his proposal to USAID, Trager specifically asked for funds to support a post-doctoral student who was experienced in the cultivation of intracellular parasites. James B. Jensen was the person invited by Trager to fill that position.

James B. Jensen (1943–) was a graduate student in the laboratory of Datus Hammond at Utah State University working on the cultivation of coccidian parasites when Dr. Hammond died of complications from

recent heart surgery. As a consequence, Jensen renewed his work on coccidian sporozoite invasion of cultured cells at Auburn University from which he received his PhD in 1975. Shortly after Jensen's arrival at Rockefeller University in January 1976, Trager and he planned their approach to cultivating *P. falciparum*: first, they selected commercially available culture media high in glucose; second, they abandoned the bicarbonate buffer system (since it was clearly inadequate to control the lactic acidosis); third, they decided to compare parasite growth in the rocker flasks and 'flow vials' and fourth, they elected to modify the gas mixture. During February 1976, they tested the suitability of commercial media using falciparum-infected red cells from an *Aotus* monkey. The parasites in the rocker flask died out within 4 days, but after the same period of time the parasite numbers were maintained in the flow vial. The numbers of parasites increased dramatically in the flow vials when fresh cells were added to a diluted sample of infected red cells, but attempts to maintain parasites in the rocker flasks failed time and time again. In the meantime Jensen decided to take some of the infected red cells and place them into 35-mm Petri dishes with a variety of media (such as RPMI 1640 and Dulbecco's Modified Eagles Medium, Ham's H-12, MEM and Medium 199), buffers, etc. When he placed the Petri dishes containing the infected red cells into a 5% CO_2 plus 95% air incubator the parasites died out after 2–3 days. It was then that Jensen decided to employ a candle jar instead of the CO_2 incubator—a method he had used to grow the cells for the cultivation of various coccidian species (and when the CO_2 cell culture incubators in the virology laboratory at Utah State University were unavailable to him). Jensen located a large glass desiccator, placed his Petri dish cultures inside and after lighting a candle, closed the stopcock; this was incubated at 37 °C for several days. At first Trager was dismayed to observe Jensen's use of a nineteenth century technology, but when he was shown the Giemsa-stained slides Trager was convinced Jensen was on to something. In the summer of 1976 Milton Friedman, a graduate student in the Trager laboratory who was working in the Medical Research Council (MRC) laboratories in The Gambia, arranged for a sample of human blood infected with *P. falciparum* to be sent to New York. This was diluted with RPMI 1640 (which turned out to be the best of the commercial media) in Petri dishes, placed in a candle jar and incubated. The line grew very well and became FCR-3, one of the most widely used strains. Later, other lines would be established using similar methods and the impact of continuous cultivation of *P. falciparum* was phenomenal: the number of publications on malaria between 1976 and 1986 was eight times that of the previous 10 years (Trager, 1997)!

William Trager (1912–2005) received his PhD from Harvard in 1933, joined the Department of Animal Pathology at the Princeton Division of the Rockefeller Institute, and worked in the laboratory of R. W. Glaser, whose forte was the axenic culture of protozoans. During his early period at Rockefeller, Trager succeeded in growing mosquito larvae in axenic culture—no small feat since this was before the advent of antibiotics—and this allowed for the determination of their nutritional requirements. His interest in malaria resulted from his World War II experiences in New Guinea as an officer in the United States Sanitary Corps where he was involved in the supervision of human trials with atabrine. Upon his return from the Pacific, Trager began laboratory studies on the nutritional requirements of *P. lophurae*. Initially, he followed parasite growth in chicken red cells, as did the Harvard group with *P. knowlesi* in monkey cells. Both groups found that pantothenate and pABA (as well as methionine) favored intracellular growth (Anfinsen *et al*., 1946 Trager, 1943, 1947). In an attempt to define the *raison d'être* for the parasite requiring a living host red cell, Trager tried to cultivate *P. lophurae* extracellularly (Trager, 1950, 1952, 1953, 1957). After many years of painstaking effort he was able to grow the parasite from the uninucleate ring stage to the multi-nucleate schizont; in the extracellular cultures he found growth required: coenzyme A (CoA) adenosine triphosphate (ATP), nicotinamide adenine dinucleotide (NAD), reduced glutathione (GSH), thiamin, malate, pyruvate, folinic acid (N^5-formyl-tetrahydrofolic acid) and a key ingredient was the addition of 30% 'red cell extract'. Later, he was able to do the same for *P. falciparum* (Williams *et al*., 1995) but before this, he turned his attention to growing *P. falciparum* within red cells.

Although *P. falciparum* grew well in Trager's 'flow vials' the method would not have been practical for growing these parasites outside of laboratories such as those at Rockefeller University: the flow vials were hand made by an expert glass blower, were cumbersome to set up, used large quantities of medium, produced very small samples of infected red cells and required expensive peristaltic pumps. The greatest value of the candle jar method was that it could be used in laboratories almost anywhere in the world where there is an incubator, a candle and a desiccator (Jensen, 2002). The successful continuous cultivation of *P. falciparum* involved more than an understanding of the idiosyncratic growth requirements of *Plasmodium*, it needed determination and the combined talents of two exceptional culturists.

The specific requirement for methionine for malaria parasites had already been demonstrated by the nutritional studies of Ball *et al*. (1948) and Trager (1947). In addition, by using ^{35}S-methionine and ^{35}S-cysteine, Fulton and Grant (1956) were able to demonstrate incorporation by *P. knowlesi*-infected red cells and concluded that the need for an extracellular source of methionine stemmed from the fact that this amino acid was unavailable in the host hemoglobin. Working at the Walter Reed Army Institute of Research (WRAIR, Washington), Polet and Barr (1968) and Polet and Conrad (1969a) confirmed this by finding that growth of the parasite was impaired in a methionine-deficient medium. However, a more striking inhibition was found when isoleucine was omitted from the medium; indeed radioactive isoleucine showed the greatest incorporation when it was omitted from the medium (Polet and Conrad, 1969b). In addition to methionine, arginine, proline and lysine were directly incorporated into proteins of the free parasites. Although it was claimed that the degree of incorporation of free amino acids was related to their abundance in hemoglobin (and this appears to be the case for methionine and isoleucine with *P. knowlesi*), the situation is more complex since with *P. lophurae* the extent of amino acid incorporation was not correlated with the amino acid composition of hemoglobin (Sherman, 1977a,b; Sherman *et al*., 1971b) and the same seems to hold for *P. falciparum* (see below). What remains of value from this work is that specific amino acids can be used to measure parasite growth as well as the effects of anti-malarials on protein synthesis.

The availability of a continuous culture system for *P. falciparum* allowed for determinations of low-molecular-weight components necessary for growth and the effects of anti-metabolites. Using a semi-defined medium (in which human serum was exhaustively dialysed) pantothenate, cysteine, glutamate, glutamine, isoleucine, methionine, proline and tyrosine were found to be necessary for continuous *in vitro* growth (Divo *et al*., 1985). Growth was markedly reduced in the absence of amino acids and deletion of isoleucine and methionine suppressed growth. Adequate concentrations of glucose and GSH were needed and ribose, mannose, galactose and maltose could not substitute for glucose. Only fructose supported growth although it was inferior to glucose (Geary *et al*., 1985). These findings, which differed from earlier nutritional studies with *P. knowlesi* (where mannose and fructose but not maltose supported growth), may have been the result of their use of short-term cultures rather than continuous culture, as well as differences in the host red cell and parasite species.

The continuous culture of *P. falciparum* dramatically altered studies of malaria parasites. In 1997, when Trager and Jensen wrote about the impact of their culture system, they noted it could be usefully applied in nearly every aspect of research: chemotherapy, drug resistance,

immunology, pathogenesis, gametocytogenesis and mosquito transmission, genetics, red cell receptors for merozoite invasion and the biochemistry of malaria. Much of this has been realized: by 1988, there were 850 citations to the uses of the continuous culture system and by 1996 the number had grown to 1924. Today there are many more thousands of citations. Perhaps most importantly the continuous *in vitro* culture of *P. falciparum* made possible molecular approaches, including the sequencing of the entire genome that enabled a giant leap forwards in understanding the biochemical properties of *Plasmodium*.

The culture of *P. falciparum* also affected the use of model systems. *P. lophurae* used so successfully for more than 40 years became obsolete. In 1988, Barclay McGhee reviewed the contributions of the avian malaria and the last literature citation for *P. lophurae* was in 1990 and concerned itself with the histidine-rich protein (Margossian *et al*., 1990). The rodent malarias, *P. berghei, P. chabaudi, P. vinckei* and *P. yoelii* (Renia *et al*., 2002) as well as *P. gallinaceum*, became useful for studies of exo-erythrocytic (EE) and sporogonic stages and *P. knowlesi* would be used primarily to investigate merozoite invasion and antigenic variation (see below). Regrettably, the success with falciparum has not been realized with other human malarias and a continuous culture system for *P. vivax* is still not available.

2. LIFE OUTSIDE THE ERYTHROCYTE

Ronald Ross, shortly after his discovery of the mosquito as a vector for *Plasmodium*, presumed sporozoites entering the blood stream burrowed straightaway into red cells. However, his rival Batistta Grassi suggested that the nucleus of the sporozoite was so different from that found in the blood stages that a considerable degree of transformation would be necessary to convert one directly into the other. Based on this, in 1901, Grassi hypothesized an intermediate stage occurred somewhere in the body—an EE form—and this would carry out the necessary transformation. Grassi's hypothesis quickly fell apart when 2 years later Fritz Schaudinn (1903) described in great detail the direct penetration of the red cell by *P. vivax* sporozoites. So persuasive was Schaudinn's description that even Grassi did not pursue the matter further and 'Schaudinn's curious delusion lay like a spell over subsequent investigators' (Harrison, 1978). However, since 'science is a study of errors slowly corrected' indirect evidence soon questioned both the observations and conclusions of Schaudinn. First, there was the failure to confirm Schaudinn's microscopic findings and second the effects of quinine were found to be markedly different in blood-induced infections from those that were sporozoite-induced. However, more telling were the observations of Huff, James and Tate, and Fairley. During World War II, with the help of army volunteers, Fairley

(1945) measured the incubation period, that is, the time it took for parasites to appear in the blood after a mosquito-induced infection; in *P. vivax* it was 8 days and in *P. falciparum* it was 5 days. He also found that during the incubation period the blood was not infectious by transfusion. Clearly malaria parasites must have been lurking somewhere in the body, but the question was where?

Beginning in the mid-1930s, Huff and Bloom (1935) and James and Tate (1938) and then others observed EE forms in erythroblasts, endothelial cells and macrophages prior to the appearance of parasites in the red blood cells in avian as well as in lizard malarias (masterly summarized by Huff, 1969). Following Huff and Coulston's (1944) landmark description of the development of *P. gallinaceum* from the entrance of sporozoites into the skin of chickens to the appearance of parasites in the blood, Huff (1947) was bold enough to suggest, 'Since indirect evidence for ... exo-erythrocytic stages in mammalian malarias is good it would appear advisable to adopt their presence in sporozoite-induced infections as a working hypothesis ...'

In 1945, Col. S. P. James told P. C. C. Garnham, then a young medical officer in Kenya, not to return from East Africa until he found EE forms in mammalian malaria. James' gentle insistence proved stimulating to Garnham and 2 years later, after James' death, Garnham found EE stages in the liver of an African monkey infected with *P. kochi* in the Medical Research Laboratory, Nairobi (Garnham, 1966; Lainson and Killick-Kendrick, 1997). Shortly thereafter, Garnham joined H.E. Shortt at the London School of Hygiene and Tropical Medicine (LSHTM, London, United Kingdom) where work began using *P. cynomolgi* in rhesus monkeys expecting that the findings would relate to *P. vivax*. There were many attempts and many failures. However, success was achieved when 500 infected mosquitoes were allowed to bite a single rhesus, and then to make sure, mosquitoes were titrated in monkey serum and also injected. Seven days later, the monkey was sacrificed and its organs taken for microscopic examination. Shortt expected that the EE stages would be found in locations similar to those described for bird malarias; however, this turned out not to be the case. Instead, the site of EE stages for *P. cynomolgi* was the liver, as had been the case with *P. kochi*. Shortt and Garnham (1948) promptly reported their findings. From that time forwards, EE stages have been described for the primate malarias (see Coatney *et al*., 1971) and in the human malarias *P. ovale* (Collins and Jeffrey, 2005), *P. malariae* (Bray, 1960), *P. vivax* (Shortt, 1948; Shortt and Garnham, 1948) and *P. falciparum* (Shortt *et al*., 1951), as well as in many of the rodent malarias (Cox, 1988; Landau, 1998).

In *P. falciparum* infections, the disappearance of parasitized red cells from the peripheral circulation (as evidenced by simple microscopic examination of a blood film) may be followed by a re-appearance of parasites in

the blood. This type of relapse, called recrudescence, results from an increase in the number of pre-existing blood parasites. *P. vivax* and *P. ovale* also relapse; however, the re-appearance of parasites in the blood is not from a pre-existing population of blood stages and occurs after cure of the primary attack. The source of these blood stages remained controversial for many years, but in 1980, the origin of such relapses was identified. In relapsing malarias, induced by sporozoites, Krotoski *et al.* (1980;1982 a,b,c) found small dormant parasites, called hypnozoites, within hepatocytes. The hypnozoites, by an unknown mechanism, are able to initiate full EE development and then go on to establish a blood infection. It is important to note that in the bird malarias, unlike those of mammals, the EE stages are capable of re-invasion into non-erythrocytic cells, as a consequence there can be continued production of EE forms with subsequent invasion of the red blood cells without hypnozoites.

Some questions particularly immunological and pathophysiological can only be addressed by using *in vivo* models of malaria (Gysin, 1998; Landau, 1998). However, the EE stages of non-human primates are difficult to study because of ethical considerations as well as scarcity of suitable species of monkeys, and the narrow range of parasite lines adapted to primates and with differing pathologies from the human and expense. Humans are more plentiful, but the numbers of volunteers willing to undergo liver biopsy are difficult to find. Moreover, even when a mosquito inoculates tens of sporozoites into a human, and these successfully invade hepatocytes and can develop into an EE form, only a few tens of EE stages may be present in a 1.4-kg organ.

A somewhat more practical approach has been to utilize smaller, more manageable and less expensive rodent malarias as well as immunodeficient mice to produce mouse–human chimeras. Using an immunodeficient/transgenic mouse, CB-17/SCID bearing a urokinase-type plasminogen activator gene controlled by an albumin promoter, it has been possible to re-populate the mouse liver with human hepatocytes. When injected with *P. falciparum* sporozoites, EE stages developed; however, the number of EE stages was not provided (Morosan *et al.*, 2006). This approach and others such as that reported by Morosan *et al*. may have promise, but at present they are complex and time consuming. As a consequence, many *in vivo* studies have used rodent species. *P. berghei* was discovered in 1948 by Belgian workers in rodents in the Congo (Vincke and Lips, 1948), but it was another 17 years before Meir Yoeli discovered that low temperature was essential for mosquito transmission; once known, EE stages were quickly described in the liver (Yoeli and Most, 1965 a,b; Yoeli *et al.*, 1965). Other rodent malarias were subsequently found (reviewed by Cox, 1988). These too have been used for studies of liver stages.

Because of the limitations in the use of *in vivo* models, attempts were made to carry out studies of EE stages *in vitro* (Beaudoin *et al.*, 1988;

Hollingdale, 1985; Jensen, 1983; Meis and Verhave, 1988). Early attempts at, and success with, the *in vitro* cultivation of avian EE stages have been summarized by Huff (1964); Davis *et al*. (1966) and Schuster (2002).

In vitro cultures of mammalian malarias initiated from sporozoites were reported as early as 1976, however, most of these were only partially successful. The discovery that the human embryonic (WI38) cell line was susceptible to *P. berghei* led to the first successful culture of mammalian EE stages (Hollingdale *et al*., 1981). Following this, *in vitro* cultivation of various rodent species of *Plasmodium* was achieved in hepatocytes; however, the necessity for repeated isolation of hepatocytes, which do not replicate or retain their differentiated functions, had restricted the value of such culture systems. This was soon overcome by the use of 'immortalized' lines such as the human hepatoma line HepG2-A16 that supports liver forms of *P. berghei* with about 8% of the sporozoites developing into EE forms (Hollingdale *et al*., 1983). Unfortunately this cell line and others used successfully with rodent malarias (i.e. HeLa, human lung cells, mouse hepatocytes), do not support *P. falciparum* EE stages with the exception of one line that supports it with low efficiency.

Complete development of EE forms of *P. falciparum* was accomplished by Mazier *et al*. (1985) as was *P. ovale* (Mazier *et al*., 1987), the simian malarias *P. fieldi* and *P. simiovale* (Millet *et al*., 1994) and *P. inui* (Nguyen-Dinh *et al*., 1980) using primary hepatocytes. Up until recently the only reliable *in vitro* system available for *P. falciparum* EE forms has required the continuous isolation of primary hepatocytes and repeated inoculation with fresh sporozoites. However, in 2006, a new continuous line HC-04 was isolated in which EE parasites attained full maturity (as demonstrated by their ability to infect erythrocytes), though infection rates were low: 0.006% and 0.041% for *P. falciparum* and *P. vivax*, respectively (Sattabongkot *et al*., 2006).

By 1976, culture techniques allowed the cultivation of the EE forms of *P. vivax* in human hepatocytes (Doby and Barker, 1976) and this was extended by Mazier *et al*. (1984) and then to *P. ovale* by Mazier *et al*. (1987) and in HepG2-A16 cells by Hollingdale *et al*. (1985a). In *P. vivax*, transformation into the trophozoite occurs by de-differentiation of the pellicle and apical organelles of the sporozoite and occurs within 24 h (Uni *et al*., 1985); the EE forms grow slowly until day 3; after day 3 nuclear division occurs and by day 5 there is a small multi-nucleated schizont, which rapidly grows to a large size with over 1000 nuclei by day 9. Also present at days 5 to 9 were small parasites approximately the size of 24-h-old parasites presumably representing hypnozoites.

Michael Hollingdale (1946–) received his bachelor of science (BSc) degree from the University of Liverpool (1967) and then went on to do a PhD at the University of London (1971) working on *Mycoplasma*. He

then spent 3 years at the LSHTM working on a delayed hypersensitivity disease caused by thermophilic bacteria. Hollingdale emigrated to the United States, joined the Harvard School of Public Health in 1974 and worked on rickettsias. In 1976, he was hired by Johns Hopkins School of Medicine to continue work on hypersensitivity, which he did not like, so that by 1978 he wanted to move to New York and would have taken any job. That year he was interviewed by Araxie Kilejian at the Rockefeller University about working on malaria, of which he knew nothing, and was hired. He began working on the tissue stages of the avian malaria *P. lophurae* and during the Rockefeller years (1978–1979) tissue stages were successfully established in *in vitro* cultures and in embryonated turkey eggs obtained from a farm in New Jersey. The starting infected tissue for the lophurae work had been received from Richard Beaudoin (1931–1990) at the Naval Medical Research Institute (NMRI, Bethesda, Maryland), and, in 1979, Hollingdale accepted a position with him to work on mammalian liver stages of *P. berghei*. After about 1 year, using WI32 cells, a human lung cell line, it was discovered that the full cycle with release of infectious merozoites took 72 h *in vitro*, 1 day longer than *in vivo*. This was the first report of culture of the full liver stage of mammalian malaria. Subsequently they used the human hepatoma cell line, HepG2-A16, obtained from Alan Schwartz (now at Washington University) and achieved fairly high infection rates. It turned out they were fortunate to use *P. berghei* for these initial studies as *P. berghei* sporozoites successfully invade and develop in many cell types, unlike *P. yoelii* or *P. falciparum*. In fact, Hollingdale and co-workers also showed that *P. vivax* would also fully develop in HepG2-A16 cells, and after 9 days' culture small uninucleated parasites similar to hypnozoites were observed. Finally, in collaboration with Ruth Nussenzweig and Fidel Zavala (Hollingdale *et al*., 1982) it was possible to demonstrate that monoclonal antibodies to *P. berghei* circumsporozoite protein (CSP) protected mice from sporozoite infection by completely blocking sporozoite invasion of these cells, confirming their *in vivo* studies' findings that anti-CSP antibodies were important mediators of anti-sporozoite protective immunity. This was also confirmed using *P. falciparum* sporozoite invasion assays (Zavala *et al*., 1985).

Clearly greater improvements in the *in vitro* cultivation of EE stages will have to be achieved before these can serve as a ready source for studies of biochemistry, the testing of drug sensitivity and the immunology of EE stages.

3. LIFE IN THE MOSQUITO

Despite the importance of mosquito stages for understanding malaria transmission, the *in vivo* and *in vitro* sporogonic processes have often been the most overlooked aspect of *Plasmodium*–host interactions. This is in part due to the small size of the mosquito vector as well as the difficulties in clean isolation of the even smaller-sized stages of the parasite. More importantly, however, has been the lack of understanding of the biochemical and environmental conditions that underlie the complex development of the malaria parasite in its vector. In brief, the processes involved in sporogonic development are: gametocytes taken up during blood feeding complete their differentiation and fertilize in the lumen of the mid-gut; the resultant zygote transforms into a motile ookinete that moves out of the blood bolus, crosses the mid-gut epithelium, where it becomes an oocyst in the subepithelial space between the epithelium and the basal lamina; within the oocyst sporogony occurs producing sporozoites that migrate through the hemolymph of the hemocoel, enter the salivary glands, and there they mature.

Early workers who investigated the *in vitro* culture of mosquito stages held to the view that cultivation of these stages would offer a clearer understanding of the relationship between the host and parasite, and that, with culture media of known composition, the nature of the dependence of the *Plasmodium* on the mosquito host would be revealed. In short, the expectation was that *in vitro* culture would reveal the biochemical systems that had been altered or lost such that the parasite had become dependent on the host to provide the essential substances (or reactions) for completion of its life cycle, that is, the *raison d'être* for parasitism. Cultivation of a parasite apart from its host would, these pioneers of *in vitro* culture believed, be an important step towards understanding the biochemical capabilities of the *Plasmodium* (Ball, 1964). Further, by understanding sporogonic development, programs directed at blocking parasite transmission, which has been an objective of malaria control programs since the time of Ronald Ross (1898), could be benefited. The demonstration that irradiated sporozoites can be effective as immunizing agents (Clyde *et al*., 1973; Nussenzweig *et al*., 1967, 1972) provided an additional incentive for culturing the mosquito stages to produce sporozoites for use as a vaccine. In addition, although 35 years later it has still not been possible to produce sporozoites by *in vitro* cultivation in sufficient amounts and of acceptable purity for use as a vaccine for humans, Sanaria Inc., a recently formed company by Stephen Hoffman, is now attempting the production of sporozoites in large quantities and of acceptable purity in mosquitoes such that they can be used to vaccinate humans.

One of the early investigators in the *in vitro* culture of sporogonic stages was Gordon Ball (1899–1982), who, in collaboration with Jowett

Chao and after 27 years of painstaking effort, was able to have a complete *in vitro* sporogonous cycle of *P. relictum* by culturing overlapping stages. Ball (1964) lamented on the lack of progress by suggesting that so few undertook the cultivation of the extracellular mosquito stages because they had a better appreciation than he did of the difficulties, technical and otherwise, connected with this approach (i.e. tedious dissection, bacterial and fungal contamination, inadequate media and a lack of knowledge of developmental triggers), or else they doubted that even when successful the results could be applied to the *in vivo* relationship. Indeed, a successful culture of sporogonic stages requires simulation or replacement of four distinctly different environments: intraeythrocytic for gametocytes, the lumen of the mid-gut for zygote (ookinete) formation, the hemolymph in the mosquito hemocoel for oocyst and sporozoite development and the salivary glands for sporozoite maturation.

Studies of the cultivation of the sporogonic stages prior to 1980 were summarized by Vanderberg, Weiss and Mack (1977) and Schneider and Vanderberg (1980). In their 1980 review, Schneider and Vanderberg wrote that there had been few significant advances in culture systems capable of supporting growth and development of insect stages of malaria. However, in a later review Vanderberg (1988) wrote 'the stepped up pace of . . . research on *in vitro* sporogony in recent years has given rise to some progress.' He noted that although the problems of inducing gametocyte formation of *P. falciparum in vitro* and supporting these to full maturation had been solved, obtaining further development of the ookinete to the oocyst with subsequent formation of mature sporozoites yielded virtually no progress. All that changed in the next decade. By 2007, *Plasmodium* spp. that had been successfully cultured from gametocyte to sporozoite included *P. falciparum* (Warburg and Schneider, 1993), *P. gallinaceum* (Warburg and Miller, 1992), *P. berghei* (Al-Olayan *et al*., 2002) and *P. yoelii* (Porter-Kelley *et al*., 2006). More recent reviews of progress (and lack thereof) can be found in Schuster (2002) and Hurd, Al-Olayan and Butcher (2003).

In 1979, Carter and Miller using a culture system combined from the methods for the *in vitro* cultivation of the blood stages of *P. falciparum* by Trager and Jensen (1976) and Haynes and colleagues (1976) published a study on the production of *P. falciparum* gametocytes in continuous culture, in which it was shown that there was an abrupt switch in the rate of production of gametocytes in culture by up to two orders of magnitude over a period of approximately 24 h at around the fifth or sixth day of a culture; this high rate of gametocyte production continued throughout the subsequent days of culture so long as no fresh red blood cells were added. Meanwhile, using the same system of *in vitro* cultivation, Carter and Beach (1977) reported that functionally mature gametocytes of *P. falciparum* could be produced from *in vitro* cultures as shown by their

ability to undergo gametogenesis (commonly referred to as exflagellation) under appropriate stimulation (see below) *in vitro*. Not long after, based on the same system of culture, methods were developed for infecting mosquitoes with the *in vitro*-produced gametocytes of *P. falciparum* (Carter *et al*., 1993; Ifediba and Vanderberg, 1981; Ponnudurai *et al*., 1982b). Addition of hypoxanthine (50 μg/ml) was reported to be necessary for maturation of the *P. falciparum* gametocytes to the point of being able to infect mosquitoes (Ifediba and Vanderberg, 1981). Later, Trager and Gill (1989) used phorbol 12-myristate-13-acetate and phorbol dibutyrate and 8-bromo cyclic adenosine monophosphate (8-bromo cAMP) to promote gametocyte production; it is not entirely clear whether these additions actually affected signal transduction but since bistratin A, an inhibitor of protein kinase C, inhibited gametocytogenesis *in vitro* it suggests that phorbol esters may be involved in switching parasite development from asexual to sexual. In 1983, Janse and colleagues achieved production of gametocytes of *P. berghei in vitro* and showed that these too could infect mosquitoes (Janse *et al*., 1985).

As noted above, gametocytes of *P. falciparum* do not begin to appear in significant numbers in Trager-Jensen cultures until 5–7 days after dilution and to reach maturity requires about a further 8 days. It appears that asexual stages have to reach high densities and to become stressed in order for gametocyte production to begin (Carter and Miller, 1979). When there are too many actively growing and dividing asexual parasites, gametocyte maturation seems to be interfered with.

A fundamental question concerning gametocyte formation is how it is determined that a merozoite will become an asexual parasite or a gametocyte following its entry into a new host red blood cell. Using lawns of red cells attached to a Petri dish surface with concanavalin A as a parasite plaque-invasion method (invented by J. L. Williams at the WRAIR in 1981) Inselburg (1983) produced results that indicated that merozoites from the same schizont of *P. falciparum* in culture had a tendency, but no absolute commitment, to produce either asexual parasites (leading to further schizonts) or gametocytes in the next generation. The question was re-examined by the same method by Marion Bruce and colleagues (1990). By this time, these workers had available monoclonal antibodies that could identify, unequivocally, the form (gametocyte or asexual) of parasites produced in plaques resulting from the invasion of merozoites from individual schizonts. Contrary to the 1983 report of Inselburg, for whom, without access to the stage-specific antibodies, precise identification of parasite stage was not possible on purely morphological grounds, the 1990 finding of Bruce *et al*. showed that schizonts of *P. falciparum* are, in fact, absolutely pre-committed to produce either all asexual parasites or all gametocytes in the next generation of merozoite invasion. Moreover, as would be expected, the proportion of schizonts committed to produce

gametocytes was dependent upon the time in the culture at which they were collected. However, regardless of when they were collected, the degree of commitment of individual schizonts remained absolute; always, the merozoites from the same schizont gave rise to all asexual parasites or all gametocytes. Most recently, using improvements in the same methodology, but by this time with antibodies that could also distinguish the sex of the gametocytes as they appeared in the invasion plaques, Todd Smith, working with Lisa Ranford-Cartwright and David Walliker, (Smith *et al*., 2000b) and Francesco Silvestrini working with Pietro Alano (Silvestrini *et al*., 2000) showed that schizonts were pre-committed, not only to the production of either all asexual parasites in the next generation, or all sexual-stage parasites, but that the sex of the gametocytes was also predetermined. Finally, it should be noted that a single cloned line of *Plasmodium* can produce gametocytes of both sexes (Walliker *et al*., 1973) and, as malaria parasites are haploid in the blood stages, a single haploid genome contains all the necessary genetic information for the production of both male and female stages . . . the ultimate hermaphrodite.

In the development of a system for the production of gametocytes of *P. falciparum* that were highly, and routinely, infectious to mosquitoes, Ponnudurai *et al*. (1982a) used purpose-designed cultivation vessels held in a cam-operated table for culture of *P. falciparum*. For routine cultures, infected blood was placed in the vessels with RPMI 1640 culture medium supplemented with 5-mM HEPES buffer plus 0.2% sodium bicarbonate and 10% human serum in an atmosphere of 3% O_2, 4% CO_2 and 93% N_2. Fresh red cells were added every 4–5 days to reduce the parasitemia to 0.3–0.5%, however, no new red cells were added when it was desired to produce gametocytes; instead, the contents were divided between two vessels after 7–8 days to reduce the parasite density further. During medium exchanges, the temperature was maintained at 38 °C in order to prevent gametogenesis. In this system, with only a single daily medium change, young gametocytes (stage II) appeared on the about the sixth day and were mature after about 11 days, a time roughly corresponding to maturation *in vivo*.

The ability to produce mature, infectious, gametocytes of *P. falciparum in vitro* has allowed for the possibility of producing 'large' amounts of sporozoites (up to approximately 10^5 per mosquito) by membrane feeding to susceptible mosquitoes. Previously the only dependable source of gametocytes were infected volunteers, neurosyphilitics treated with 'malaria fever therapy' and naturally infected individuals. Although there are at least 66 different anopheline vector species for *P. falciparum* for laboratory studies the most commonly used species are *Anopheles stephensi, An. freeborni* and *An. gambiae*. Three- to six-day post-emergent mosquitoes are the best blood feeders. With *P. falciparum*, mosquitoes can be infected with as few as 50–100 gametocytes/mm^3, but for heavy infections 300/mm^3 are required. Theoretically, *in vitro* it is possible to produce

P. falciparum gametocytes as high as 100,000/mm^3 (assuming a gametocytaemia of 2% and a red cell count of 5 million/mm^3); however, in practice the numbers are usually 2,000/mm^3. A flask with 1% parasitemia containing 700 μl of red cells can provide enough gametocytes to infect at least 200 mosquitoes by means of a membrane-feeding device (Vanderberg, 1988). It should be noted that infectivity to mosquitoes by cultured gametocytes can be highly variable and often unpredictable, however, there are laboratories where consistency and productivity are high.

Jerome Vanderberg (1935–) as a sophomore biology major at City College of New York (CCNY, New York) in the autumn of 1952 took invertebrate zoology as his first advanced biology course following freshman biology. Out of a relatively small class, three people became professional parasitologists: Jerry, Paul Basch, a schistosoma specialist who spent his career at Stanford University (Palo Alto, California), and the author of this work, Irwin Sherman. Vanderberg surmises it might have been something in the water that produced such inclinations! After courses in ecology, entomology and field zoology with the most eminent lepidopterist in the United States, Alexander Klots, Jerry decided that he wanted to be an entomologist. But a course in parasitology (1954) convinced him that he wanted to work on things that had medical significance. Thus, it was not butterflies but mosquitoes that turned him on. Klots, like many of his generation, had spent World War II in the United States Army, in his case doing mosquito surveillance and control in the South Pacific. Therefore, Vanderberg worked in his laboratory at the American Museum of Natural History on the mosquitoes that he had collected during the war there. After receiving his bachelor of science (BS) degree (1955) from CCNY he did graduate work at Penn State and then at Cornell with Barnie Travis and received his PhD (1961) for a thesis entitled '*The role of gonadotropic hormone in protein synthesis in* Rhodnius prolixus.' After post-doctoral work at The Johns Hopkins University, Jerry joined the faculty of the Department of Medical Parasitology at the New York University (NYU, New York) School of Medicine in 1963 where Harry Most was the chair. Studies of an attenuated sporozoite vaccine were initiated in collaboration with Ruth Nussenzweig in 1966. Vanderberg has remained at NYU for all of his professional life and his research is currently focused on the biology of the sporozoite and immunity against sporozoites.

Defining the controls of gametogenesis is crucial to understanding the transition of malaria parasites from the blood circulation of the vertebrate host into the mid-gut lumen of the mosquito vector during

a blood meal. As now understood *in vivo* (i.e. within the mid-gut of a female mosquito that has just engorged upon gametocyte-infected blood) triggering of exflagellation in malaria parasites is induced by the presence of a mosquito exflagellation-inducing factor (MEF), in the mosquito mid-gut, now known to be xanthurenic acid (XA), a breakdown product of mosquito eye pigment together with a very slight drop in temperature below that of the blood circulation. The activity of MEF is also affected by the pH of a blood meal, which may be critically important to successful exflagellation *in vivo*. The story behind our present understanding is as follows.

From the earliest observations of the blood stages of malaria parasites it had been regularly noted that the stages that we now know to be gametocytes spontaneously undergo a dramatic physical transformation when a drop of gametocyte-containing blood is placed upon a glass slide and examined through a cover slip under a microscope. This was first witnessed by Laveran in the blood of malaria patients (see p. 3) a century ago, and was indeed, the basis for his discovery of malaria parasites as the causal agents of the disease. The process of exflagellation has fascinated malariologists of every succeeding generation. In 1897, Ross studied it in India as he moved towards his insight into the mosquito transmission of malaria (see p. 5). In the same months, on the other side of the globe, MacCallum was the first to recognize that 'exflagellation' represented the formation of male gametes in prelude to an act of fertilization. It was Ross, however, who appreciated that what took place spontaneously in a drop of blood exposed to air on a glass slide, represented the first essential step to the establishment of the parasites in a mosquito vector following a blood meal.

For many years, there were little further published attempts to understand the process of exflagellation. Marchoux and Chorine (1932) working with *Haemoproteus*, a blood parasite of birds related to the genus *Plasmodium*, found that a drop in temperature below that of the host's blood circulation (40–42 °C in the case of most birds) was not needed for spontaneous exflagellation to take place on exposure of the gametocyte-infected blood to air; this also was Ross's surmise. However, in blood kept under an atmosphere of CO_2, gametocytes failed to exflagellate, but did so shortly upon exposure to atmospheric air. As a consequence of the loss of CO_2 from the blood, was the expected rise in the pH of the blood from pH 7.3–7.4 to around pH 7.75 within 5 min of its exposure to air. When the pH of the blood exposed to air was held at 6.3 (by addition of 'Tyrode's solution') no exflagellation occurred, whereas in blood whose pH was kept at 7.6 (by re-alkalinization of the Tyrode's solution), the parasites exflagellated. Thus, Marchoux and Chorine proposed that the factor that triggers exflagellation of gametocytes in blood exposed to air is the rise in pH following loss of CO_2.

Subsequent observations by other workers began to cast doubt, however, upon this conjecture as the explanation for the factors that trigger exflagellation. In 1948, Micks and colleagues reported that there was no rise in pH in blood meals in the mosquito vectors of the canary parasite, *P. elongatum*, above the pH of the blood in the circulation of the canary host (Micks *et al*., 1948). Nevertheless, these workers found that exflagellation did occur readily in the mid-guts of engorged vector mosquitoes (*Culex* and *Aedes* spp.). Later, Ann Bishop and Elspeth McConnachie (1956), working at the Molteno Institute (see p. 21), measured the pH of blood meals of *A. aegypti* fed on chickens, the host of *P. gallinaceum*. Many mosquito blood meals did exceed pH 7.5, at which pH exflagellation had been observed to occur by Marchoux and Chorine. Nevertheless, in an extensive series of observations upon infected blood re-suspended in Tris-buffered saline solutions at different pH, Bishop and McConnachie were unable to reach a firm conclusion regarding the contribution of pH to the triggering of exflagellation by *P. gallinaceum* gametocytes in chicken blood. They did, however, conclude that chicken blood itself contained a factor(s) upon which the ability to exflagellate depended absolutely and which was absent in the simple Tris-buffered saline solution.

Following up on this, Bishop and McConnachie (1960) conducted an extensive investigation into the composition, and particularly the ionic composition, of chicken serum. This time, their experiments led to a clear and unequivocal finding, that is, in the presence sodium and chloride ions bicarbonate was the only other ion absolutely necessary and sufficient for gametocytes to be able to be triggered to exflagellate *in vitro*. No further comment was made on the role of pH in this process, neither was any further consideration given to the possibility that any other factors might have a role in exflagellation as it occurred within a vector mosquito.

In 1976, Richard Carter and Mary Nijhout working at the National Institutes of Health (NIH, Bethesda, Maryland), began to examine these questions using *P. gallinaceum* in chickens as their experimental system (Carter and Nijhout, 1977). The task they undertook was to attempt to resolve the roles of pH and/or CO_2 in triggering gametocytes to exflagellate *in vitro*. Using a gas-tight perfusion chamber, the Dvorak-Stotler chamber, designed with an observation window for high-power microscopy, Nijhout and Carter perfused suspensions of *P. gallinaceum*-infected chicken blood with bicarbonate saline solutions at different pH, bicarbonate ion concentrations and CO_2 gas tensions, such that a wide range of physiological values of each was represented. Precise values of pH and CO_2 tensions were measured in the solutions perfusing the chambers using a blood/gas analyzer. The results showed, unequivocally, that, in the presence of bicarbonate ion in any concentration within a physiological range between 15 and 100 mM, there was a very tight relationship between the pH and the triggering of exflagellation. Below a pH of 7.6 no

exflagellation ever took place. At pH greater than 7.5 exflagellation was increasingly frequent, reaching a peak at pH 8.0; above this pH exflagellation rates declined again, reaching zero at and above pH 8.5. This has been confirmed with *P. berghei* gametocytes *in vitro* (Billker *et al*., 2000). The values for CO_2 and bicarbonate per se, bore no correlation with exflagellation. However, in the total absence of bicarbonate ion, that is, in a simple Tris-buffered saline solution, no exflagellation occurred at any pH value. Their findings, together with those of Bishop and McConnachie (1960) showed that the *in vitro* triggering of exflagellation in blood exposed to air is absolutely dependent upon the presence of bicarbonate ions in the blood and is determined by its attained pH, being triggered once this rises above about 7.7. Although the absolute tension of CO_2 in the blood does not in itself determine the triggering of exflagellation, it is unquestionably the release of CO_2 from blood upon its exposure to atmospheric air that is responsible for the rise in pH.

The question of what really triggers exflagellation in the mid-gut of a blood-fed mosquito had not, however, been resolved. The measurements of pH in the chicken blood meals of *A. aegypti* (a vector of *P. gallinaceum*) by Bishop and McConnachie (1956) had shown most of these, especially when measured within 20 min of the start of blood engorgement (range pH 7.66–7.90), to lie within the range at which Carter and Nijhout (1977) had shown that bicarbonate-dependent triggering of exflagellation does occur *in vitro*. In contrast, Micks *et al*. (1948) had suggested that factors in the mosquito might be involved although neither they nor Bishop and McConnachie (1956) had been able to obtain direct evidence for the activity of such factor(s). Nijhout now conducted a simple experiment that showed the mosquito blood meal not only contained a factor that triggers exflagellation, but that it did so quite independently of the pH/bicarbonate system involved in exflagellation of blood simply exposed to air (Nijhout, 1979). With *P. gallinaceum* gametocyte-infected chicken blood washed entirely free from all plasma components and re-suspended in the simple Tris-buffered, glucose-containing, saline solution at pH 7.4, routinely used in the experiments by Carter as a means of keeping gametocytes viable but untriggered for exflagellation, she introduced it by enema into the mid-guts of male and female *Anopheles* and *Aedes* mosquitoes. On dissection of the mid-guts, the gametocytes were observed to be vigorously exflagellating. Control and other experimental manipulations showed beyond doubt that the mosquitoes produced an extremely potent exflagellation-inducing factor that was not bicarbonate ion. Other than the mosquito mid-gut, Nijhout's experiments showed that the only tissue in which the factor could be identified was the head of the mosquito. She named the factor mosquito exflagellation factor MEF.

In 1998, using the rodent malaria parasite *Plasmodium berghei*, and following advances in mass spectrometry, Oliver Billker and colleagues

working in the laboratories of Robert Sinden and H. E. Morris, identified the mosquito-produced factor (MEF), which they referred to as gametocyte-activating factor (GAF), to be XA, a breakdown product of mosquito eye pigment (Billker *et al*., 1998). XA fulfils all the known biological properties described for MEF, including its activation of gametocytes.

In 2000, Billker *et al*. (2000) found that in contrast to the *in vitro* situation, blood ingested by the mosquito undergoes only a small shift in pH and this is suboptimal for the induction of exflagellation by *P. berghei* (when compared to *in vitro* where the value is at least 7.8 for half-maximal activity). This suggests that XA is able to reduce the size of the pH change required for the induction of gametocytogenesis. Interestingly there are mosquito species differences in their production of XA (quoted in Siden-Kiamos and Louis, 2004), while different species of malaria parasite also differ in their sensitivity to having exflagellation induced by XA. *P. gallinaceum* gametocytes exflagellate when the concentration of XA is in the range of 10^{-7} M, *P. falciparum* needs 25 times and *P. berghei* and *P. yoelii* 100 times this amount (Arai *et al*., 2001).

The role of temperature in the control of gametogenesis has not been formally reported upon since the clarification of the bicarbonate/pH control of the spontaneous exflagellation of *Plasmodium* gametocytes in blood exposed to air and the induction by XA of gametogenesis in mosquitoes. Unpublished laboratory records of Carter attest, however, that the induction of exflagellation, for example, by gametocytes of *P. gallinaceum* and *P. falciparum*, cannot be effected by any known means, bicarbonate/pH or MEF, so long as the gametocytes are not allowed to fall below the circulating blood temperature of their natural host species. Once, however, a fall in temperature has taken place by as little as 2 °C, or perhaps less, below that of circulating blood, exflagellation can be immediately induced (e.g. Table 7.3 in Carter and Graves, 1988). Comparable findings for temperature dependence of exflagellation in *P. gallinaceum* and *P. yoelii nigeriensis*, have been described (Sinden and Croll, 1975), although in these data the required temperature drop for exflagellation was in the range of 5 °C and 10 °C, respectively, below the normal temperature of the host's circulation (see Fig. 23 in Sinden, 1983).

Although chemotaxis of male and female gametes has not been witnessed in exflagellating blood on a slide under a cover slip, red blood cells and female gametes are drawn in sudden shooting movements into the centre of an exflagellating male gametocyte. Before long, six or so extracellular female gametes can be seen being jostled in the same center along with a 'rosette' of red blood cells. Aggregates form by an interaction with sialic acid residues on the red cells (Templeton *et al*., 1998) by both male and female gametes and it is within the aggregate in the blood bolus that fertilization and zygote formation take place and formation of the ookinete occurs. The ookinete moves towards the edge of the blood bolus

where it encounters the peritrophic matrix and the microvillar network that covers the mid-gut epithelium. After traversing through or between the epithelial cells, the ookinete comes to lie below the basal lamina where it transforms into an oocyst (Siden-Kiamos and Louis, 2004).

A method for *in vitro* production of ookinetes from zygotes in *P. gallinaceum* was reported by Carter *et al*. (1979) and used in the biochemical and electron microscopic characterization of ookinetes (Carter and Kaushal, 1984); Aikawa *et al*.'s (1984) *in vitro* production of ookinetes from *in vitro* or *in vivo* gametocytes following culture for 20–24 h was described for *P. berghei* by Janse *et al*. (1985). Syafruddin *et al*. (1992) found that in this species the development from gametocyte to oocyst occurred in a medium consisting of only RPMI 1640 or Grace's medium supplemented with 10% or 20% bovine serum albumin (BSA) or 5% silkworm serum. The presence of insect cells (*Aedes* or *Toxorhynchites* spp.) increased the number of oocysts by about six-fold compared to those in the absence of cells, but sporozoites did not develop. In contrast, ookinete culture of *P. gallinaceum* and *P. falciparum* was achieved in supplemented RPMI 1640 at a pH of 7.8–8.0 (Sinden *et al*., 1996).

Ookinetes of *P. berghei* can be isolated from cultures by Nycodenz density gradients or magnetic isolation (Carter *et al*., 2003) with the latter producing cleaner preparations more quickly. As noted above, transformation of *P. berghei* zygotes was achieved using the methods of Sinden *et al*. (1996) and did not require the presence of insect cells, however, transformation rates were increased significantly when mosquito cell lines and *Drosophila* L2 cells were present (Mazzacano *et al*., 1998). Although the role of insect cells for transformation remains unclear, it has been suggested that they may provide a continuing supply of unstable compounds that are rapidly utilized or degraded. Some have suggested that these materials could be laminin-γ and collagen type IV.

Warburg and Schneider (1993) reported *in vitro* ookinete development of *P. falciparum* using lectin agglutination to remove erythrocytes, plus addition of the basement membrane-like substrate Matrigel and *Drosophila* cells, however, this system has been reported to have low reproducibility (Hurd *et al*., 2003). In 1992, Warburg and Miller described the transformation of *P. falciparum* and *P. berghei* into sporozoites when Matrigel and a co-cultured cell line (*Drosophila* S2 cells) were present. Sporozoite development took 12–16 days with *P. falciparum*. However, only 10–30% of the oocysts developed to maturity, very few sporozoites were produced and these were not infective for mosquitoes. By contrast, Siden-Kamos *et al*. (2000) were able to develop *P. berghei* oocysts within a variety of insect cells as well as extracellularly; in this case Matrigel was not required. In a follow up study, Al-Olayan *et al*. (2002) were able to obtain mature oocysts in 15 days using Schneider's medium, a medium more closely akin to mosquito hemolymph, at a pH of 6.8, and with

co-cultured cells and Matrigel. The addition of increased concentrations of *p*-aminobenzoic acid (pABA; from 11 to 44 nM) enhanced oocyst yield and speed of growth. By day 15, oocysts were mature. The need for Matrigel they suggested was because it mimicked the basal lamina of the mid-gut epithelium. In support of this Vlachou *et al*. (2001) found the mosquito laminin-γ to bind to the ookinete surface proteins P25 and P28 as well as to secreted ookinete adhesive protein (SOAP; Dessens *et al*., 2003) and circumsporozoite- and thrombospondin-related adhesive protein (TRAP)-related protein (CTRP; Mahairaki *et al*., 2005) and when Arrighi *et al*. (2005) used a double-stranded ribonucleic acid (RNA) construct targeted against the *LANB2* gene (laminin-γ1) of *An. gambiae* there was a significant reduction in the number of oocysts coincident with a reduction in *LANB2* expression.

In vivo it is necessary for the ookinete to pass through the mid-gut epithelium, however, this migration is not obligatory for oocyst development. More than 50 years ago, A. B. Weathersby (1952; 1954), working in Huff's NMRI laboratory, showed that sporogony could be completed in the hemocoel of susceptible mosquitoes when parasites were directly injected into this hemolymph-containing space. In spite of this, it has been impossible to culture all of the sporogonic stages without the presence of a co-cultured insect line. Why is it that ookinete to oocyst development can take place *in vitro* with only cells and does not require added extracellular matrix? In many of the cell lines used it may be the cells themselves that provide the extracellular matrix. Indeed, in *Drosophila* the genes encoding extracellular matrix proteins were found to be essential for development to the adult. By contrast, Carter *et al*. (2007) recently demonstrated, using ookinetes that had been obtained *in vitro* as a starting point, that transformation will occur in the absence of any source of basal lamina and that adhesion to a solid substrate or other cells was not required in an ookinete culture medium (OCM). The OCM is a complex medium containing 23.8-mM sodium bicarbonate, 3.68-μM hypoxanthine, antibiotics (streptomycin, penicillin, gentamycin), 44-μM pABA, 15% heat-inactivated foetal bovine serum, 0.2% lipid/cholesterol and 85% Schneider's medium. (Schneider's medium, described in 1968, has a higher osmolarity with altered concentrations of inorganic salts and sugars and lacks organic acids and phosphate buffer.)

Carter *et al*. (2007) speculated that in *P. berghei* ookinete transformation may be driven by soluble factors derived from the hemolymph. Transformation appeared not to be a simple time-dependent phenomenon and an environmental stimulus (not the basal lamina!) was required. The first stage in the transformation was bicarbonate dependent, however, full transformation required suitable nutrients and a suitable pH (pH 7–8).

'A simple *in vitro* system (for sporogonic stages) could be an indispensable tool in the development of transmission-blocking antibodies as

well as anti-sporozoite vaccines' (Hurd *et al*., 2003). *In vitro* development of sporogonic stages has been achieved, however, the system remains far from simple and many of the factors required remain to be identified. There is still a need to achieve reproducibility and consistency in the *in vitro* models used. As the last decade has shown, understanding the basis for culture failure may be as important as knowing the factors that have led to success. What remains for the future are answers to: Why are some *Plasmodium* spp. or lines of a particular species more amenable to culture than others? What are the critical components of the culture media? What are the environmental and chemical stimuli that underlie development? How does *in vitro* development relate to that *in vivo*? What are the biochemical characteristics of the sporogonic stages?

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CHAPTER 6

Malaria Pigment

I was born in a small private hospital on the eastside of lower Manhattan on 12 February 1933. My parents were immigrants from Russia who had lived in the same village (Okna) and met and married after coming to the United States. My father was a factory manager and my mother was a housewife. Our family was distinctly at the lower end of the middle class. I attended the local public schools (P.S. 93 and Hermann Ridder Junior High School and James Monroe High School). After graduating from high school, I enrolled at the City College of New York (CCNY, New York) where I majored in biology and education. CCNY was famous for its academic rigor and a student body composed of high achievers. As the time of graduation approached, several of my biology professors, especially James Dawson (the Biology Department's chair and a protozoologist), William Tavolga (a behaviorist/histologist) and Herman Spieth (a *Drosophila* geneticist/behaviorist) encouraged me to go on to graduate school. Through personal contact between Tavolga and Spieth I was introduced to Libbie Hyman at the American Museum of Natural History and Dawson and Spieth arranged that W. C. Allee (who, because of his age, had been forced to retire from the University of Chicago and was now heading the Zoology Department at the University of Florida (Gainesville, Florida)) accept me as a graduate student; Allee in turn arranged a research assistantship for me with the protozoologist James B. Lackey in the Department of Sanitary Engineering.

My first independent research project involved a survey of oligotrich ciliates (protozoans) in the Gulf of Mexico. Just as I was becoming proficient with ciliate taxonomy and behavior I received a letter from the Selective Service Board—I was drafted into the United States Army. This interruption of my graduate studies turned out to be an opportunity to learn some parasitology and to travel the world at Uncle Sam's

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expense. After infantry basic training (Fort Dix, NJ) I was sent to Fort Sam Houston in Texas and then to Valley Forge Army Hospital in Pennsylvania to be trained as a medical technologist. I was then shipped overseas, where, through good fortune, I was assigned to medical laboratories in Salzburg, Austria and Darmstadt and Heidelberg, Germany. In Germany and Austria, I worked in clinical laboratories doing hematology, parasitology, microbiology, phlebotomy and blood chemistry. Upon my discharge from the United States Army (where by an act of Congress I received the exalted rank of Private First Class) I decided not to return to Florida to continue my graduate studies; instead I began to teach science and mathematics in a junior high school in Yonkers, NY. At the same time I took a few graduate courses at CCNY and assisted in the teaching of the evening session introductory biology laboratories at CCNY. Knowing of my interests in research, William Etkin, an endocrinologist/behaviorist and a professor at CCNY, encouraged me to return to graduate school on a full-time basis. He said, 'If you want to run with the hounds and do some original research get yourself an advanced degree.' It was sage advice.

During the summer of 1957, thanks to a CCNY Biology Club Scholarship, I took the invertebrate zoology course at the Marine Biological Laboratory (MBL) in Woods Hole (Massachusetts). At the MBL I was able to spend all day, every day, for 10 weeks studying live invertebrates. The field trips were extraordinary, especially for a South Bronx slum kid, and the faculty was exceptional: Clark Read (Rice University, Houston, Texas), Howard Schneiderman (Case Western Reserve University, Cleveland, Ohio), John Buck (National Institutes of Health (NIH), Bethesda, Maryland), Theodore Bullock (University of California, Los Angeles, UCLA), Grover Stephens (Minnesota) and Ralph Smith (University of California, Berkeley, California). At the end of that summer I was invited to be the laboratory assistant for the course. I readily accepted and for the next two summers shared the work with another young parasitologist, Frank Friedl, from the University of Minnesota.

The experiences in the United States Army and the MBL crystallized my interests in combining protozoology with clinical disease. I scoured university catalogues for those offering the greatest number of courses in parasitology and eventually settled on Northwestern University (or more accurately, they settled on me). I was awarded an Abbott Laboratory Fellowship with Robert Hull (a student of the eminent protozoologist R. R. Kudo). Hull (who had little training either in parasitology or biochemistry) had an NIH grant and together with a graduate student (Father Truong, a Vietnamese priest) they had begun a malaria project with *Plasmodium lophurae* and chickens.

When I was at the start of my graduate studies very few laboratories in the United States were working on malaria biochemistry and those who

had done so previously (see p. 12) were carrying out biochemical research on other subjects. Hull's laboratory had minimal instrumentation: there was an ancient Warburg manometer, an assortment of low-speed centrifuges, a Klett colorimeter, a DU spectrophotometer and a paper electrophoresis apparatus but there was no possibility for working with radioisotopes. Hull, who did no bench work, appeared not to be very knowledgeable about malaria and was more interested in his favorite organism the suctorian, *Tokophrya*, so no specific malaria project was assigned to me. It soon became apparent that if I wanted to study the biochemistry of malaria I would have to do it on my own by learning more about the parasite and then I would have to find mentors who could teach me biochemistry. My first year as a graduate student was spent reading the literature and taking notes.

Hemozoin, the brown–black pigment found within organs such as the spleen and liver as well as in erythrocytes of those infected with malaria, has long been a source of fascination for students of the disease (Sullivan, 2002a). Laveran's finding (1880) of pigmented bodies in the blood of a soldier suffering with fever led to his discovery of the malarial parasite (see p. 3) and when Ross examined the stomachs of mosquitoes that had fed on a malaria patient and found 'each of these bodies contained . . . granules of black pigment absolutely identical in appearance . . . with the . . . characteristic pigment of the parasite of malaria' it provided the critical clue to mosquitoes as vectors of the disease (see p. 5).

At Northwestern University, during my time as a graduate student, *P. lophurae* was maintained in chickens by intravenous inoculation. The method is rather simple, however, the amount of blood obtained from chicks (relative to ducklings) is small (5–10 ml), and because of age immunity the animals would not provide sufficient numbers of parasites when they were older than 5–6 weeks and weighed more than 200 g. As a result, most of my time during the first year involved passing the infection by blood inoculation every 3–4 days, measuring blood volumes, determining whether there were statistically significant differences between blood smears and trying to block natural immunity using carbon ink. By taking courses in biochemistry (with Lazlo Lorand) and biophysics (with Irving Klotz) I became more familiar with biochemical techniques and approaches. I first became curious about the composition and mode of formation of hemozoin when I peered through a microscope to examine a blood film taken from a chicken infected with *P. lophurae* and saw the parasites filled with refractile golden-brown granules. As a consequence, one of my doctoral thesis projects became a characterization of *P. lophurae* hemozoin.

In 1891, Carbone reported that hemozoin had spectral properties similar to the iron-containing heme of hemoglobin and not melanin as had been proposed by Meckel (1847). Brown (1911) rediscovered this

work and confirmed that it was hematin or ferriprotoporphyrin (FP). When Brown found intravenous injections of hematin produced a malaria-like paroxysm he suggested the pigment was involved in the production of the febrile attack. Later studies were consistent with FP being a constituent of hemozoin, however, there was other work—particularly that of Deegan and Maegraith (1956 a,b) at the Liverpool School of Tropical Medicine (LSTM, Liverpool, United Kingdom)—that indicated malaria pigment was more than monomers of FP. Deegan and Maegraith's reasoning was that hemozoin could not be hematin since the latter would inhibit parasite succinoxidase, an enzyme vital to parasite survival. They proposed that vigorous extraction procedures (used by others) had cleaved the heme from its associated protein and by using milder extraction procedures found evidence for hemozoin being heme plus a proteinaceous moiety. Enlisting the support of Professors Lorand and Klotz, I was able to characterize malaria pigment spectrophotometrically and was able to carry out studies employing analytical ultracentrifugation (Sherman and Hull, 1960). The conclusion from this work with *P. lophurae* reaffirmed the work of Deegan and Maegraith.

Years later (when newer biochemical/immunological methods were available), I once again became interested in malaria pigment (Yamada and Sherman, 1979). Together with a graduate student, Kenneth Yamada, we isolated hemozoin: erythrocyte-free parasites were homogenized using a French pressure cell; the suspension was centrifuged, the pellet recovered, sonicated and ultracentrifuged through a 1.7-M sucrose cushion. Using the techniques of peptide mapping, polyacrylamide gel electrophoresis (PAGE), gel (Sephadex) chromatography, as well as immuno-double diffusion we found hemozoin to consist of a complex of monomers and dimers of hematin, a 14-kDa protein, a 21-kDa protein (presumed to be of parasite origin coupled to heme) and methemoglobin. Electron microscopy of the purified hemozoin showed identity to that seen in sections of intact parasites. Supporting such work were reports by Ashong *et al*. (1989) who claimed protein was associated with FP, and the findings of Goldie *et al*. (1990) that hemozoin was a mixture of native and denatured globin. However, others claimed the non-covalently bound proteins resulted from contaminants (Fitch and Kanjananggulpan, 1987; Homewood *et al*., 1975) and when these were removed the pigment was identical to β-hematin. Even analyses by sophisticated biophysical methods did not completely clarify the nature of hemozoin: Morselt *et al*. (1973) using microspectrophotometry on intact cells found the pigment to differ from pure hematin, whereas the work of Bohle *et al*. (1997), Wood *et al*. (2003) and Pagola *et al*. (2000) found hemozoin to be identical with a cyclic dimer of β-hematin. Once hemozoin was equated with β-hematin subsequent investigations concerned themselves with its mode of formation.

The literature is now replete with methods to synthesize β-hematin in the laboratory (Fitch, 2004; Sullivan, 2002a). Slater *et al*. (1991) postulated that hemozoin formation involved the 'enzymatic polymerization of heme into a non-toxic crystal' (i.e. β-hematin), however, later studies showed that a 'heme polymerase' did not exist and FP conversion to β-hematin could occur spontaneously in the absence of biological materials with solutions high in acetate concentrations and low in pH, but to increase the rate of formation the temperature had to be raised to 60 °C (Egan *et al*., 2000, 2001). However, formation of β-hematin did not occur under physiological conditions or at pH 5 with preparations from uninfected red cells; it did form at pH 5 with preparations from infected erythrocytes. A more recent study has shown that under acid conditions β-hematin assembles rapidly and spontaneously near long-chain alcohol/water and lipid/water interfaces (Egan *et al*., 2006). However, the morphology of the dimers is not identical to natural hemozoin.

Although a considerable body of work describing the role of β-hematin in immunological and pathological events now exists (see below) I have continued to believe hemozoin is not identical to β-hematin. Indeed, many of the discrepancies regarding the chemical nature of hemozoin do not reflect the analytical techniques used, but largely depend on the manner by which the pigment is isolated and purified. For example, if hemozoin is isolated by physical means (mechanical breakage of cells or isolated food vacuoles (FVs) containing the pigment granules followed by density-gradient centrifugation) and the colored material is solubilized in alkali (0.1 N NaOH) the absorption spectrum is consistent with heme coupled to protein; further, as noted above immunological and chemical analysis of the associated protein (proteins?) has shown it to be different from native globin. However, when hemozoin is isolated by extensive proteolytic digestion and lipid extraction then the resultant product consists of crystals of β-hematin. Hemozoins from various plasmodia, when examined by electron microscopy (see Noland *et al*., 2003; Scheibel and Sherman, 1988) are so variable in morphology it is difficult to conceive of hemozoin as being pure crystals of β-hematin. This notion of lack of equivalence of β-hematin and hemozoin has received support from two recent studies. Parroche *et al*. (2007) found malarial deoxyribonucleic acid (DNA) to be a component of hemozoin when isolated using a strong magnetic field; in addition there was a 'ladder of Coomasie -stained proteins.' In another study when hemozoin, prepared from disrupted *P. falciparum*, was ultracentrifuged through a 1.7-M sucrose cushion (as we had done previously with *P. lophurae*) a paucity of proteins was found, however, the pigment granules were enveloped by neutral lipid (NL) nanospheres (Pisciotta *et al*., 2007).

What roles does hemozoin play and how is it made? Coy Fitch began to study hemozoin in the late 1960s when he was at Walter Reed Army

Institute of Research (WRAIR, Washington, DC). After finding that a chloroquine-resistant strain of *P. berghei* was deficient in a high-affinity receptor for the drug (Fitch, 1969, 1970), he went on to identify FP as the receptor (Chou *et al*., 1980; Fitch *et al*., 1974); later he found hemozoin to be dimerized FP similar to β-hematin (Fitch and Kanjananggulpan, 1987). Recently, he speculated that unsaturated fatty acids (FAs) and their mono- and diglycerides in the FV serve to concentrate the monomeric FP and keep it in a state favourable for dimerization (Fitch *et al*., 2003; Fitch and Russell, 2006).

Coy Fitch (1934–) as a medical student at the University of Arkansas School of Medicine (Little Rock, Arkansas), became interested in biochemistry and was provided research space and other resources. He earned an master of science (MS) in biochemistry as well as a doctor of medicine (MD) in 1958 and remained at the School of Medicine as a resident in medicine and Russell M. Wilder-National Vitamin Foundation Fellow in biochemistry (1958–1962). By the time the residency was completed he had developed an interest in membrane transport processes, and he remained on the faculty of the University of Arkansas as an assistant professor of medicine and biochemistry studying such. Five years later, he moved to the Saint Louis University School of Medicine (Saint Louis, Missouri) as Associate Professor of Internal Medicine and Biochemistry. That move made him vulnerable to military service. As a consequence, he was promptly drafted into the Army and assigned to the Division of Biochemistry of the WRAIR.

The director of the Division of Biochemistry at WRAIR allowed Coy to have an independent laboratory but stipulated that it had to be devoted to malaria research. He decided to study the biochemistry of chloroquine (CQ) accumulation in erythrocytes infected with malaria parasites. This decision was based on a recent report that mouse erythrocytes infected with a CQ-susceptible strain of *Plasmodium berghei* accumulated more CQ than erythrocytes infected with a CQ-resistant strain. He thought he would be studying a membrane transport process; however, he soon realized that the movement of chloroquine across biological membranes was far too fast to measure with the centrifugation techniques available for his use. Nevertheless, by the end of his Army career (1967–1969), he had learned that CQ-resistant malaria parasites are deficient in a high-affinity receptor (target) for the drug.

Returning to Saint Louis, Coy continued to study CQ accumulation by malaria parasites, focusing on the receptor. In 1974, he and his coworkers were able to describe the specificity and affinity of the binding site, so the receptor could be recognized when isolated. After

describing the high-affinity binding site, several years were spent searching for a high-affinity protein receptor for CQ in malaria parasites, only to find none. In 1980, his group discovered that ferriprotoporphyrin IX (FP) had the specificity and affinity characteristics of the CQ receptor as had been suspected earlier by Macomber, Sprinz and Tousimis (Macomber and Sprinz, 1967). His group also found that FP and its complex with CQ are toxic for erythrocytes and malaria parasites (Banyal and Fitch, 1982; Fitch *et al*., 1982). These observations led Fitch and co-workers to propose that FP mediates the chemotherapeutic action of CQ and to ask how malaria parasites escape FP toxicity while ingesting and metabolizing hemoglobin. Believing the lack of toxicity to result from FP sequestration in hemozoin, they decided to characterize the pigment of hemozoin better. This pigment appeared to be an aggregate of FP that was similar, perhaps identical, to β-hematin (Fitch and Kanjananggulpan, 1987). Several years later, β-hematin was found to be aggregated dimers of FP (Pagola *et al*., 2000).

Since FP is rendered non-toxic by converting it to β-hematin, it was now logical to study this process. In 1992, Chou and Fitch discovered that FP dimerization could be measured easily and that CQ treatment reduces FP dimerization *in vivo*. At first, Chou and Fitch and others (Slater and Cerami, 1992) assumed that an enzyme catalysed the reaction; however, that assumption was wrong. Instead, it was found that the reaction is catalysed in malaria parasites by unsaturated FAs, probably predominantly linoleic acid. Furthermore, Fitch and co-workers (2003) obtained evidence that CQ treatment causes this catalyst to be masked (i.e. unavailable to catalyse FP dimerization) in CQ-susceptible malaria parasites, thus explaining the accumulation of toxic, undimerized FP in response to CQ treatment.

The next task was to explain why unsaturated FAs are masked after CQ treatment. Based on their findings, Fitch and Russell (2006) proposed that the CQ–FP complex inhibits endosomal maturation and, thereby, reduces the release of unsaturated FAs from the membranes of erythrocytoid bodies. These bodies are found in endosomes early in the feeding process of malaria parasites. This explains, Coy believes, how CQ treatment results in masking of unsaturated FAs, reduces FP dimerization, causes toxic undimerized FP to accumulate and be available to bind CQ, and also causes denatured hemoglobin to accumulate in vesicles in malaria parasites (Fitch *et al*., 2003; Macomber and Sprinz, 1967; Warhurst and Hockley, 1967b). In this scenario, the toxic, undimerized FP that binds CQ and mediates its chemotherapeutic effect is derived from hemoglobin as it is denatured or hydrolyzed in immature endosomes. Currently, Coy is devoting his time to determining how the CQ–FP complex inhibits endosomal maturation.

Fitch's work on β-hematin synthesis (Fitch, 2004; Fitch *et al*., 1999; 2000) has recently been extended by Pisciotta *et al*. (2007). Hemozoin, isolated from saponin-purified *P. falciparum* trophozoites, followed by osmotic lysis and sonication and sedimentation through a sucrose cushion, was associated with lipid nanospheres consisting mainly of saturated lipids monostearic glycerol (MSG) and monopalmitic glycerol (MPG) with the principal diacylglycerols (DAGs) being distearic glycerol and 1-stearic-3-palmitic glycerol; no monooleic glycerol (MOG) or monolinoleic glycerol (MLG) was found.

The source of these may be the neutral lipid bodies (NLBs) containing triacylglycerol (TAG) and DAG, which are associated with the FVs (Jackson *et al*., 2004). The scenario proposed is: the double-membrane vesicle produced during cytostomal feeding (see p. 171) has its outer membrane fused with the FV membrane while its inner membrane is degraded by phospholipase C and/or lysosomal acid lipase (and found in the falciparum genome); the breakdown products are assembled into TAG and its precursors serve as promoters of β-hematin formation.

Although Fitch *et al*. (1999) found TAG to be inactive in β-hematin formation Jackson *et al*. (2004) found MOG as well as mono- and dimyristoyl glycerol to be effective. In contrast, Pisciotta *et al*. (2007) found MPG to be a potent promoter of heme crystallization as was the combination of 1-stearic-3-palmitic glycerol. A NL blend of MPG/MSG/dipalmitic glycerol (DPG)/dioleic glycerol (DOG)/dilinoleic glycerol (DLG) (2:4:1:1:1) produced heme crystals rapidly. Of some interest is their observation, 'the lipid blend crystals did not exactly replicate hemozoin made by *P. falciparum* (and) may require the presence of non-specific proteins or other molecular species.'

What is the role of hemozoin? Due to the massive degradation of hemoglobin a large amount of free heme is produced. Because free heme may intercalate with plasmodial membranes to lyse parasites and plasmodia lack the enzymes necessary to degrade the porphyrin ring the incorporation of the heme moieties into insoluble hemozoin has been postulated to be a detoxification mechanism (Fitch and Chevli, 1981; Fitch *et al*., 1982). Other postulates are peroxidative degradation within the FV (Loria *et al*., 1999) and glutathione (GSH)-mediated heme degradation in the cytosol (Ginsburg *et al*., 1998).

Hemozoin (in the form of α- and β-hematin) has been reported to suppress erythropoiesis (Casals-Pascual *et al*., 2006), and after endothelial cell ingestion suppresses ICAM-1 and PECAM-1 expression, as well as production of interleukin (IL)-6 (Taramelli *et al*., 1998), and it can lyse red blood cells by incorporation into the membrane (Omodeo-Sale *et al*., 2005). In its native form, hemozoin has been shown to inhibit the function and maturation of dendritic cells (Millington *et al*., 2006; Skorokhod *et al*., 2004; Urban and Todryk, 2006) and to enhance matrix metalloproteinase activity as well as tumor necrosis factor (TNF)-α production in monocytes (Prato

et al., 2005). Murine phagocytes fed natural hemozoin or FP plus supplemental interferon (IFN)-γ increased their expression of nitric oxide (NO) synthase and NO (Jaramillo *et al*., 2003), however, human monocytes were neither able to generate NO nor to increase expression of NO synthase under the same conditions even with IFN-γ (Skorokhod *et al*., 2007b). Further, there was little evidence of NO killing *P. falciparum in vivo* and in a Papua New Guinea study no correlation between NO levels and parasitemia was found in human subjects (Boutlis *et al*., 2004). Clearly, extrapolation of murine data to human malaria may not be justified and caution should be exercised when doing so.

Others report that β-hematin does not promote such activities directly, but that it is the heme-catalysed peroxidation of cellular debris that results in a toxic gradient of primary and secondary FA oxidation products (15-hydroxyeicosatetraenoic acid (15-HETE) and 4-hydroxynonenal (HNE)) that not only activates the innate immune response (Carney *et al*., 2006) the HNE-protein adducts but also acts to decrease red cell deformability (Skorokhod *et al*., 2007a), promotes their removal and leads to anaemia.

Coban *et al*. (2005) contend that β-hematin and hemozoin (prepared from a sonicated pellet of parasites, treated with proteinase K, washed in sodium dodecylsulphate (SDS), incubated with 6-M urea) on their own activate the toll-like receptor (TLR)-9 to mediate immune activation. By contrast, Parroche *et al*. (2007) report that β-hematin is immunologically inert, whereas natural hemozoin (isolated as described above) activates the TLR. Further, Parroche *et al*. have provided evidence that the natural pigment acts as a carrier for malarial DNA and that this results in it being targeted to TLR-9. (Malarial DNA on its own was found not to be immunostimulatory.) Although TLR-9 is known to recognize GC-rich areas that are typically found in bacterial DNA, and falciparum DNA is exceedingly AT-rich, there are small GC-rich regions in plasmodial DNA that are able to stimulate TLR-9.

Clearly, the varied and inconsistent pathological and immunological findings result from a lack of equivalence of the 'malaria pigment' used. In my view, despite more than half a century of biochemical research on malaria pigment, much is yet to be learned about its natural composition and its varied roles. Sometimes there is a great temptation on the part of investigators to use a laboratory-synthesized compound that is easy to prepare and mimics the natural product, but extrapolation of the activities of a synthetic product such as β-hematin with natural hemozoin can be misleading. In addition, although β-hematin may have some utility in describing the relationships between different types of anti-malarials (Basilico *et al*., 1998; Egan and Ncokazi, 2005; Ncokazi and Egan, 2005; Solomonov *et al*., 2007; Trang *et al*., 2006) to conclude that the mimic and the authentic malaria pigment are identical is more often than not without adequate justification.

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CHAPTER 7

Chloroquine and Hemozoin

Drug accumulation by lysosomes had first been noticed in mammalian cells by workers at the NIMR and applying this concept to CQ accumulation, Homewood, Warhurst, Peters and Baggaley (1972) were able to explain how CQ could accumulate in the acidic FVs of the *Plasmodium*. Warhurst and co-workers also found that quinine and WR142,490 competitively inhibited the CQ-induced morphological changes in *P. berghei*; later, WR142,490 would be developed as mefloquine (Warhurst and Thomas, 1975).

In 1963, **David Warhurst (1938–)** who received his doctor of philosophy (PhD) at Leicester University, United Kingdom (1964) took a position at National Institute for Medical Research (NIMR, London, United Kingdom) in the Division of Parasitology headed by Frank Hawking. Working under a grant from the World Health Organization (WHO, Geneva, Switzerland) he was drawn to the problem of the mode of action of chloroquine (CQ) by its morphological effects on *Plasmodium berghei*-infected red cells, that is, food vacuole (FV) swelling and microscopic clumping of hemozoin due to engulfment of vacuoles in an autophagic vacuole (Warhurst and Hockley, 1967a,b). The clumping effect was seen with other species of malaria (*P. cynomolgi* but not with *P. falciparum* where digestion already occurs in a large single vacuole) and with other 4-aminoquinolines related to CQ, and this correlated with anti-plasmodial activity. In addition, a proportionality was found between anti-malarial activity for 4-aminoquinolines and quinine and the inhibition of β-hematin formation (Warhurst, 1987; Warhurst *et al*., 2003).

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In the early 1960s the mode of action of chloroquine was generally believed to be through binding to deoxyribonucleic acid (DNA), and the evidence, particularly from Hahn's group at the Walter Reed Army Institute of Research (WRAIR, Washington), was thought to be conclusive (Ciak and Hahn, 1966). However, as discussed above, subsequent studies by Warhurst and colleagues demonstrated that the mechanism was incorrect. Since that time various other theories have been postulated as to the mechanism of action of CQ (Fitch, 2004; Jiang *et al*., 2006; Sullivan, 2002b). Chou and Fitch (1980) and Orjih *et al*. (1981) found that ferriprotoporphyrin (FP) and its complex with CQ were toxic for erythrocytes and malaria parasites; since CQ treatment reduced FP dimerization *in vivo* in CQ-sensitive *P. berghei*-infected red cells, Chou and Fitch (1993) claimed this explains the accumulation of toxic, undimerized FP after CQ treatment, and that the chemotherapeutic effect is due to toxic, undimerized FP that binds CQ. Sullivan (2002b) suggests that CQ binds to the FP and caps the growing crystal thereby preventing further FP incorporation; in this theory FP binding to the growing face of the hemozoin crystal is the crucial feature for the action of CQ, and this is only seen when the crystal is growing (proven by incorporation of radiolabelled drug into the hemozoin). Non-covalent binding of CQ to hemozoin would remove CQ from solution equilibrium and lead to its accumulation in the FV. Although this notion was very convincing when the polymer structure of hemozoin was believed, it is presently less so with the varied new 4-aminoquinolines that require a range of possible sites on the hemozoin (Warhurst, personal communication).

The worldwide spread of CQ resistance has led to a significant resurgence of malarial morbidity and mortality concomitant with interest in the manner by which resistance develops. Investigations into the mechanisms of CQ resistance have generated several different models, including reduced influx of CQ, increased efflux of CQ, pH effects on drug accumulation and/or receptor availability, glutathione (GSH) degradation of hematin or formation of CQ-hematin complexes. It is generally accepted that CQ enters the acidic FV by passive diffusion as an uncharged species and becomes trapped in the vacuole in its di-protonated, membrane-impermeable form; the di-protonated CQ is retained in the FV as a hematin-CQ complex. If resistance involves restricted access of CQ to hematin such that there are reduced drug levels in the digestive vacuole, then possible models for how this might be achieved are: (1) efflux of CQ from the digestive vacuole *via* an energy-coupled mechanism, (2) leakage of CQ out of the digestive vacuole down its concentration gradient with energy driving the vacuolar proton pump such that a concentration gradient of protonated CQ is maintained (rather than energy being coupled to drug movement per se), (3) a pH-dependent reduction in CQ accumulation and (4) passive outwards movement of protonated

CQ through a gated aqueous pore (O'Neill *et al*., 2006). It is generally thought that differences in the digestive vacuole pH are not primarily responsible for CQ resistance, therefore, other models have received much more attention and all likely involve the *P. falciparum* chloroquine transporter (PfCRT; Valderramos and Fidock, 2006).

The *PfCRT* gene was identified through the analysis of a genetic cross between a CQ-resistant and a CQ-sensitive clone (Fidock *et al*., 2000). The 45-kDa PfCRT protein has been localized to the digestive vacuolar membrane and it shows extraordinary amino acid sequence diversity among geographic isolates involving as many as 15 residues and in a single resistant line there can be four to eight individual mutations. Indeed, when the K76T (lysine to threonine) mutation is removed resistant parasites become sensitive to CQ. A recent study suggests that the outwards movement of CQ from the digestive vacuole (thereby reducing the binding of CQ to FP) in resistant parasites is not directly coupled to the energy supply (Bray *et al*., 2006). Therefore, an efflux pump and/or an active carrier-mediated transport mechanism is unlikely. The model that best suits the experimental findings is that in CQ-resistant lines a gated aqueous pore permits a passive outwards movement of the protonated form of CQ.

David Fidock (1965–) received his PhD in microbiology from the Institute Pasteur (Paris, France) in 1994, having worked for 5 years in the group of Pierre Druilhe, an expert on *Plasmodium* exo-erythrocytic (EE) stages and malaria immunology. There, Fidock worked on mechanisms of protective immunity induced by irradiated sporozoites, and was awarded the prestigious Bourse Roux that led to his being recruited as a 'Pasteurien' scientist during his graduate years. His sabbatical to the United States led him first to work with Anthony James (University of California, Irvine, Irvine, California) on sporozoite biology and later with Thomas Wellems (National Institutes of Health (NIH), Bethesda, Maryland) on the genetic basis of CQ resistance. It was at NIH that Fidock disproved the leading gene candidate for CQ resistance (CG2) and discovered *PfCRT*, the gene that is now known to be the primary determinant of CQ resistance in *P. falciparum*. In 1999, he set up his own laboratory at the Albert Einstein College of Medicine in the Bronx (New York, New York) and in 2007 moved to Columbia University (New York, New York) where his major research focus continues to be on understanding the genetic and molecular basis of drug resistance in *P. falciparum*, exploring the cell biology of the *P. falciparum* digestive vacuole, and identifying new drug targets and lead anti-malarials.

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CHAPTER 8

Isoenzymes

The winter before I completed my doctor of philosophy (PhD, 1960) I spent the Christmas holidays in New York City visiting my parents. During that time I dropped in to see Frank Friedl, with whom I had shared the invertebrate zoology laboratory assistant duties during the summers of 1958 and 1959. Frank had finished his PhD and was now a post-doctoral fellow at the Rockefeller University (New York, New York) working in the laboratory of the distinguished helminthologist Norman R. Stoll, author of many papers, including the classic '*This wormy world*.' Frank suggested that I stop in and see William Trager on the third floor of Theobald Smith Hall, just across from Stoll's laboratories. Trager was the 'dean' of malaria researchers and although I had no appointment, he graciously listened to my account of what I was doing and heard my future plans. This accidental encounter affected my scientific career for the next 45 years, when Trager offered me a National Institutes of Health (NIH, Bethesda, Maryland) post-doctoral fellowship at Rockefeller University beginning in the autumn of 1960.

As noted earlier, Trager's laboratory was concerned with nutritional studies of *Plasmodium lophurae*. He maintained the infection by blood passage in ducklings, where (unlike that in chickens) the developmental cycles were synchronous, the degree of infection high (as much as 90% of the red cells were infected in 4 days) and the large body size of the duckling (1–2 kg) allowed for the possibility of obtaining up to 100 ml of blood from each duckling. The biochemical equipment consisted of a brand new Beckman DU spectrophotometer (placed in a broom closet!), which Trager was very anxious be used, an assortment of power supplies for electrophoresis, refrigerated centrifuges, fraction collectors, water baths and so on. When I arrived at Rockefeller, my research interests were vague. This was soon to change, largely as a result of Trager having

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attended a conference in Florida where the other speakers were Julius Marmur (Brandeis) and Ernest Bueding (Johns Hopkins). As early as 1955, Bueding (1910–1986) and his co-workers had shown, using immunological methods, the 'Einheit' in biochemistry did not hold: there was enzyme heterogeneity in schistosome as well as in the tissues of the host (Bueding and Mackinnon, 1955; Henion *et al*., 1955). Of course, in this he was at odds with some of the most influential biochemists of the time (Bueding, 1962). During the meeting, Bueding asked Trager, 'Do you think malaria parasites have enzyme heterogeneity?' Returning from the meeting, Trager 'volunteered' me to provide Marmur, who was carrying out a survey of the nucleotide base composition of deoxyribonucleic acid (DNA) from a variety of protozoans using the 'melting' properties of DNA, with a large quantity of erythrocyte-free *P. lophurae* as well as *Trypanosoma lewisi*. He also suggested that I not concern myself with *lophurae* DNA but instead concentrate on the possible heterogeneity of lactate dehydrogenase (LDH) in *P. lophurae* and its duckling host.

By 1959 Markert and Moller (1959) had already demonstrated the presence of isoenzymes in various tissues using an overlay method after zone electrophoresis in starch gels and in 1960 Elliot Vesell, in the Rockefeller laboratory of Alexander Bearn, was separating LDH isoenzymes electrophoretically by starch gel electrophoresis and then directly visualizing the enzyme in the gel. With the help of Vessel and another Trager post-doctoral investigator, Philip D'Alesandro, I was soon able to separate *lophurae* LDH from that of the red blood cell by starch gel and starch block electrophoresis (Sherman, 1961) and to Trager's delight the Beckman DU spectrophotometer was fully utilized. When Marmur's colleagues at Brandeis, Nathan Kaplan and Allan Wilson, heard of the availability of gram quantities of relatively pure malaria parasites they too wanted some for their own work. Wilson, a post-doctoral fellow with Kaplan, was interested in molecular evolution and would go on to become world famous for establishing a 'molecular clock' for human evolution. (Wilson died in 1991 at the age of 56 years while undergoing a bone marrow transplant for leukemia.)

Kaplan received his PhD in 1943 with David M. Greenberg at University of California at Berkeley (Berkeley, California) working on phosphate metabolism in rat liver using ^{32}P and then did post-doctoral work with Fritz Lippmann (who had apprenticed with Otto Meyerhoff in Germany) where he was instrumental in determining the structure of coenzyme A and helped establish its role in the tricarboxylic acid (TCA) cycle. By the time Kaplan established his laboratory at Brandeis University (Waltham, Massachusetts; 1957), he was concentrating on enzymes that required nicotinamide adenine dinucleotide (NAD) as a co-factor. The Kaplan laboratory was actively synthesizing analogues of NAD and had found that the ratio of enzymatic activity with NAD and its various analogues

(especially the acetyl pyridine NAD) was a very sensitive measure of the differences of various dehydrogenases (especially LDH) in different species and in different organs. Wilson had received some of the frozen *P. lophurae* that I had sent to Marmur but found the preparation had lost all its catalytic activity through several freeze–thaw cycles. During a trip to the New York Zoo to pick up a variety of species of birds that had died (and which he intended to study LDH in their muscles) he stopped by to visit us at Rockefeller. He invited me to bring a fresh preparation of packed frozen *P. lophurae* to Brandeis and to present a seminar. During that visit to Brandeis it was discovered that *lophurae* LDH had an exceptionally high affinity for the acetylpyridine analogue of NAD (Sherman, 1966a). This proved to be the case for other species of *Plasmodium* and today LDH activity with acetylpyridine-NAD serves as the basis for the diagnostic tests OptiMal and Malstat (Chiodini *et al*., 2007; Makler *et al*., 1998) for human malaria infections and also as an immunodiagnostic (Tomar *et al*., 2006). It has also been used to measure the adherence of falciparum-infected red cells *in vitro* quantitatively (Prudhomme and Sherman, 1999) and sequestration in comatose children (Seydel *et al*., 2006).

The demonstration of a specific plasmodial LDH settled the question of whether the increased LDH activity found for infected red cells was due to the parasite or stimulation of the LDH of the red cell. Further, it also demonstrated the unique properties of the plasmodial enzyme, that is, it was more active at low pH and at high pyruvate concentrations whereas the red cell enzyme had optimal activity at high pH and low pyruvate concentrations. Presumably this acid sensitivity of LDH (and also phosphofructokinase) is advantageous to the growing parasite as there is an excess of lactic acid formed. It is of some interest to note that Trager (1952, 1953, 1957) found the extracellular growth of *P. lophurae* to be best at pH 6.8–6.9. Similar findings were made for *P. berghei* (Phisphumvidhi and Langer, 1969), *P. yoelii* (Momen, 1979) and *P. falciparum* (Vander Jagt *et al*., 1981, 1982) LDH isozymes. The *LDH* gene from *P. vivax* has been cloned and sequenced (Turgut-Balik *et al*., 2004) and shown to have more than 90% homology to that from *P. falciparum*. The crystal structure of LDH from *P. vivax* with dihydronicotinamide adenine dinucleotide (NADH) and dihydroacetylpyridine adenine dinucleotide (APADH) has shown the active sites and co-factor binding pockets to be similar to that of *P. falciparum* and distinct from the human enzyme (Chaikuad *et al*., 2005). In addition, the *LDH* genes from all four human species of *Plasmodium* have been cloned, sequenced and kinetic properties of the proteins compared (Brown *et al*., 2004) and a series of azole-based compounds that inhibit the LDH of *P. falciparum* has been described (Cameron *et al*., 2004).

Although I was quite happy at Rockefeller—truly a researcher's paradise—I still longed to see whether I could carry out an independent research program and I also wanted an opportunity to teach. In 1962,

I was offered the position of assistant professor of biology at the University of California, at Riverside (UCR, Riverside, California) where one of my former professors from CCNY, Herman Spieth, had moved in 1954 to head up the Department of Biology. At the time I interviewed at UCR, Spieth was the chancellor and it was upon his recommendation that I was eventually selected to join the UCR faculty.

During my early years at UCR, I continued using *P. lophurae* grown in ducklings because it allowed us to obtain large quantities of erythrocyte-'free' parasites each week very inexpensively. At seminars, in discussing our research, I had a standard line that went: a gram of parasites that costs only a buck a duck. I was convinced (as was Trager) that the *lophurae*-duckling system was an appropriate model for human malaria; moreover, it would be another 15 years before *P. falciparum* would be grown *in vitro* in continuous culture.

In addition to LDH, we studied the heterogeneity of several other dehydrogenases, including malic dehydrogenase (MDH) and glutamic dehydrogenase (GDH). We found the cytosolic MDH isozymes from *P. lophurae* and *P. berghei* to differ from those of the host (Sherman, 1966d). We speculated that MDH might play a role in re-oxidizing NADH formed at the triose phosphate dehydrogenase step of glycolysis. Later, MDH from *P. falciparum* was purified (Lang-Unnasch, 1992) by conventional biochemical methods and through the use of an antiserum was shown to be cytoplasmic and not mitochondrial (Lang-Unnasch, 1995). The *falciparum MDH* gene, located on chromosome 6, has been cloned and expressed (Tripathi *et al*., 2004) and has high homology with bacterial LDH and MDH. In support of our earlier work with *P. lophurae*, the *P. falciparum* MDH did not use nicotinamide-adenine dinucleotide phosphate (NADP), but was NAD/NADH specific and used the acetylpyridine analogue of NAD; falciparum MDH could complement the function of falciparum LDH.

The demonstration that glutamate was one of the products of CO_2 fixation (see p. 92) that was accumulated by *P. lophurae*-infected red cells and could be metabolized (Sherman *et al*., 1971b) prompted us to investigate GDH (Sherman *et al*., 1971a). The *lophurae* GDH was shown to be cytosolic and NADP specific. We suggested that in the conversion of glutamate to α-ketoglutarate 'a product of the reaction that may be of considerable significance is the formation of reduced NADP . . . which can be used in reductive synthesis.' (Our other hypothesis that α-ketoglutarate might be a supplemental source of energy by feeding into the Krebs cycle would subsequently turn out to be false.) Later, Walter *et al*. (1974) described a GDH isozyme in *P. chabaudi* and Vander Jagt *et al*. (1982), supported by funds from United States Agency for International Development (USAID), showed the falciparum enzyme to be specific for the parasite and thus a useful tool for monitoring the contamination of

sub-cellular fractions (intended for use as antigens). Vander Jagt *et al*. (1989) using conventional biochemical methods found GDH purified from *P. falciparum* to have a relative molecular mass (M_r) of 230 kDa and capable of producing nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) at about 10% of the capacity of the red cell with activity increasing throughout plasmodial development. The falciparum GDH was not inhibited by chloroquine. They went on to suggest that since the isocitric dehydrogenase (IDH) had higher activity it could offer the parasite an additional pathway for NADPH synthesis. Further characterization of the falciparum GDH has come from cloning and expression in *Escherichia coli*. It is a homohexamer with a subunit M_r of 49.5 kDa; its 470 amino acids show 23% sequence homology with the human enzyme and 50% homology with that from lower eukaryotes and bacteria. The enzyme has been crystallized and the most obvious differences between the human and plasmodial enzyme are the subunit interfaces of the hexameric protein and the unique *N*-terminal extension of the falciparum GDH (Wagner *et al*., 1998; Werner *et al*., 2005). Although these investigators did not demonstrate directly that the source of glutamate was glutamine and not CO_2 fixation, their proposal receives support from the following observations: there is a 100-fold increase in glutamine accumulation by falciparum-infected red cells (Kutner *et al*., 1982) and addition of glutamine to the RPMI 1640 medium enhances the *in vitro* growth of *P. falciparum*. According to their proposal (Wagner *et al*., 1998), the glutamine would be converted to glutamate by the parasite synthase, glutamine amide α-ketoglutarate amidotransferase (GOGAT):

$$\text{glutamine} + 2\alpha - \text{ketoglutarate} + \text{NADPH} \rightarrow 2\text{glutamate} + \text{NADP}.$$

Although the GOGAT reaction consumes one NADPH it produces two molecules of glutamate that can yield, *via* GDH, two NADPH that can in turn serve as an electron source for the anti-oxidative enzymes glutathione reductase (GR) and thioredoxin reductase (TrxR; see p. 264–266); glutamine could also be used in pyrimidine biosynthesis. (The glutamate metabolic pathway can be found online at http://sites.huji.ac.il/malaria/maps/glutamatemetpath.html; last accessed 16 July 2008.)

Prior to the advent of recombinant DNA techniques 'enzyme typing' was used to analyze the genetics of *Plasmodium*. Studies on the genetics of malaria parasites began in 1971 at the Institute of Animal Genetics at Edinburgh University (Edinburgh, United Kingdom) when David Walliker and Richard Carter, supported by Medical Research Council (MRC) funds and using isozymes, were able to demonstrate recombination between two lines of *P. yoelii*. A battery of isozymes (adenylate kinase, MDH, glucose phosphate isomerase (GPI), LDH, GDH and 6-phosphogluconate dehydrogenase) were used to differentiate

morphologically indistinguishable samples of *P. falciparum* and to study species differences in the murine malarias *P. berghei, P. yoelii, P. vinckei*, and *P. chabaudi* as well as the variation among parasites in individual wild caught rodents harboring these species (see below). Since that time the genetic structure of *P. falciparum* isolates in Africa, as well as *P. vivax* isolates in India, have been studied using isozymes (Joshi *et al*., 1997). Indeed, as Richard Carter has said, isozymes have been, and continue to be, a powerful taxonomic and genetic tool to unravel the outstanding epidemiological questions.

Richard Carter (1945–) always wanted to lead a life in biology. Recognizing its growing importance in the understanding of life, Carter chose chemistry for his major study at the Edinburgh University. Though not in itself his favorite subject, chemistry, particularly in the form of biochemistry, carried the promise of exciting things to come. In the late spring of 1967, shortly before graduating with a bachelor of science (BSc) degree in biochemistry, Carter set off one day for the Institute of Animal Genetics (which doubled as the Genetics Department of Edinburgh University) and the laboratory of Geoffrey Beale. Beale was one of the first to pick up upon the contributions that biochemical analysis, in the form of protein, and more precisely enzyme electrophoresis, could make to the investigation of genetic differences between organisms. He had just initiated a project to study the genetics of malaria parasites and was in need of a 'biochemist'. That afternoon, Carter appeared at his office door, looking to do a PhD in 'something interesting' in biology. Beale said, 'If you get a good degree, you have the place.' In addition, that is how it all began. Carter's task was to conduct starch gel electrophoresis analysis of 'enzyme variation' in malaria parasites of rodents with the objective of obtaining 'genetic markers' for use in crossing experiments between the parasites.

He learned almost everything on the job. Andrew Tait, a graduate from the same Edinburgh University Biochemistry Department (now a professor in the Veterinary Department at the University of Glasgow, Glasgow, United Kingdom) was Beale's first 'biochemist'. One year senior to Carter, Tait was using starch gel electrophoresis analysis of enzyme variation in *Paramecium* to study their genetics and he showed Carter the craft. His side-by-side partners in the malaria genetics project were David Walliker, a recent post-doctoral scientist, and Andrew Sanderson, who had been acquired as a team from the Department of P. C. C. Garnham, professor of medical protozoology at the London School of Hygiene and Tropical Medicine (LSHTM, London, United Kingdom).

Some time in 1968, Carter saw his first 'enzyme variants'—beautiful little bands of sky blue on a China white gel (a flat slab of starch gel on a glass plate that had been laid between electrodes), each band at a precise and different position on the gel according to the 'strain' of malaria parasite whose content had been extracted for 'electrophoretic separation' (Carter, 1970). They were the biochemical genetic markers of two strains of *Plasmodium berghei yoelii* (as then defined), 17X and 33X that Walliker needed in order to be able to analyze a genetic cross between these two parasites.

Although there were now two genetically distinguishable 'strains' of malaria parasite several questions remained. How many genetically distinct lines of parasite might there really be in a single line, or 'strain', of the parasites isolated in the laboratory? It was, on the face of it, impossible to tell except that Carter now had a genetic marker, the GPI isoenzymes, that could provide an insight into this purely practical question. All 'strains' examined so far showed only one or the other, but never both, of the two enzyme variants. It seemed unlikely that the individual 'strains' contained numerous genetically different parasites within them or surely some would have shown the presence of both enzyme variants. The way forward was to collect more isolates of the rodent malaria parasites. Through Irene Landau and professor A. G. Chabaud at the Museum of Natural History in Paris, who had made major contributions in collection and characterization of rodent malaria parasites in Africa, two consignments of thicket rats, captured in the vicinity of Bangui, in central Africa, were delivered to the laboratory in Edinburgh. Walliker, Sanderson and Carter set about the process of isolation and characterization of the malaria parasites from this collection. Subsequently further isolations from other regions of central tropical Africa were added. By 1974, with a now expanded arsenal of enzyme markers, four unequivocal species of rodent malaria parasites were distinguished, namely *P. berghei, P. yoelii, P. vinckei* and *P. chabaudi*. In most (except *P. berghei*) two to three subspecies were defined, based on their distinctive enzyme types and their different places of geographic origin in tropical Africa (Carter, 1978; Carter and McGregor, 1973; Carter and Walliker, 1975). These taxonomical, and implied phylogenetical, relationships have been upheld by recent molecular genetic analyses based on DNA sequence (Perkins *et al*., 2007).

Carter now concerned himself with his original question, 'How many genetically distinct lines of parasite are there in individual infections?' The thicket rats harboured simultaneously often up to three of the different species of the parasites which by this time had been distinguished. These, and their specific genetic types, as defined by enzyme type, came and went in different combinations in the blood of the wild-caught

thicket rats over the weeks and months of their captivity. Naturally infected animals contained, in short, a small zoo of malaria parasites. Assisted by Laurie Piper, a quantitative geneticist working in the department, Carter devised a mathematical approach to quantify parasite genetic diversity in the blood of an infected animal and applied it to his data. The result implied that typically one to three genetically distinct clones of any given species of the parasites could normally be detected in the blood of naturally infected thicket rats at any given time, for example, when the blood was being isolated into mice.

While all of this was happening Carter met another individual who became very influential in his scientific life. At a laboratory meeting at the LSHTM, in 1972, as he stood beside his poster on some aspect of enzyme variation in rodent malaria parasites, he was approached by Alistair Voller, a senior lecturer at the LSHTM. 'Why aren't you doing this on human malaria parasites?' he asked, to which Carter replied that he would love to. 'It can be done,' said Voller, an experienced worker on human malaria in Africa, and forthwith plans began for a trip to collect human malaria-infected blood from Africa for enzyme analysis. Back in Edinburgh, Carter spoke of the proposal with Beale. He was tolerant but not obviously enthusiastic. Then the situation took an unexpected turn. Beale had been invited by Ian McGregor, who was, in 1972, head of the United Kingdom MRC Laboratories in The Gambia, West Africa, to visit them. He did so and returned with his luggage packed with 177 freeze-dried samples of *P. falciparum*-infected blood that McGregor had collected over several years from human patients. Having had nothing to do with the decision to bring the samples from The Gambia, and knowing nothing about it until they had arrived in Edinburgh, Carter was faced with a painful dilemma. He immediately let Voller know what had happened, and put the case, an entirely valid one, that collections from other areas of Africa would be as important as that from The Gambia. Voller was understandably unhappy, to say the least, at being scooped. 'The job is done,' was his response and their expedition was called off. Carter proceeded to work on the Gambian collection. The material was of superb quality and yielded results that made full use of enzyme typing and of the method of mathematical analysis that he had developed with Laurie Piper. *P. falciparum* was shown to be as genetically diverse in The Gambia as in any of the collections of rodent malaria parasites. The number of detectible genetically distinct clones was likewise shown to be 'a few' - around two per isolate on average (Carter and McGregor, 1973).

In due course Voller was persuaded of the value of continuing the studies in another part of Africa. The expedition he had originally proposed was made, in mid-1973, to Tanzania on the east coast of Africa. Collection and analysis of a few tens of samples of *P. falciparum*-infected

blood showed that the same GPI and LDH diversity existed in *P. falciparum* from Tanzania as from The Gambia and that the variants themselves were in comparable frequencies and combinations in both locations (Carter and Voller, 1975). This suggested the possibility of a freely inter-breeding, or 'panmictic', population of the parasites from the west to the east coast of Africa.

By late 1975, Carter had left rodent malaria parasites and malaria genetics behind, returning to each, by stages, only many years later. In the mean time, the rodent malaria parasites themselves have continued to provide the material of much basic research in malaria, particularly in cell biology, immunology and, of course, genetics. First, using isoenzyme typing, and later employing molecular techniques, the population genetics of *P. falciparum* and other malaria parasites of humans has become a vigorously investigated field.

Today, it is an accepted fact that *Plasmodium* has isozymes with unique biochemical properties some of which may serve as selective chemotherapeutic targets. This property—unrecognized for parasites until the 1950s when Ernest Bueding (Bueding and Mackinnon, 1955; Bueding and Mansour, 1957) broke with the accepted dogma of unity in biochemistry—provides grist for the drug designers' mills.

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CHAPTER 9

The Road to the *Plasmodium falciparum* Genome

During my 2 years as a post-doctoral fellow at Rockefeller University (New York, New York) I became acquainted with malaria deoxyribonucleic acid (DNA). This was not so much the result of my familiarity with Avery, McCarty and McLeod, who had identified the transforming principle as DNA at the Rockefeller Institute, or because I was aware that the Watson-Crick double-helix structure of DNA had made sense of Chargaff's rule, namely that the base composition (A + T) may vary from species to species but the amount of A always equals T and the amount of G always equals C; rather, it was the result of my providing samples of *Plasmodium lophurae* to Julius Marmur (1926–1996). Marmur had carried out post-doctoral work with Paul Doty at Harvard and was able to show that heat denaturation ('melting') could be used to separate the helical strands of DNA and that the specific 'melting temperature' was related to DNA base composition by virtue of its G + C content. In 1960, Marmur moved to the Biochemistry Department at Brandeis University (Waltham, Massachusetts) where Nathan Kaplan was chairperson and Allan Wilson was a post-doctoral investigator. Perhaps influenced by their thinking on taxonomy and evolution, Marmur recognized that the base sequences of different organisms might be 'a valuable asset in their classification' and proceeded to acquire samples of various protozoans. Marmur asked Trager for samples of parasitic protozoans and he in turn assigned me the task. I provided Marmur with *P. lophurae* and when the results were published (Schildkraut *et al.*, 1962) Wilson and Sherman were thanked for 'useful discussions' but unexpectedly the data for *P. lophurae* DNA were not included. When I inquired about the omission, Marmur simply replied, 'It was so low in G + C that it didn't fit.' (A *T. lewisi* sample,

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which I also provided to Marmur, grouped nicely with the other hemoflagellates at 54–59% G + C, and so it was included!) I thought little of this experience until 1966 when my graduate student Charles Walsh began studies of the nucleotide base composition of *P. lophurae* DNA, found it to be exceedingly A + T rich (80%) and showed that the nucleus of the duckling red cell did not provide building blocks for *P. lophurae* DNA, something Delphine Clarke (1952) had shown earlier for *P. gallinaceum* growing in chicken red cells.

When we published (Walsh and Sherman, 1968a) the unexpectedly elevated A + T content of the *P. lophurae* genomic DNA (confirming Marmur's unpublished findings 6 years earlier) we were told by others who were studying plasmodial DNA that it had no 'real' significance since this was a bird malaria with little in common with mammalian malarias such as *P. falciparum* and *P. vivax*. It was gratifying to learn, some 10 years later when the *P. falciparum* DNA base composition was found to be 18–20% G + C (Pollack *et al*., 1982) that the *lophurae* DNA shared greater affinity with the human parasite relative to rodent (24% G + C) and the simian (37% G + C) malarias (Goman *et al*., 1982; Gutteridge *et al*., 1969).

The chromosomes of *Plasmodium* do not condense during mitosis and hence they are not visible by light microscopy. Detailed information on the number and size of malaria parasite chromosomes became available only after the development of pulsed- field gel electrophoresis (Schwartz and Cantor, 1984) a clever technique that permits separation of megabase-sized molecules. Using this novel approach, it was possible to separate the 14 chromosomes of *P. falciparum* (Kemp *et al*., 1985; Van der Ploeg *et al*., 1985). All species of plasmodia studied have 14 chromosomes although the size of individual chromosomes can vary amongst isolates.

In the early 1970s, recombinant DNA technology made possible the isolation of genes; later technology enabled mapping these same coding sequences to particular chromosomes. The method (as originally developed by Boyer and Cohen) used plasmids—small circles of naked DNA. Both strands of a circular plasmid double helix could be severed at a specific location with molecular scissors known as restriction enzymes. When mixed in the same test tube with a second DNA (such as *Plasmodium* DNA) that had been similarly cleaved, and in the presence of another enzyme (called ligase), the snipped ends could be 'glued' together; the end result was the creation of a hybrid or recombinant plasmid. These ligation mixtures could then be used to infect bacteria. After bacterial growth in a Petri dish containing antibiotics, only the recombinant plasmids would enable the bacteria to survive. In some instances the bacteria containing the recombinant plasmid would express the protein encoded by the foreign DNA.

Expression or complementary DNA (cDNA) libraries can be constructed using genomic DNA or circular DNA (prepared from messenger

ribonucleic acid (mRNA) by reverse transcriptase). Genomic expression libraries are often constructed from sheared DNA or DNA partially digested with nuclease, including restriction enzymes. Theoretically, genomic libraries will have all the sequences present at equal frequency. A cDNA expression library is somewhat more difficult to prepare because it first requires the isolation of mRNA; however, in this library the sequences are present in proportion to their abundance as mRNA molecules and thus represent differentially expressed genes. Although plasmid-based vectors were used early on, later it was found that engineered bacteriophage (especially λgt11) were more efficient vectors and the number of clones generated was greater than that found for plasmids. In addition, handling of large numbers of phages and screening of recombinant clones was far easier. John Scaife's group in Edinburgh prepared the first *Plasmodium* genomic library (Goman *et al*., 1982).

John Scaife (1934–1991) was a founding father of molecular parasitology and his laboratory was the first to produce a *P. falciparum* cDNA library. Upon graduating with a bachelor of science (BSc) in botany from University College, London, Scaife arranged to do a doctor of philosophy (PhD) with William Hayes who had just established a Medical Research Council (MRC) Microbial Genetics Unit at Hammersmith Hospital, London. Scaife's PhD thesis (awarded in 1964) was on the origin and behavior of integrated autonomous sex factors in *Escherichia coli* and in this he provided evidence for the circularity of bacterial plasmids and for their insertion into the bacterial chromosome by a single genetic exchange. After completion of his PhD, he spent time in laboratories in Moscow and Leningrad and thereafter 3 years at the Harvard Medical School (Boston, Massachusetts) with Jonathan Beckwith who was engaged in investigations on the lac operon. Together they isolated and characterized the first promoter mutants in the lac operon. Upon his return to the United Kingdom, Scaife joined the Hammersmith group in its move to Edinburgh, where, as the MRC Molecular Genetics Unit, it comprised half of the Department of Molecular Biology. In Edinburgh, he turned his attention to the genetic control of the key transcription enzyme, ribonucleic acid (RNA) polymerase, and showed that certain subunits of RNA polymerase are within an operon that also codes for ribosomal proteins. In 1974, on the retirement of Hayes, he became a senior lecturer, was appointed a reader in 1978, and in 1984 was appointed to a personal chair.

In the late 1970s, he and other bacterial geneticists sought new challenges and Scaife recognized the potential of applying the emerging techniques of recombinant DNA technology to understanding the nature of parasitic organisms. Influenced by his colleagues at

Edinburgh University (Edinburgh, United Kingdom), the *Paramecium* geneticist Geoffrey Beale and the *Plasmodium* geneticist David Walliker, he took the bold decision to set up a group to study the molecular biology of the malaria parasite. His major goal was to search for antigens suitable for the development of vaccines. Awarded his first MRC grant in 1980, Scaife and his group involved themselves in cloning the genes for a number of parasite proteins; by combining the use of monoclonal antibodies with molecular genetic techniques they were able to characterize several blood-stage antigens as well as initiate the search for a key folate pathway enzyme, dihydrofolate reductase (DHFR), target of the major anti-malarial drug pyrimethamine. (The DHFR work was subsequently carried forwards by his former collaborator John Hyde—see later.) The success of Scaife's work was recognized by an invitation from the MRC to head a new section at the National Institute for Medical Research (NIMR, London, United Kingdom), but unfortunately coincident with this he learned he was HIV positive. As a result, he elected to remain in Edinburgh. Despite declining health he continued to work in the Institute of Cell and Molecular Biology until 6 months before his death and was still talking science with the members of his research group until his last week.

The accomplishment of the Scaife laboratory in Edinburgh was followed by the Australian group at the Walter and Eliza Hall Institute (WEHI). WEHI was established in 1915 and its principal focus has been immunology, immunopathology, transplantation biology and virology. Malaria research at the WEHI arose at a time when advances in recombinant DNA technology made possible their application to parasites and funding agencies began to support the study of neglected tropical diseases. In the mid-1970s, Graham Mitchell, an immunologist who had turned his attention to the study of host–parasite interactions, established an immunoparasitology unit at WEHI. A biochemist, Russell Howard, joined the unit and, working with Mitchell, developed several mouse models of malaria. A meeting with Michael Alpers of the Papua New Guinea Institute of Medical Research and funding from the Rockefeller Foundation precipitated the move to human malaria. Recruitment of Robin Anders and Graham Brown led to the establishment of the *in vitro* culture system for *P. falciparum*, methods of immunochemical analysis, access to serum from residents of malaria-endemic countries and methods for preparation of affinity-purified antisera.

By 1980, investigations on the molecular biology of malaria under the direction of David Kemp had begun. Two PhD students, Ross Coppel and Alan Cowman, were able to construct cDNA libraries with Ross working on *P. falciparum* and Alan on *Babesia bovis*. Work on *P. falciparum* was

hampered by the small scale and low yield of parasites from *in vitro* cultures and the inefficiency of enzymes such as reverse transcriptase and terminal transferase, incredibly valuable and difficult-to-come-by reagents, which at that time could be obtained from only one laboratory in the world. Malaria parasites had to be cultured continuously for months to harvest enough parasites to allow a single cDNA synthesis to be performed. In contrast, *Babesia* was obtained from the blood of infected cows at high parasitemia and in virtually unlimited amounts. In this way, cloning and screening methods could be developed and perfected in the *Babesia* system before application to the malaria system. Although a malaria cDNA library was available by 1982, progress was slow since the vector used, pBR322, expressed antigens in very small amounts. Indeed, the only way to detect expression was to use the incredible sensitivity of the mouse immune system: random lysates of clones from the library were injected and antibody production to malaria antigens assessed. These studies led to the identification of a schizont antigen, subsequently known as the glycophorin binding protein (a thoroughly misleading name as it turned out). However, such methods did not provide a great enough throughput for a meaningful attack on the repertoire of important protective antigens.

The turning point was the introduction into the laboratory of the bacteriophage vector λgt11 by Robert Saint, a post-doctoral fellow who joined the WEHI group following a stint at Stanford University (Palo Alto, California), where the vector had been developed in the laboratory of Ronald Davis. With all the steps required for construction and screening of cDNA libraries already in place, the group was quickly able to take advantage of the new vector. Expression levels were at least 1000-fold higher than pBR322 could provide and direct screening of libraries became possible. Improvements to the screening procedure by Robin Anders, including the preparation of affinity-purified pooled human serum provided sufficient sensitivity. Hundreds of phage plaques expressing epitopes of proteins recognized as immunogenic by residents of endemic areas were found, many of which remain uncharacterized even today. The clones could then be used to raise monospecific sera either by immunization of laboratory animals or by affinity purification on expressing clones. In turn, the monospecific antisera could be used to define properties of the corresponding malaria protein by immunoblotting, biosynthetic labelling and immunofluorescence and immunoelectron microscopy. The requirement for affinity-purified serum was circumvented by Coppel's development of methods for screening using individual sera. This allowed comparisons between exposed individuals who differed in their level of immunity to malaria. Clones expressing many defined malaria proteins, including RESA, S antigen, the circumsporozoite protein-related antigen CRA, AMA1, MSP-1, MSP-2, KAHRP, MESA, SPAM/MSP-3, hsp70-1 and BIP, MSP-4 and MSP-5 (and many

other acronyms beloved by malariologists) were defined by these studies and reports characterizing their role and utility in inducing host protective immunity were published over succeeding years. Other advances included: the first applications of pulsed field gel electrophoresis (revealing the presence of the chromosomes of *Plasmodium*), the use of this and yeast artificial chromosome libraries in early attempts at mapping the location of genes within the malaria genome, and the development of the techniques of inverted polymerase chain reaction (PCR) and colorimetric identification of PCR reaction products by David Kemp.

Ross Coppel (1952–) graduated in 1976 from the University of Melbourne (Melbourne, Vic, Australia) with a medical degree and interned at the Royal Melbourne Hospital (Parkville, Vic, Australia). During his medical course, he had undertaken a year of research for the bachelor of medicine (BM) degree researching the function of radioresistant macrophages with Jacques Miller and Graham Mitchell at the WEHI. He went on to do post-graduate studies in tropical medicine at the London School of Hygiene and Tropical Medicine (LSHTM, London, United Kingdom, 1976–1979) and in 1979 he returned to Australia to join Graham Mitchell at WEHI in the recently established Immunoparasitology Unit. After an unsuccessful attempt to study the surface antigens of microfilariae, Ross was given the project of applying recently developed techniques in molecular biology to the study of the malaria parasite. Ross extended many of the still rudimentary approaches to mRNA extraction and cDNA synthesis and expression screening to successfully work with the small amounts of material available in the malaria system. He constructed the first cDNA expression libraries for the asexual stage of the parasite, but existing expression methods were incapable of expressing sufficient amounts of protein for most purposes. However, these studies allowed the group to exploit better vectors such as λgt11 rapidly as they became available. Ross published a number of studies with co-workers, including the first cloning and characterization of a number of blood-stage proteins, including S antigen, RESA and a number of merozoite surface and rhoptry proteins. In addition he refined methods of screening of expression libraries that allowed application of these techniques to the study of human autoimmune diseases and allergy. After his doctorate he continued to work at WEHI first as a post-doctoral fellow and then as a senior research fellow. His research interests in malaria included descriptions of the protein repertoire of the asexual-stage invasive forms, elucidation of interactions and functional consequences of malaria proteins binding to the red cell membrane skeleton and design of malaria vaccines. In 1993, he was

appointed professor and head of the Department of Microbiology at Monash University (Melbourne, Vic, Australia). He has continued his research into malaria there. Ross for many years curated the World Health Organization (WHO, Geneva, Switzerland) malaria sequence database, one of the earliest organism-specific databases. This led to an involvement in the creation of PlasmoDB (see p. 295). He has taken an increased interest in more general applications of information technology to biomedical research and was appointed director of the Victorian Bioinformatics Consortium in 2001.

Initially, the WEHI group functioned in an integrated fashion and a significant infusion of Australian government money supporting development of a malaria vaccine led to a large increase in numbers of workers and efforts at defining host-protective malaria proteins. In time, however, the group became riven with dissent and many researchers departed to work elsewhere. Despite this, many of the original team continues to be productive in malaria research and a malaria research group remains at WEHI under the direction of Alan Cowman.

Alan Cowman (1954–) began his PhD at Walter and Eliza Hall Institute (WEHI) with David Kemp as supervisor in 1980. At the time, Kemp was working on immunoglobulin genes and Cowman's project was to analyze the structure of Cμ immunoglobulin transcripts in T cells. This involved making circular deoxyribonucleic acid (cDNA) libraries. Because the Kemp laboratory was very close to those of Robin Anders and Ross Coppel, with the former working on, among things, *Giardia*, while the latter was beginning a PhD on malaria in the Immunoparasitology Unit, Kemp became interested in malaria and convinced Cowman to start a PhD on *Babesia bovis*. Together, Kemp and Cowman began working on expression of cDNAs in *E. coli* for ultimately screening sera from humans (in the case of malaria) or *B. bovis* (in the case of cows). During this time they worked out how to express parasite proteins in *E. coli* as β-galactosidase fusion proteins that were screened for antigens using sera. This was the first step into malaria. Cowman wrote his PhD thesis on *B. bovis* (1984) and then started working full time on malaria. As a post-doctoral student his first project was to sequence two S-antigen genes from different strains of *P. falciparum*. This resulted in a manuscript published in *Cell* (Cowman *et al*., 1985). A second project, finished first, was identification of RESA (Cowman *et al*., 1984) and this was his first 'first author' paper on malaria. Cowman then did a post-doctoral study on *Drosophila* with Gerald Rubin at University of California at Berkeley (California). In 1987, he returned to WEHI and began

working again on malaria and decided to concentrate on drug-resistance genes. The main reason he did that was because he was keen to look at transfection of *P. falciparum* and wanted genes that could be used as selectable markers. This led to the identification of the genes for DHFR and DHPS.

In retrospect, it became clear that the screening approach used, although particularly good at identifying repetitive antigens and proteins containing linear epitopes, was much less efficient at identifying the important subgroup of cysteine-rich proteins. Though successful at identifying AMA1, MSP-4 and MSP-5, the approach used proved incapable of identifying *P. falciparum* erythrocyte surface protein-1 (PfEMP1), the family of reticulocyte-binding protein homologues, the EBA family and the cysteine knot family of merozoite proteins. Working as they were at the beginning of the molecular biology/biotechnology revolution the WEHI group was one of the first to encounter the complexities involved in commercialization of basic research and struggled with the resulting problems of direction and management that come from large-scale funding of big science projects, a failure to understand the regulatory requirements, as well as the complexities of bringing a recombinant protein formulation to market. Nonetheless, the technological contribution of the WEHI group was enormous and by building an atmosphere of excitement and optimism about malaria research it attracted a new generation of talented scientists.

David Kemp (1945–) graduated in 1969 from the University of Adelaide (Adelaide, SA, Australia) with a BSc and went on to obtain a PhD (1973) in biochemistry from that institution. In a time before recombinant DNA techniques had been developed, he purified mRNA and using nucleic acid hybridization techniques was able to demonstrate a multi-gene family that encoded feather keratin. After his doctorate, he continued to work as a post-doctoral fellow on the same project for about 1 year. In 1975, he had a further post-doctoral at Commonwealth Scientific and Industrial Research Organization (CSIRO, ClaytonSouth,VIC, Australia) where he worked with *Drosophila*. In 1976, Kemp went to Stanford University (Palo Alto, California) to study with David S. Hogness, a leading expert in the study of gene regulation in bacteriophage, and who had recently started using this expertise to study *Drosophila*. At that time, Stanford was one of the few places in the world where it was possible to do recombinant DNA work, mainly because most of the Biochemistry Department worked on enzymes of nucleic acid metabolism. Kemp's project was to isolate developmentally regulated genes and this involved construction and

analysis of cDNA libraries. While at Stanford, Kemp and colleagues invented the Northern blotting technique for detecting RNA (Alwine *et al*., 1977), their publication becoming one of the most widely cited papers as a basic recombinant DNA technique.

By the late 1970s it had become clear to the biological community (and to Kemp) that recombinant DNA techniques would become important in many aspects of medical research. In 1978, he returned to Australia and joined WEHI starting as a research fellow working on immunoglobulin genes. In 1979, he joined the Parasitology Program at WEHI and began to supervise a number of students. He became the supervisor for Alan Cowman, a PhD candidate, and as such was responsible for teaching him, among other things, central molecular biological techniques. The first successful work in this area began in 1980, when Kemp and Cowman developed a method for detecting antigens expressed in *E. coli*, by screening with antibodies. Although methods for doing this had been described in the literature, when Kemp had tried one of these earlier— the Broome and Gilbert method— it had failed. The Kemp-Cowman method (1981a,b) turned out to be a more generally applicable procedure and it was soon included in standard laboratory handbooks.

In 1981, Kemp became a senior research fellow at WEHI where he and colleagues spent the next 2 years developing a system for cloning malaria antigens. The goal of cloning malaria genes was to try to make a vaccine and the approach used was to identify malaria genes critical to immunity by screening expression libraries with antibodies from immune subjects. Using a modification of their colony screening system many hundreds of specific clones were identified. In 1984, Kemp received a John D. and Catherine T. MacArthur Foundation grant and took up the position of head of the MacArthur Laboratory of Molecular Parasitology at WEHI. From 1986 to 1990 Kemp was a principal research fellow at WEHI and from 1990 to 1992 senior principal research fellow and head of the Immunoparasitology Unit. Over this period, the group built up to about 60 people, most working on malaria using the cloning procedures that had been described in the early 1980s. During that period, a gene for a malaria antigen, apical membrane antigen-1 (AMA-1), which shows potential as a malaria vaccine antigen was discovered. After WEHI, and then several years in Darwin studying aspects of Aboriginal health, Kemp moved to the Queensland Institute of Medical Research (Herston, QLD, Australia) where, as laboratory head, he continues to direct research on the molecular biology of malaria parasites and scabies mites.

Powerful as the recombinant methods are, there are significant limitations: it requires growing large volumes of bacteria to amplify the DNA of interest as well as a considerable investment in time and research funds to

identify recombinant clones of interest. By 1985, it was largely replaced by the PCR, a technique called 'molecular photocopying.' In brief: two short (17–20 nucleotides) stretches of single-stranded DNA (called primers), corresponding in nucleotide sequence to the regions bracketing a DNA expanse of interest, such as a specific gene, are synthesized. The primers are added to the DNA template, that is, total genomic DNA or a cDNA population of interest, and the DNA is 'melted' (à la Doty and Marmur) by heating to 90–95 °C to separate the helical strands. Upon cooling, the primer can bind to its complementary stretch of single-stranded template DNA. Also present in the test tube is a DNA polymerase and all four nucleotide bases. The polymerase will only begin incorporating bases where the DNA is already double stranded, and so it begins adding nucleotides at the end of the primer and synthesizes the DNA region that follows. By using a thermal cycler, one that heats ('melts') and cools ('anneals'), the process can be repeated every 5 min and the stretch of DNA of interest will be copied again and again; in 2 h the DNA of interest will be increased about 34 million-fold. This amplified DNA can then be sequenced by a variety of methods.

In the Sanger sequencing method, the strands of DNA are duplicated by means of DNA polymerase in the presence of a mixture of the normal nucleotides A, T, G and C plus some dideoxy A (ddA), T (ddT), G (ddG) or C (ddC). If the polymerase incorporates the normal base the DNA chain grows but when it encounters a dideoxy nucleotide it stops lengthening. The result isfour different samples, each containing a series of DNA chains of varying length depending where in the growing chain the different dideoxy nucleotides were incorporated opposite the complementary T, A, C or G template bases during the replication process. Each sample is placed on a gel and the fragments separated in an electric field: short chains move faster and longer chains slower. The positions can be read off such that the shortest fragment will contain the first base, the next larger the second and so on. Later this four-lane method of manual sequencing would be replaced by one that was automated and used a single lane: a different colored dye for each type of chain-terminating dideoxy nucleotide was added to the polymerase mix and incubated. By subjecting the single-lane sample to an electric field, all the DNA pieces can be sorted according to size. With ultraviolet light illumination each fragment fluoresces differently depending on its terminal dideoxy nucleotide; by scanning the fluorescent pattern and feeding this into a computer a sequence can be printed out.

Using such an automated system half a million bases can be sequenced in a day. In the succeeding decades many genes of *Plasmodium* would be cloned and sequenced. Indeed, in 1996, an international consortium of scientists from more than 12 institutions set out to determine the 23 million base pairs in the nuclear DNA of *P. falciparum*. The 14 chromosomes were physically

separated by pulsed field electrophoresis. Then the DNA was mechanically sheared into random fragments; after the fragments were cloned in bacteria they were sequenced using high-throughput, automated dideoxy-sequencing and the nucleotide order determined for individual chromosomes by assembling overlapping sequences using a computer. In 2002, the results of the *Plasmodium falciparum* genome project were published: 5279 genes were postulated with 60% being of unknown function as they match no other gene sequences in the international data banks (Gardner *et al*., 2002). Mass spectrometry identified peptides/proteins encoded by 2391 of the genes. When the genome of *P. yoelii* was sequenced (Carlton *et al*., 2002), more than 60% were similar to that found in *P. falciparum*, enabling the cataloguing of genes for general cell function as well as coding regions specific to the parasitic life cycle.

Malcolm Gardner (1958–) became interested in biology in middle school with the first biology course he took. His interest in molecular and cellular biology started in Dickinson College, a small liberal arts college in Carlisle (Pennsylvania), from which he graduated in 1980. He continued this interest in a doctoral program at Oak Ridge National Laboratory and received his PhD (1985) working in W. K. Yang's laboratory on endogenous murine retroviruses and oncogenes. Entry into the malaria field as a post-doctoral was completely serendipitous. Because of family connections he sought a post-doctoral position in the United Kingdom. Applications for post-doctoral positions in tumor virology in the United Kingdom failed but he had sent one application to Robin Holliday at the NIMR. That too was rejected, but unknown to him his curriculum vitae had been circulated around the Institute. He received a handwritten note from Iain Wilson, who indicated that he was looking for a molecular biologist to work in his laboratory on malaria. Iain recognized that Gardner had not worked on malaria before, but wrote 'perhaps you might find one of these projects to be of interest?' Two projects were suggested, one on S antigens and the other on the 35 kb circle, the 'mitochondrial' genome. Knowing virtually nothing about malaria, he sought out a new post-doctoral in the Oak Ridge group who had trained in Dan Colley's laboratory at Vanderbilt University, and he gave him an introduction to tropical diseases. Gardner also reviewed the recent publications in the molecular biology of malaria parasites. He accepted Iain's offer and started at the NIMR in December 1985 and spent the next 6 years studying the 35 kb circle in collaboration with the Iain Wilson and Don Williamson group. Although initially it was thought that the 35 kb circle was the mitochondrial genome, later it was found that it had plastid-like features, including the *rpo*B and *rpo*C genes encoding subunits of

RNA polymerase. This work eventually led to the identification of the apicoplast a few years later. Doing a great deal of manual sequencing and learning how to use computer programs to collect and analyze sequence data were skills that would prove useful later.

After Mill Hill, Gardner spent 2 years in Wilbur Milhous's group in the Experimental Therapeutics Division at the Walter Reed Army Institute of Research (WRAIR, Washington), where he learned about drug development and then he moved to Stephen Hoffman's group at the Naval Medical Research Center (NMRC, Silver Spring, Maryland) and worked on *P. yoelii* DNA vaccines for about 3 years, which provided an introduction to immunology and vaccine development. It was while working in Hoffman's group that the opportunity to work on the *P. falciparum* genome sequence arose. Craig Venter, the founder of The Institute for Genomic Research (TIGR), had published the first microbial genome sequences in 1995. He called Hoffman and expressed an interest in sequencing the genome of the malaria parasite. Gardner and Hoffman went over to TIGR, discussed the project with Craig and Mark Adams, and agreed to initiate an effort to obtain funding for the project. Dan Carucci, fresh from his PhD at LSHTM, arrived soon thereafter, and all worked to obtain funding. Colleagues in the United Kingdom, led by Chris Newbold were simultaneously launching a genome sequencing effort with the Sanger Centre (Cambridge, United Kingdom). Due to the large size of the *P. falciparum* genome and the cost of the project, an international consortium to sequence the genome was formed, using a chromosome-by-chromosome shotgun sequencing approach. In February 1997, Gardner moved to TIGR to pursue the genome project full-time, and the TIGR-NMRC team published the first *P. falciparum* chromosome sequence in November 1998. Sanger finished chromosome 3 soon afterwards, and the consortium began the long, difficult process of 'completing' the entire *P. falciparum* genome sequence, which was published in 2002.

Gardner worked on several other genome projects at TIGR, including the *Theileria parva* genome, but after eight years of genomics he decided to return to laboratory work and moved to the Seattle Biomedical Research Institute (Seattle, Washington) in 2005. There he is developing a program to study the function of the apicoplast, while working with his wife Ruobing Wang, whom he met in Stephen Hoffman's laboratory, on antigen discovery and vaccine development. He hopes to launch a genome curation effort to ensure that the *P. falciparum* genome sequence and associated annotation is updated on a regular basis to maximize its usefulness to the malaria research community.

The next step in understanding *Plasmodium* gene function would come from DNA microarrays where single strands of DNA (representing genes or gene fragments) are placed on a silicon sliver the size of a small computer chip. If a particular mRNA is tagged with a fluorescent marker and then the DNA chip is flooded with the RNA, only the spots to which the tagged mRNA has bound by complementary Watson-Crick pairing will emit fluorescence under ultraviolet light. In effect, by using microarrays it would be possible to assess when in the *Plasmodium* life cycle specific genes are turned on or off at the DNA transcriptional level. The study of global gene expression using microarray technology is called transcriptomics (see p. 280), and it is finding utility in both understanding the complex life cycle of the malaria parasite and in providing a large array of gene targets for *Plasmodium* therapeutics.

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CHAPTER 10

Carbohydrate Metabolism

Contents

1. GLYCOLYSIS AND THE TRICARBOXYLIC ACID CYCLE

Developments in nuclear energy post-World War II improved the production and detection of radioactive compounds. The first isotopes for biochemical use were spin-offs following construction of cyclotrons in the United States and the United Kingdom. By the 1940s, studies using ^{32}P were possible and later ^{14}C-labeled compounds were introduced. Between 1945 and 1950, Schneider introduced a modification of the Schmidt-Thannhauser procedure for the fractionation of labeled compounds and this, coupled with various chromatographic methods, made it possible to study the incorporation of metabolites into acid-soluble nucleosides and nucleotides, nucleic acids and proteins. The use of paper and ion-exchange chromatography (much of it developed at the National Institute for Medical Research (NIMR), London, United Kingdom) provided a simple and effective means for the separation of products from substrates.

The earliest studies on plasmodial respiration using radiolabeled glucose were those of Fulton and Spooner (1956) with *Plasmodium berghei*. When P. T. Grant, who had completed a doctor of philosophy (PhD) at NIMR and had collaborated with Fulton in describing the sulphur requirements of *P. knowlesi* (Fulton and Grant, 1956) using radiolabeled methionine, moved to the Department of Biological Chemistry at Aberdeen University (Aberdeen, United Kingdom), it was logical that one of his students

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(I. B. R. Bowman) would study the effects of atabrine on the metabolism of *P. berghei* growing in rat reticulocytes using uniformly labeled ^{14}C glucose as well as glucose labeled in the C-1 and C-6 positions (Bowman *et al*., 1961). The reason for the use of C-1- and C-6-labeled glucose is that by measuring $^{14}CO_2$ from glucose labeled in the C-1 position it is possible to determine activity of the pentose pathway since the CO_2 released is derived from the decarboxylation of the C-1 carbon of glucose-6-phosphate, whereas CO_2 released from glucose labeled in the C-6 position represents that from glycolysis. Bowman *et al*. (1961) found the major end product from radiolabeled glucose was lactate with small amounts of citrate, fumarate, malate, oxaloacetate (OAA) and α-ketoglutarate. Atabrine depressed glucose catabolism but did not affect the ratio of CO_2 from (1-^{14}C) or (6-^{14}C) suggesting that the drug did not affect the pentose pathway. Further, since the percent (U-^{14}C) glucose converted to $^{14}CO_2$ was unaffected it suggested the drug acted on glycolysis; based on the redistribution of products they suggested the most sensitive enzyme to be 6-phosphofructokinase and to a lesser extent hexokinase. (Earlier studies by Bovarnick *et al*. (1946) claimed that atabrine interfered with the phosphorylation reaction and this was consistent with the findings of Speck and Evans (1945) who found hexokinase to be most strongly inhibited.) The selective inhibition of 6-phosphofructokinase by atabrine suggested to Bowman *et al*. that 'the 6-phosphofructokinase of this parasite differed from that of the host cell' and this possibility was supported by Bueding's finding (Bueding, 1959; Bueding and Mansour, 1957) that the 6-phosphofructokinase of the blood fluke *Schistosoma mansoni* was 80 times more sensitive to tetravalent antimonials than the enzyme in mammalian host tissues. Indeed, the prescience of Bowman *et al*. (1961) would be borne out when isoenzymes were discovered in *P. lophurae*, *P. berghei*, *P. knowlesi* and *P. falciparum* (see p. 63).

Some years later Bryant, Voller and Smith (1964) and Scheibel and co-workers (Scheibel and Miller, 1969; Scheibel and Pflaum, 1970) confirmed the findings of Bowman *et al*. for *P. knowlesi*. However, working with *P. lophurae* and finding significant increases in the liberation of $^{14}CO_2$ and ^{14}C-labeled intermediates from labeled glucose (and still operating under the influence of earlier findings/suggestions that avian malarias have a tricarboxylic acid (TCA) cycle) we claimed this was evidence for a TCA cycle in *P. lophurae* (Sherman *et al*., 1970). Similarly, Shakespeare *et al*. (1979a) at the NIMR suggested that in *P. knowlesi* the production of CO_2 from 6-^{14}C glucose 'was the result of breakdown of pyruvate catalyzed by reactions of the Krebs tricarboxylic acid cycle.' Upon reconsideration we would concur with the view of Herman *et al*. (1966) who, after studying the metabolism of ^{14}C glucose with *P. gallinaceum*, questioned the existence of a TCA cycle in avian malarias. Thirty years later, studies of the mitochondrial genome of *Plasmodium* (see p. 96) provided convincing evidence against a functional TCA cycle and supported the conclusions of Herman *et al*.

In 1988, William Scheibel made sage comments about the early studies on carbohydrate metabolism, the action of anti-malarials and some of the anomalous findings. He said some of this could be due to contamination, damage to the parasites and limitations in analytical methods and he went on, 'It is impossible to assess quantitative transformations of substrate into each end product ... pathways of minor importance may have been misinterpreted as having major significance. Assessment of growth conditions and substrate requirements ... by measuring oxygen uptake actually provide no insight into whether or not an agent enters the economy of the cell ... a metabolic pathway ... cannot be established by merely identifying the component enzymes of that pathway, much less does the reporting of a single enzyme. Similarly, pharmacological agents have frequently been employed at excessively high concentrations and their mechanism of action ... misinterpreted'.

Finally, perhaps an explanation for the beneficial effects of coenzyme A (CoA), malate and pyruvate for the extracellular *in vitro* growth of *P. lophurae* found by Trager (1952) and interpreted by Moulder (1962) to 'neatly explain the shift in pattern of carbohydrate metabolism accompanying liberation of parasites from the host cell'... (The) 'lack of CoA in free parasites logically explains the lessened rate of pyruvic acid oxidation *via* the Krebs cycle. It is difficult to escape the conclusion that the inability of plasmodia to synthesize CoA extracellularly results in extensive dislocations in glucose metabolism, which in turn contribute heavily to the restriction of the malarial parasite to an intracellular habitat' is this: malate and pyruvate could be linked to the generation of dihydronicotinamide adenine dinucleotide (NADH) for glycolysis, and a CoA deficiency could limit activity in pathways other than the TCA cycle.

Most of the glycolytic enzymes found in bird and simian malarias by standard biochemical assays have been found in *P. falciparum*-infected red cells (Roth *et al.*, 1988) and increased enzyme activity is, in general, proportional to the parasitemia. By 2007, almost all of the genes for the glycolytic isoenzymes of *P. falciparum* had been cloned and sequenced (see http://sites.huji.ac.il/malaria/maps/glycolysispath.html; last accessed 16 July 2008, and Woodrow and Krishna, 2005).

2. THE PENTOSE PHOSPHATE PATHWAY

The pentose phosphate pathway (PPP) is the major pathway for recycling nicotinamide adenine dinucleotide (NAD) to nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) and for the production of ribose-5-phosphate that is needed for the synthesis of nucleotides. The function of the PPP depends on the synthesis of nicotinamide-adenine dinucleotide phosphate (NADP) and thiamin pyrophosphate, a coenzyme

of the PPP enzyme, transketolase. In red blood cells the PPP contributes to the maintenance of reduced glutathione (GSH) and prevents the accumulation of methemoglobin. Further, individuals whose red cells are deficient in the first enzyme of the pathway, glucose-6-phosphate dehydrogenase (G6PD), have been shown to be resistant to falciparum malaria (see p. 91).

Early studies using C-6- and C-1-labeled glucose with *P. lophurae*-, *P. gallinaceum*-, *P. knowlesi*- or *P. berghei*-infected red cells (Bryant *et al*., 1964; Scheibel, 1988; Shakespeare and Trigg, 1973) found very little (usually <20%) of the C-1 metabolized to CO_2 and with 'free' *P. lophurae* and *P. knowlesi* the amount was so low that it appeared the PPP was either absent or if present would play a minor role. Shakespeare *et al*. (1979a) erroneously concluded from their analysis using radioactive glucose 'that the parasite possesses its own complement of glycolytic enzymes but may obtain at least some of the pentose pathway enzymes from the host erythrocyte.' Considerable confusion has also arisen regarding PPP enzymes: although the third enzyme in the PPP (6-phosphogluconate dehydrogenase) was consistently found, G6PD was not (reviewed in Sherman, 1979) and although Hempelmann and Wilson (1981) did identify G6PD in simian and rodent malarias it was not found in *P. falciparum* (Roth *et al*., 1988) or *P. lophurae* (Sherman, 1965).

Thanks to the availability of *in vitro*-grown *P. falciparum* there is no longer any confusion concerning the existence of the PPP. PPP activity in *P. falciparum*-infected red cells has been shown by the evolution of $^{14}CO_2$ from (1-^{14}C) glucose and incorporation of the C-1 into nucleotides (Atamna *et al*., 1994). There was a large increase in PPP in infected red cells with 82% being contributed by the parasite, whereas the host cell PPP activity was activated 24-fold as a result of what was presumed to be parasite-induced oxidative stress. The falciparum gene coding for G6PD has been cloned (O'Brien *et al*., 1994) and the biochemical properties of the isolated enzyme characterized (Kurdi-Haidar and Luzzatto, 1990; Yoshida and Roth, 1987). G6PD is encoded by a hybrid gene that also contains 6-phosphogluconolactonase the second enzyme in the PPP (Clarke *et al*., 2001). Activity of ribose phosphate diphosphokinase (Reyes *et al*., 1982) and levels of its product 5'-phosphoribosyl pyrophosphate (PRPP) have been measured for *P. falciparum*-infected erythrocytes (Roth *et al*., 1986). The levels of PRPP were increased 56-fold in infected cells bearing trophozoites compared to uninfected cells.

Using a transcriptome database (see p. 280) that contains the relative abundance of messenger ribonucleic acid (mRNA) for every hour of the parasite developmental cycle, Bozdech and Ginsburg (2005) were unable to find the genes for transaldolase and NAD kinase that are essential to the PPP. During early stages of parasite development the activity of the PPP involves only the oxidative arm and is geared for NADP reduction and ribose-5-phosphate production, whereas later in the cycle the

non-oxidative arm (that uses fructose-6-phosphate and glyceraldehyde-3-phosphate supplied by glycolysis as well ribose-1-phosphate generated by salvage of purines) is deployed and this would allow for fuller production of ribose-5-phosphate.

The G6PD deficiency allele probably arose within the past 4,000–12,000 years and spread with agriculture and malaria. Recent population-based studies have demonstrated a protective effect of a deficiency of red blood cell G6PD in *P. falciparum* infections in hemizygous males and homozygous females but not heterozygous females (Guindo *et al*., 2007). Perhaps the most important mechanism for such protection is selective removal of oxidant-damaged, ring-infected red cells in those individuals whose erythrocytes are uniformly deficient by phagocytosis (Cappadoro *et al*., 1998). However, with the finding that there is reduced expression of plasmodial G6PD in deficient red cells derived from natural populations (but not in laboratory strains grown in deficient red cells) it may be that oxidant damage affects parasite gene transcription and it is this that impairs parasite growth and reproduction (Sodeinde *et al*., 2003). It has been speculated that a specific inhibitor of plasmodial G6PD might be an effective anti-malarial; however, none has been developed.

3. PYRIDINE NUCLEOTIDES

At UCR, the availability of an Aminco-Bowman spectrofluorimeter allowed for the measure of pyridine nucleotides in *P. lophurae* (Sherman, 1966c). The results agreed with the earlier findings of Speck and Evans (1945) for *P. gallinaceum* and Ball (1948) for *P. knowlesi*., that is, that the parasites contained NAD. Nagarajan (1964) found substantial (nine-fold) increases in the reduced pyridine nucleotides in *P. berghei*-infected red cells; however, in *P. lophurae* the increase was only 1.5-fold. Zerez *et al*. (1990) demonstrated a 15-fold increase in the levels of NAD in *P. falciparum-infected* erythrocytes as well as a three-fold increase in nicotinate phosphoribosyl-transferase (NAPRT) activity. These results suggested that the plasmodia were capable of NAD synthesis. The presence of abundant nicotinamidase activity in falciparum-infected red cells indicated that the parasite was able to synthesize NAD from nicotinic acid and nicotinamide (both present in the RPMI-1640 culture medium used for growing *P. falciparum*); however, since the genes coding for nicotinamide phosphoribosyl-transferase and NAD pyrophosphorylase were not found in the genome (Gardner *et al*., 2002) it seems that NAD is synthesized from nicotinamide and not nicotinic acid: nicotinamide is deamidated to nicotinic acid and then by NAPRT forms nicotinate *D*-ribonucleotide, which is adenylated to deamino-NAD by nicotinate-nucleotide adenyltransferase and then amidated by NAD synthetase.

In *P. falciparum*, the concentration of NADP was one-tenth that of NAD (Zerez *et al*., 1990). There was, however, a paradoxical finding: the gene for NAD kinase (essential for the production of NADP) could not be found, yet the parasite is able to synthesize NADP. Further, the reason for the biphasic transcriptional profiles for nicotinamidase, nicotinamide adenyltransferase and NADP transhydrogenase remains unclear (Bozdech and Ginsburg, 2005).

4. CARBON DIOXIDE FIXATION

By 1960, the traditional Warburg manometer was obsolescent, biochemicals labeled with radioisotopes were commercially available and separation techniques were simple and practical. Knowing the value of good collaborators I enlisted the support of a UCR colleague, Irwin P. Ting (a plant physiologist), who was working on CO_2 fixation in corn. Ting assured me: all organisms fix CO_2! Unlike the graduate student experiences at Northwestern University, our laboratories, now well equipped with liquid scintillation spectrometers, made possible investigations using radioisotopes. Working with *P. lophurae* we found incorporation of radioactive bicarbonate into α-ketoglutarate, OAA, aspartate, glutamate, lactate, alanine and citrate (Sherman and Ting, 1968; Ting and Sherman, 1966). This supported the exceptional finding (only one out of four experiments showed fixation) by Silverman *et al*. (1944) of CO_2 fixation in *P. gallinaceum*. The United States Armed Forces Epidemiologic Board (AFED) supported a malaria vaccine program at Stanford Medical Center under Quentin M. Geiman and Wasim Siddiqui (who had been a post-doctoral investigator with Trager at Rockefeller University (New York) when I was also in the laboratory) using *P. knowlesi* in rhesus monkeys; later, when Geiman and Meagher were able to adapt *P. vivax* to the New World owl monkey *Aotus* (1966), followed by two lines (FVO, Falciparum Vietnam Oak Noll and FUP, Falciparum Uganda Palo Alto) of *P. falciparum* (Geiman and Meagher, 1967) and *P. malariae* (Geiman and Siddiqui, 1969) the program would shift to human malarias. To discover whether CO_2 fixation occurred with other species of *Plasmodium*, Ting and I assembled our equipment and radiolabeled bicarbonate in UCR and when Siddiqui telephoned to say that he had a monkey infected with *P. knowlesi* we flew to Palo Alto, removed the infected blood from the monkey, incubated the cells with isotope ($NaH^{14}CO_3$), and found that *P. knowlesi* fixed CO_2 (Sherman and Ting, 1968). The products were essentially the same as those found with *P. lophurae*, that is, aspartic and glutamic acids and alanine (Ting and Sherman, 1966).

Co-ordinated with the work on CO_2 fixation was an analysis of the free amino acid pool of uninfected duckling red cells, *P. lophurae*-infected red

cells and duckling blood plasma before and during infection that was carried out in collaboration with J. Brian Mudd (a colleague in the Department of Biochemistry who had a brand new Beckman 120B amino-acid analyzer). Almost no changes occurred in the plasma of heavily infected ducklings but dramatic increases were found for glutamic and aspartic acids, alanine, lysine and proline in the infected red cells; most of this was attributable to the free amino acids found in the parasites (Sherman and Mudd, 1966). We suggested that the increase in free amino acids was probably by way of CO_2 fixation rather than proteolysis of hemoglobin.

Following our work, CO_2 fixation was demonstrated in *P. berghei* and *P. falciparum* (Blum and Ginsburg, 1984). The CO_2-fixing enzymes phosphoenolpyruvate (PEP) carboxylase ($CO_2 + PEP \rightarrow OAA$) and PEP carboxykinase (PEPCK) ($OAA \rightarrow PEP + CO_2$) were both found in *P. berghei*. The gene for PEPCK was found by microarray analysis (Hayward, 2000) but only PEPCK was found in *P. berghei* (McDaniel and Siu, 1972). Blum and Ginsburg (1984) unable to find CO_2 released from glutamate by *P. falciparum*, suggested the parasites lacked α-ketoglutarate dehydrogenase activity.

The precise role of CO_2 fixation in the economy of the parasite remains uncertain (see Scheibel, 1988), but the up-regulation of PEPCK in the early stages of sexual development in *P. falciparum* has been suggested to be important during parasite transition to the mosquito vector (Hayward, 2000). Another possibility is that CO_2 fixation supplies aspartate for pyrimidine biosynthesis as well as NADPH (*via* glutamate dehydrogenase) and NAD (*via* malic dehydrogenase).

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CHAPTER 11

Pyrimidines and the Mitochondrion

In 1968, my graduate student Charles Walsh and I addressed the following question: What are the pyrimidine sources for nucleic acid synthesis by *Plasmodium lophurae*? We found the parasite synthesized pyrimidines *de novo* (Walsh and Sherman, 1968b). The evidence for a *de novo* synthesis was the presence of the key enzymes, thymidylate synthetase and orotidine-5-monophosphate pyrophosphorylase, as well as the demonstration of the incorporation of ^{14}C-bicarbonate into cytosine, uracil and thymine. Finding a *de novo* pathway for the synthesis of pyrimidines by the malaria parasite would, in the next three decades, provide a biochemical basis for the mechanism of action of anti-folate anti-malarials as well as contributing to an understanding of the unique properties of the malaria parasite mitochondrion.

Electron microscopic studies of asexual parasites from a variety of malaria species have shown mitochondria with a sac-like appearance and very few cristae (Aikawa, 1988; Krungkrai, 2000). The mitochondria of sexual stages, in contrast, had a more conventional appearance. Based on these observations, it was assumed that in the asexual stages the mitochondrion did not function as an energy-yielding metabolic pathway involving a Krebs tricarboxylic acid (TCA) cycle in conjunction with oxidative phosphorylation, whereas in the sexual and mosquito stages it would function in the same manner as in most aerobic eukaryotes. This notion of mitochondrial function would change when Gutteridge, Dave and Richards (1979), working on a collaborative project between the University of Kent (Canterbury, United Kingdom), and the Wellcome Research Laboratories (Beckenham, United Kingdom), discovered that the conversion of dihydroorotate (DHO) to orotate by dihydroorotate

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dehydrogenase (DHOD), a key reaction in the *de novo* synthesis of pyrimidines (a pathway identified by Walsh and Sherman in 1968 (Walsh and Sherman, 1968b), was inhibited by several respiratory chain inhibitors, one of which was menoctone, an anti-malarial, and an analogue of ubiquinone, coenzyme Q. Neither we nor Gutteridge *et al*. formally determined the sub-cellular location of the enzymes; however, on the basis of the particulate nature of the activity they boldly suggested: electrons were fed into a cytochrome chain at about the ubiquinone level, and since menoctone acts at the mitochondrial level, the electron transport chain would probably be present in this organelle.

The isolation of intact, contaminant-free mitochondria from *Plasmodium* had been a major obstacle in understanding the function of this organelle until Fry and Beesley (1991) working in the Wellcome Research Laboratories were able to isolate functional mitochondria from *P. yoelii* and *P. falciparum*. They found reduction of cytochrome c by dihydronicotinamide adenine dinucleotide (NADH), α-glycerophosphate and succinate, as well as low rates of oxidation of proline, dihydroorotate and glutamate. Although oxidation of either α-glycerophosphate or succinate was inhibited by standard mitochondrial electron transport inhibitors, including a number of complex III inhibitors, the mitochondria were unable to oxidize nicotinamide adenine dinucleotide (NAD)-linked substrates. In addition, there was insensitivity of NADH oxidation by rotenone in the presence of fumarate, with or without cytochrome c, suggesting absence of complex I of the respiratory chain.

In 1971, a time when Win Gutteridge was working at the National Institute for Medical Research (NIMR, London, United Kingdom) with Peter Trigg, they found a minor deoxyribonucleic acid (DNA) component (with a base composition of about 20% G + C) in addition to a major component in *P. knowlesi* and *P. falciparum*. They suggested the major component represented nuclear DNA and the minor 'might arise from mitochondrial-like organelles' (Gutteridge *et al*., 1971). Following this, Kilejian (1975) at the Rockefeller University (New York) and Wilson, Williamson and Gardner at the NIMR (Gardner *et al*., 1988; Williamson *et al*., 1985) confirmed the 19% G + C base composition in the minor DNA component of *P. lophurae*, *P. falciparum* and *P. knowlesi* and finding it to consist of closed circles with a contour length of 10–12 μM stated 'our results are consistent with the view that (such) a size range ... may be characteristic of the mitochondrial DNA.' It would turn out that they were incorrect. Indeed, what was believed to be mitochondrial DNA was in fact the DNA from a rather unique organelle, the apicoplast (see later).

The DNA of the *Plasmodium* mitochondrion is much smaller than that of most other eukaryotes at 6 kb in length, and is linear rather than circular. It was first isolated in 1987 (Vaidya and Arasu, 1987) and its

sequence was characterized as mitochondrial by the Vaidya and Wirth laboratories 2 years later (Aldritt *et al*., 1989; Vaidya *et al*., 1989).

Akhil B. Vaidya (1947–) received his doctor of philosophy (PhD) in 1972 from the University of Bombay (Mumbai, India), then did research at the Institute for Medical Research in Camden, New Jersey (1972–1977), and subsequently joined the Department of Microbiology, Hahnemann University (Philadelphia, Pennsylvania; now Drexel University College of Medicine). He was trained as a retrovirologist at a time when molecular biology was moving into its recombinant DNA phase. His laboratory at Drexel University was involved in constructing genomic libraries and fishing out endogenous retroviruses. William Weidanz, a colleague in the department who worked on rodent malaria parasite immunology, suggested that he construct genomic libraries of malaria parasites to identify antigen genes. A newly starting graduate student, Prema Arasu, did just that, and at Vaidya's suggestion, checked the quality of the library by screening it with a probe made from labeled total parasite DNA; the idea was that repeated sequences in a eukaryotic genome would be represented frequently in a good-quality library and detectable by the labeled total DNA. Prema's library was quite good and many clones lit up, apparently containing the repeated sequences. These clones were put aside for a future leisurely look. In 1988, the sequence of one of these clones was obtained. Using ARPANET (the precursor of the Internet), the sequences were submitted for a homology search to IntelliGenetics (there was no NCBI at that time). Surprisingly, the 6-kb tandemly arrayed 'repeated' sequence encoded mitochondrial electron transport genes. Vaidya had literally stumbled on to the mitochondrial genome of malaria parasites!

The discovery of the smallest mitochondrial genome, its unusual gene content and organization altered Vaidya's scientific career. He gave up his funded retrovirus program, cancelled the subscription to the *Journal of Virology*, and focussed his laboratory's interests on these unusual mitochondria. Vaidya found that the sequence of the electron transport chain proteins were significantly divergent from their mammalian counterparts so as to explain selective toxicity of anti-malarials targeting the parasite mitochondrion. The malarial mitochondrion is a validated target for anti-malarial drugs and the parasite genome is revealing additional potential targets in mitochondrial functions. Recently, Vaidya's laboratory found that the mitochondrial electron transport in blood stages of *P. falciparum* is maintained for just one purpose: to regenerate CoQ to be used with dihydroorotate dehydrogenase (DHOD) during pyrimidine biosynthesis. Transgenic parasites expressing yeast DHOD that does not

use CoQ were completely resistant to ALL electron transport inhibitors (Painter *et al.*, 2007). This now provides the means to examine closely the mitochondrial physiology in malaria parasites.

The *P. falciparum* mitochondrion contains 30–100 copies of the genome and encodes only three proteins (cytochrome b and subunits I and III of cytochrome oxidase) and ribosomal ribonucleic acid (rRNA). The rRNA genes are fragmented and scrambled; it does not encode any transfer ribonucleic acid (tRNA), ribosomal proteins or ATPase subunits. As such, the *Plasmodium* mitochondrion 'denotes an endpoint in the slimming down process whereby the ancient progenitor of all mitochondrial genomes began the slow march to near total dependence on host nuclei' (Williamson, 1998).

In metazoans, the electron transport chain consists of four integral membrane complexes localized to the inner mitochondrial membrane: complex I (NADH-ubiquinone oxidoreductase), complex II (succinate-ubiquinone oxidoreductase), complex III (ubiquinol-cytochrome c oxidoreductase) and complex IV (cytochrome c oxidase), plus coenzyme Q (ubiquinone) and cytochrome c. As first shown by Fry and Beesley (1991), the plasmodial electron transport chain differs from the metazoan system in lacking complex I; however, a single subunit NADH dehydrogenase is present and is homologous to that found in plants, bacteria and yeast but not in animals (Krungkrai, 2004; Vaidya, 2004, 2005; van Dooren *et al.*, 2006).

Electron flow in the mitochondrion of *Plasmodium* involves the substrates NADH, succinate, malate, DHO and glycerol-3-phosphate, located within the inter-membrane space or the matrix, being acted upon by the NADH dehydrogenase (rotenone-insensitive (alternative) complex I), succinate dehydrogenase (complex II), malate-quinone oxidoreductase, DHODH, and glycerol-3-phosphate dehydrogenase present within the inner membrane. The electron acceptor of all these dehydrogenases is ubiquinone, oxidized by complex III (cytochrome bc_1 complex)—the complex that transfers electrons to cytochrome c. The reduced cytochrome c is oxidized by complex IV (cytochrome c oxidase) that transfers electrons to oxygen. It is the oxidation of cytochrome c by molecular oxygen that explains why malaria parasites are microaerophilic. Complexes III and IV also generate a proton gradient and membrane electropotential across the inner membrane.

However, is it possible that malaria parasites also have a functional Krebs cycle? Gene expression profiles of blood-stage parasites have indicated an apparent coordinated expression of genes encoding TCA cycle enzymes; however, since the expression is so low, it is understandable why biochemical assays failed to detect such activities. (Unexplained is how Speck, Moulder and Evans (1946) were able to detect TCA cycle enzymes.) Since plasmodial mitochondria lack pyruvate kinase the

possibility for a conventional generation of acetyl coenzyme A (acetyl-CoA)within the mitochondrion cannot occur, and as a consequence, a block exists in the step necessary for initiation of the TCA cycle.

Malate dehydrogenase (MDH), an enzyme found in the mitochondria of eukaryotes is cytosolic in *Plasmodium* (Lang-Unnasch, 1992; Sherman, 1966d) and probably converts OAA to malate but, isocitrate dehydrogenase (IDH), citrate synthetase and malate-quinone-oxidoreductase—an enzyme previously found only in prokaryotes—have been localized to the mitochondrion using *N*-terminal targeting signal sequences tagged green fluorescent protein (GFP). The falciparum gene for IDH has been cloned and sequenced; it is localized on chromosome 13, has a molecular weight of 51 kDa and shows 55–57% homology to the mitochondrial IDH of mammals (Wrenger and Muller, 2003). It is unlikely that IDH is a part of the TCA cycle and since under exogenous oxidative stress there is an up-regulation and messenger ribonucleic acid (mRNA) and protein levels are increased, its major role may be in the maintenance of the intra-mitochondrial redox balance rather than in energy metabolism (as was shown for mouse mitochondrial nicotinamide-adenine dinucleotide phosphate (NADP)-dependent IDH).

The synthesis of adenosine triphosphate (ATP) in the mitochondrion is carried out by a multi-protein enzyme complex, F_0F_1 ATP synthase (complex V), that utilizes the proton gradient for this purpose. The genome of *P. falciparum* lacks the genes for encoding subunits a and b in the F_1 ATP synthase and hence the enzyme cannot function to generate ATP (Vaidya and Mather, 2005; van Dooren *et al*., 2006). (This confirms the work of Fry and Beesley, which suggested that the mitochondrion contributes little to the ATP pool of the parasite.) Because of this it is unlikely that even in the insect stages there is a functional TCA cycle. Perhaps a key point against the presence of a canonical TCA cycle in all asexual stages of malaria is that found 50 years ago: essentially all of the glucose consumed ends up as lactate.

Since the TCA cycle enzymes do not appear to function for ATP production, what role might they play? It has been suggested that they function biosynthetically providing succinyl-CoA for heme biosynthesis using the Shemin pathway. In this pathway succinyl-CoA is conjugated to glycine by δ-aminolevulinate synthetase to form δ-aminolevulinate (δ-ALA) the first substrate in the heme synthetic pathway. δ-ALA synthetase has been localized to the mitochondrion, however, since all the other enzymes in the heme biosynthetic pathway are found in the apicoplast (see later) it poses a problem as to the mechanism for shuttling substrates between these two organelles. A way around this may be the findings of Varadharajan *et al*. (2002) in which ferrochelatase was localized to the apicoplast. Surprisingly, since the cytosol of the parasite contains all the heme biosynthetic enzymes (which are host derived) possibly when provided with the mitochondrial-formed δ-ALA heme assembly takes

place there. Although such a scheme has attractive aspects it is not free from problems: how are the reactions regulated, how are the apocytochromes chaperoned, how is free heme maintained prior to import into the mitochondrion and what mechanisms serve for heme transport? Finally, yet to be discovered is the source of iron used by ferrochelatase.

It is now clear that the *Plasmodium* mitochondrion is not a significant source of ATP, however, its electron transport system is still critical for parasite survival. Its primary metabolic function, as shown in a series of elegant experiments from Vaidya's laboratory, is to regenerate (through complex III) the oxidized ubiquinone required as the electron acceptor for dihydroorotate dehydrogenase (DHOD), an enzyme essential for the *de novo* synthesis of pyrimidines (Painter *et al*., 2007). Although mitochondrial electron transport can be dispensed with by providing a cytosolic bypass for orotate synthesis, mitochondrial membrane potential is still required for other metabolic steps relegated to the mitochondrion.

CHAPTER 12

The Road to Atovaquone

Shortages of quinine during World War II provoked an interest in developing new anti-malarials (see earlier). After screening thousands of compounds, several hundred hydroxy-naphthoquinones were synthesized and some, when administered orally in ducklings infected with *Plasmodium lophurae*, had greater activity than quinine; they were also found to inhibit the respiration of the parasite at exceedingly low concentrations. During this same period, Wendel (1946) found that 2-hydroxy-3-alkyl-naphthoquinones affected the respiration of *Plasmodium* and when Ball *et al*. (1947) found that 2-hydroxy-3-alkylnaphthoquinones inhibited the respiration of beef heart succinoxidase they concluded the inhibition was due to 'some step in the chain of electron transport below cytochrome c and above cytochrome b.' During World War II, sponsored by the Malaria Research Committee, Fieser *et al*. (1948) synthesized an array of naphthoquinones and found that menoctone (3-(8-cyclohexyl)-octyl-2-hydroxy-1, 4-naphthoquinone) was a potent inhibitor of dihydronicotinamide adenine dinucleotide (NADH)- and succinate-cytochrome c reductases of yeast and sub-mitochondrial particles (Fieser *et al*., 1967). However, little further work was done with hydroxy-naphthoquinones and malaria until the 1960s when, lapinone was found to have clinical activity against *P. vivax*; however, poor absorption after oral administration required high doses and intravenous administration and, as a consequence, it was not developed any further (Aviado and Will, 1969; Fawaz and Haddad, 1951). (Retrospectively, it would be discovered that testing of naphthoquinones in animal models for malaria would not predict efficacy in humans.)

During the late 1960s, the ubiquinones of malaria were found to differ from those of the host: *P. lophurae*, *P. cynomolgi* and *P. berghei* synthesized (from *p*-hydroxybenzoic acid) ubiquinones 8 and 9, whereas the mitochondria of mammalian and avian cells contained ubiquinone 10. Based on these

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differences it was suggested that structural analogues of ubiquinone would act as anti-malarials by acting as inhibitors of the electron transfer mechanisms in *Plasmodium* (Porter *et al*., 1973; Porter and Folkers, 1974; Rietz *et al*., 1967; Schnell *et al*., 1969; Skelton *et al*., 1969; Wan *et al*., 1974). Indeed, atovaquone has structural similarity to ubiquinones and studies with BW58C (and BW566C) showed that the inhibition of respiration of mitochondria isolated from plasmodia was 1000-fold more sensitive than were mammalian and avian mitochondria (Hudson *et al*., 1985). Subsequently, it was shown that atovaquone acts on the cytochrome bc_1 complex (in which electrons are transferred from ubiquinol to cytochrome c) to block either ubiquinol oxidation or ubiquinone reduction (Fry and Pudney, 1992; Kessl *et al*., 2003).

The use of naphthoquinones as chemotherapeutics continued to languish for decades until it was discovered that menoctone was effective against the protozoan causing East Coast fever (*Theileria parva*) (McHardy, 1978). Structural studies of analogues of menoctone using *in vitro* cultures of *T. parva* resulted in the identification of a more suitable and effective agent, 2-cyclohexyl-3-hydroxy-1,4-naphthoquinone (parvaquone) (McHardy and Morgan, 1985). At the Wellcome Research Laboratories in the United Kingdom, this finding, together with the discovery that menoctone blocked electron transport/pyrimidine biosynthesis in *Plasmodium* (Gutteridge *et al*., 1979), led to re-newed interest in the possibility of using naphthoquinones for other protozoan infections, including malaria, and so several analogues of parvaquone were synthesized. The compound 1,2-(4-*t*-butylcyclohexyl)-3-hydroxy-1,4-naphthoquinone, coded as BW58C, had exceptional activity against several murine malarias, as well as *Toxoplasma* and *Eimeria* (Hudson *et al*., 1985). However, BW58C was dropped from further consideration when it was tested in humans and was shown to be rapidly converted to an inactive red-colored metabolite that appeared in the urine. (The metabolism of BW58C was replicated *in vitro* using human liver microsomes.) Retrospective testing of other naphthoquinones made in this system identified a chlorophenyl substitution of the cyclohexyl ring, coded as BW566C, which was metabolically stable. At the same time, with *P. falciparum* available both *in vitro* and *in vivo* (*Aotus* monkeys), it became possible to evaluate the structure and clinical activity of BW566C against human malaria (Fry and Pudney, 1992). It was more active than the standard anti-malarials in *in vitro* cultures of *P. falciparum* and against the murine malarias *P. berghei* and *P. yoelii* and it was curative when administered orally in *Aotus* monkeys infected with *P. falciparum*. BW566C was named atovaquone (Hudson, 1993). It was effective against *Pneumocystis carinii* infections in acquired immune deficiency syndrome (AIDS) patients and was registered as Mepron for this indication. In combination with proguanil, it was highly active against malaria and for this indication was registered as Malarone. High production

costs of the drug has precluded its use in developing countries, but its exquisite safety and potent activity mean that it is now the benchmark anti-malarial chemoprophylactic for the traveler market.

Winston (Win) Gutteridge (1941–), born in the county of Yorkshire (United Kingdom) had his interest in biochemistry stimulated by Phillip Randall (later Sir Phillip Randall, FRS, of insulin mode of action fame) whilst reading Part I of the Natural Sciences course at Cambridge University (Cambridge, United Kingdom). He received a bachelor of arts (BA) in biochemistry (1963) and then a doctor of philosophy (PhD, 1967) and a doctor of science (ScD, 1986) in biochemical parasitology at the same university. His graduate research with Bruce Newton investigated the mode of action of the anti-trypanosomal drug, pentamidine. Results were inconclusive then and despite subsequent work by others not only against trypanosomes but also *Pneumocystis* and *Plasmodium*, to this day no-one knows exactly how this drug works. The project, however, led to a life-long interest in parasites and their chemotherapeutic agents. In 1967, he joined the Division of Parasitology at National Institute for Medical Research (NIMR, London, United Kingdom) as a biochemical parasitologist (Frank Hawking, father of the physicist Stephen Hawking was division head at that time). He began collaborative projects on trypanosomes (Jim Williamson), helminths (Bridget Olgilvie) and malaria (Peter Trigg). Trigg and Gutteridge's strengths were complementary to one another and their goal was to understand the biochemical nature of the malaria parasite better, how it might be cultured *in vitro* and how anti-malarial drugs worked. Their first publication (1969) looked at deoxyribonucleic acid (DNA) base composition, and later studies were concerned with the enzymes of pyrimidine biosynthesis and the effects of chloroquine and pyrimethamine on the synthesis of plasmodial DNA and ribonucleic acid (RNA). In 1971, Gutteridge left the NIMR and became a founding member and ultimately reader in biochemical parasitology at the University of Kent where his research was primarily with *Trypanosoma cruzi* and the chemotherapy of Chagas' disease. In 1982, Win left academic research and joined the Wellcome Research Laboratories, initially as head of the department of parasitology and later as principal research scientist and head of biosciences. Initially he continued a joint University of Kent/Wellcome project on pyrimidine biosynthesis involving graduate student Dilip Dave and Wellcome's Harry Richards and Colin Ginger (who had begun work at Wellcome in 1972). At the time of his arrival in Beckenham there was suggestive evidence for a connection between folate metabolism, dihydrofolate reductase (DHFR), pyrimidine biosynthesis (TS) and the action of some 2,4-diaminopyrimidines such as

pyrimethamine, but the specific link had not been found. The result of this collaboration was their classic paper, 'Conversion of dihydroorotate in parasitic protozoa' (Gutteridge *et al*., 1979) which noted, 'dihydroorotate dehydrogenase is ... probably mitochondrial, and intimately connected to the electron transport chain ... passes electrons directly at the ubiquinone level ... and since menoctone is a good inhibitor of the enzyme, could represent a useful chemotherapeutic target'. In time, and with the collaboration of other Wellcome scientists, this would lead to the synthesis of atovaquone, discovery of its activity against *P. carinii* and ultimately registration as Mepron/Wellvone. Win also found synergy between atovaquone and proguanil and this led to registration as Malarone for the treatment and chemoprophylaxis of malaria.

In 1995, when Wellcome was taken over by Glaxo, Gutteridge moved to the World Health Organization/Special Programme for Research and Training in Tropical Diseases (WHO/TDR, Geneva, Switzerland) where he became the chief of product research and development and was responsible for identifying potential drug and vaccine leads that have led, among others, to the approval of artemotil for parenteral treatment of severe malarial and regulatory submission of chlorproguanil-dapsone, subsequently approved for the treatment of uncomplicated malaria. In 2001, he retired from WHO/TDR to become a founding board member of the Medicines for Malaria Venture (MMV, Geneva, Switzerland) and in 2004 also assumed the chair of its Expert Scientific Advisory Committee. Presently he is a consultant on neglected infectious diseases.

Atovaquone was not a panacea in the treatment of malaria. When it was used as a monotherapy for *P. falciparum* infections there was a 30% treatment failure rate with atovaquone-resistant parasites emerging 28 days after treatment (Looareesuwan *et al*., 1996). Resistance appeared to involve mis-sense mutations in a region of cytochrome b especially near the highly conserved PEWY sequence, a peptide sequence important for the way ubiquinone (or an inhibitor) fits into the binding site of the malaria cytochrome bc_1 complex (Kessl *et al*., 2003; Srivastava *et al*., 1999; Vaidya, 1998). To counter the problem associated with treatment by atovaquone alone it has been combined with proguanil hydrochloride (Malarone) and the combination has been found to be more effective than either component alone or mefloquine, chloroquine, or sulphadoxine-pyrimethamine in areas where parasites are resistant to these drugs (Kessl *et al*., 2007).

CHAPTER 13

The Ring Road to the Apicoplast

Until the 1980s all or most mitochondrial DNAs were regarded as circular. In fact, this 'rule of the ring' dogma was erroneous and impeded the appreciation of the existence of a third genome in *Plasmodium*. Delays in understanding due to scientific dogma are well known—in the words of the evolutionary palaeontologist George Gaylord Simpson, 'Science is a study of errors slowly corrected.' Indeed, this is what happened with the astonishing discovery of an unexpected unique organelle in malaria parasites, the apicoplast (Ralph, 2005; Sato and Wilson, 2005; Wilson, 1998, 2002, 2005).

In 1975, Araxie Kilejian, a post-doctoral scientist in William Trager's laboratory at New York (Rockefeller University, New York), carried out sub-cellular fractionation of *Plasmodium lophurae*. Her intention was to gain greater understanding of the nature of the mitochondrial satellite DNAs that had been reported for *P. knowlesi* and *P. berghei* and why such satellite DNAs were not found in *P. lophurae* and *P. gallinaceum*, which had perfectly good mitochondria. Kilejian (1975) resolved the conundrum by proposing that the 'mitochondrial deoxyribonucleic acid (DNA) from *P. lophurae* had the same buoyant density as nuclear DNA'; in this way the satellite DNA was masked. On finding a discrete population of circular molecules by electron microscopic examination of the 'mitochondrial DNA' fractions from *P. lophurae* she suggested it would be of interest to characterize the satellite DNAs in rodent and primate malarias previously identified by Gutteridge, Trigg and Williamson (1969, 1971). In 1985, Williamson *et al*. (1985) showed the minor A + T-rich species of DNA carried by *P. knowlesi* to be composed of covalently closed circles and presumed them 'to be of mitochondrial origin'. So too were those of *Toxoplasma gondii* observed about the same time (Borst *et al*., 1984).

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Three years later, similar 35 kb circular molecules were described from *P. falciparum* and these too were assumed to be mitochondrial (Gardner *et al*., 1988). However, the laboriously hand-sequenced ribosomal ribonucleic acids (rRNAs) found on the 35 kb circle of *P. falciparum* were unlike those on other mitochondrial DNAs: they were uninterrupted, full length, duplicate and arranged in the form of an inverted repeat. In 1996, Wilson *et al*. (1991) described the complete genomic map of the plasmodial plastid-DNA. Such an arrangement, wrote Wilson *et al*., 'has never been found in mitochondrial genomes, however, (they) are features of chloroplast genes.' Prior to the work of Wilson, Williamson and Gardner a plastid was not on anyone's horizon (neither was its existence readily accepted by many in the scientific community) but after a decade of persistent long-term research and with provision of more and more persuasive evidence, it eventually became clear that malarial parasites did contain a vestigial algal plastid with its own genome (Feagin, 1994; McFadden and Waller, 1997; Ralph, 2005; Williamson, 1998; Wilson, 2002, 2005; Wilson *et al*., 1996).

Iain Wilson (1939–) obtained his doctor of philosophy (PhD) in 1964 in the National Institute for Medical Research (NIMR, London, United Kingdom) Division of Parasitology for his research on the allergic responses to nematodes. He probably would have continued with the immunology of nematodes were it not for a chance encounter at Mill Hill with Ian McGregor who, convinced that a malaria vaccine was just 5 years away, encouraged Wilson to join him in The Gambia to work on protective malarial antigens. For several years, Iain worked in Africa and defined the strain-specific S-antigens of *P. falciparum*, but after returning to NIMR in 1971 he was told by its then director, Arnold Burgen (later Sir Arnold), to wean himself off S-antigens. In the course of subsequent annual trips to The Gambia he studied the relationship between genetic disorders (particularly glucose-6-phosphate dehydrogenase (G6PD) and hemoglobinopathies) and parasite invasion of erythrocytes and (with Geoffrey Pasvol) described the protective effect of fetal hemoglobin (HbF). Later Iain worked with Walter Gratzer in London (United Kingdom) on the erythrocyte cytoskeleton. They showed that ghosted human red cells could be made reversibly susceptible or resistant to malarial invasion by adjusting the intracellular level of adenosine triphosphate (ATP). This phase of work in the 1980s overlapped the period when the first AT-rich *P. falciparum* genes were being cloned and Iain's interests shifted again. During a meeting at the World Health Organization (WHO, Geneva, Switzerland) Iain asked John Scaife (see earlier) how he accounted for the low- and high-density DNAs

that had been found in *P. knowlesi* and *P. berghei* by his colleagues at NIMR (Gutteridge *et al*., 1969). Scaife suggested the low-density A-T-rich DNA was from the parasite (as with *P. falciparum*) whereas the heavier G-C-rich DNA was probably contaminating host cell DNA. Wilson already knew that the minor DNA from the mammalian and avian malarias had been claimed to be mitochondrial and he felt that cloning and sequencing might better resolve the relationship between organellar DNA and possible contaminants. With the help of the 'mitochondrionologist' Don Williamson (head of the Division of Cell Propagation at NIMR) the G-C-rich DNA in *P. knowlesi* was clearly shown to be plasmodial and not a host nuclear contaminant. Based on this work, Wilson and Williamson obtained WHO funds to clone the minor A-T-rich species of *P. knowlesi*. In collaboration with Malcom Gardner (see earlier), A-T-rich organellar DNA was found in the first clones, but just as Williamson was about to drop out of the collaboration (because he thought the project would be a boring exercise in sequencing just another mitochondrial genome), their continued cloning, sequencing and restriction mapping suggested that the circular molecule with which they were working was atypical and possibly not mitochondrial. They applied for continued funding from WHO but ran into a problem—the WHO decided they would not continue to support research on *P. knowlesi* and so Wilson and Williamson re-directed their efforts to separating the A-T-rich organellar and nuclear DNAs of *P. falciparum* by density-gradient centrifugation—the only approach then available for separation. Williamson eventually achieved clean separations and when genes for ribosomal proteins and ribonucleic acid (RNA) polymerase were identified in the clones, and these most closely resembled those of chloroplasts, it became clear that this minor DNA represented a third genome of *Plasmodium*. They boldly hypothesized that the plastid DNA had arisen by endosymbiosis of an algal cell and was packaged in a novel organelle, later termed the apicoplast (Kohler *et al*., 1997).

The apicoplast in asexual trophozoite stages of *P. falciparum* is a tubular structure measuring 0.35 μm × 1.6 μm that apparently has a triple-membraned wall and is closely adherent to the mitochondrion; the interior matrix contains ribosome-like granules and a network of fine-branched filaments (Hopkins *et al*., 1999). Most of the proteins in the apicoplast are now encoded by genes in the nucleus. As a result, they must be targeted from the cytosol back to the organelle. Using a bioinformatics approach, McFadden's group found that more than 540 gene products have been predicted to be targeted to the apicoplast (Gardner *et al*., 2002; Ralph, 2005; Ralph *et al*., 2004). Nuclear-encoded

apicoplast proteins include housekeeping enzymes involved in DNA replication and repair, transcription, post-translation modifications, co-factor synthesis, protein and metabolite import, and protein turnover. Despite great progress in identifying apicoplast proteins, the functions of about 60% of the putative apicoplast-targeted proteins remain unknown. In addition to the nuclear-encoded proteins, there are 23 proteins encoded by the apicoplast genome itself. In *P. falciparum*, the apicoplast includes genes for three subunits of a bacteria-like RNA polymerase, 17 ribosomal proteins, the translation factor Tuf, a likely chaperone Clp, and *SufB*—this last protein of unknown function is involved in the assembly of Fe–S clusters (Gardner *et al*., 2002). Intriguingly, the presence of *SufB* on the plastid genome is diagnostic of a red as opposed to a green algal origin. Microarray analysis of the *P. falciparum* transcriptome (Bozdech *et al*., 2003) has shown that most of the apicoplast-related genes reach maximal levels of messenger ribonucleic acid (mRNA) transcription as the parasite develops from the late trophozoite to the early schizont stages in the erythrocytic cycle.

Because the apicoplast of *Plasmodium*, a slimmed down chloroplast that has 'forgotten' how to photosynthesize, has no counterpart in the host, it continues to be an attractive target for novel chemotherapeutic agents. The apicoplast is an essential *Plasmodium* organelle: parasites die after treatment with drugs that interfere with a variety of apicoplast functions and mutant parasites lacking an apicoplast are not viable. However, the specific causes of parasite death remain obscure. One of the great mysteries associated with apicoplast function is delayed death: why do parasites continue to grow normally after interruption of apicoplast function but die when the parasites infect a new host cell (Ralph, 2005; Ralph *et al*., 2004).

The literature on the absence of *de novo* fatty acid (FA) synthesis by *Plasmodium* is both abundant and compelling; indeed, earlier studies found poor incorporation of radiolabeled precursors into plasmodial lipids, and where incorporation did occur, it was interpreted to be due to chain elongation of scavenged FAs (Beach *et al*., 1977; Holz, 1977b). This view would later be challenged by the discovery of genes involved in FA synthesis in the *P. falciparum* genome, as well as the demonstration that acetate is incorporated into its FAs (see later). Further, using a bioinformatics approach it has been possible to predict the existence of a complete biosynthetic pathway for lipids in the apicoplast (Goodman and McFadden, 2007; Ralph *et al*., 2004; Sato and Wilson, 2005; Wiesner and Jomaa, 2007).

In apicoplast FA biosynthesis, the main carbon substrate is acetyl-coenzyme A (acetyl-CoA) generated from acetate by the action of acetyl-CoA synthase or from pyruvate by the pyruvate dehydrogenase (PDH) complex. Within the plastid, pyruvate is formed by the action of

pyruvate kinase on phosphoenolpyruvate (PEP) imported from the cytosol. Enzymatic activity of the PDH complex requires three co-factors: Co A, lipoic acid and thiamin pyrophosphate (TPP) and the latter two have been shown to be synthesized and assembled in the plastid. Most animals, including humans, have a type I fatty acid synthase system (FAS I), however, in malaria parasites the genes specifying FAS I are missing. Instead, *Plasmodium* has a FAS II system that produces straight chain C10, C12 and C14 FAs to be used in membrane formation and possibly in signaling pathways.

Acetyl CoA is used as the first step of the FAS II pathway, alternatively, the FAS II system can utilize malonyl-CoA formed by the action of the biotin-dependent acetyl-CoA carboxylase (ACCase) on acetyl-CoA (for details see Ralph *et al*., 2004; Wilson, 2005; as well as http://sites.huji.ac.il/malaria/maps/facidsynthesispath.html; last accessed 16 July 2008) in *Plasmodium* the plastidic ACCase is encoded on the nuclear genome. The ACCase of grasses and that of *P. falciparum* are susceptible to herbicides of the arylyphenoxy-propionate class such as Clodinop, however, the concentration needed for anti-malarial activity is too high to make such compounds useful clinically (Sato and Wilson, 2005). Although almost all apicoplast isoenzymes of the FAS II pathway have been characterized in *P. falciparum*, only a limited number of inhibitors have been developed and evaluated. For example, the antibiotic thiolactomycin^{TM}, which binds to the FAS II enzyme β-ketoacyl-acyl carrier protein (ACP) synthase, has been shown to inhibit the *in vitro* growth of *P. falciparum* (Waller *et al*., 1998). In addition, although tricolsan, an inhibitor of the FAS II enol-ACP (acyl carrier protein) reductase, has been shown to have a rapid action and can cure malaria infections in mice by blocking the incorporation of ^{14}C FAs in cultures of *P. falciparum*, only two of 20 analogues inhibited the purified *P. falciparum* enzyme and parasite growth *in vitro* was not reduced significantly (Sato and Wilson, 2005).

Another primary metabolic pathway of the apicoplast is isoprenoid biosynthesis (Ralph *et al*., 2004; Sato and Wilson, 2005; Wiesner and Jomaa, 2007). Isoprenoids are composed of repeated isopentenyl pyrophosphate (IPP) units. The plasmodia synthesize IPP using 1-deoxy-*D*-xylulose-5-phosphate (DOXP) enzymes *via* a non-mavelonate or DOXP pathway. The DOXP pathway is widely distributed in bacteria and the plastids of algae and plants but not mammals. The starting compounds for IPP biosynthesis are pyruvate and glyceraldehydes-3-phosphate, not mavelonate. The first enzyme in the DOXP pathway, DOXP synthase, generates DOXP required for the synthesis of pyridoxal and TPP, as well as serving as the basis for the formation of ubiquinones and dolichols. (The TPP is necessary for the activity of the PDH complex as well as for DOXP synthase.) The product of subsequent enzymatic reactions yields IPP and dimethylallyl

pyrophosphate, the building blocks for isoprenoids that are used to form dolichols (essential for the transfer of glycosylphosphatidylinositol (GPI) anchors onto membrane-bound proteins) and the prenyl-containing ubiquinones of the mitochondrion. Modifications of transfer ribonucleic acid (tRNA) for the translation of apicoplast-encoded proteins are also a function of the DOXP pathway.

The discovery of a prokaryotic DOXP pathway associated with the apicoplast has stimulated efforts for the development of novel anti-malarials for this drug target (reviewed by Goodman and McFadden, 2007). The antibiotic, fosmidomycin, an inhibitor of the DOXP pathway, blocked the *in vitro* growth of *P. falciparum* and was used in a clinical trial with infected humans. Although parasitemia and fever cleared initially there was a high rate of recrudescence and gametocytemia (see Sato and Wilson, 2005). These results indicate that fosmidomycin should not be used as a monotherapy; however, this study provides encouragement that fosmidomycin-like inhibitors could be effective as anti-malarials.

Recently, it has been found that the slow-acting tetracycline doxycycline impairs the expression of apicoplast genes (Dahl and Rosenthal, 2007; Dahl *et al*., 2006) with such a loss of function the progeny of treated parasites were damaged. Treatment of patients with anti-malarial combinations that included azithromycin have shown promise; the molecular target of the antibiotic seems to be the plastid's ribosomal protein rpl4 (Sidhu *et al*., 2007). In contrast, despite the demonstration that ciprofloxacin, an inhibitor of DNA gyrase, was able to block plastid replication, it provided no clinical benefit. The hunt continues in the hope of finding effective inhibitors that can fit into rational therapeutic regimens, providing both immediate clinical value, as well as helping to delay the onset of resistance to the components of anti-malarial drug cocktails.

CHAPTER 14

Ribosomes and Ribosomal Ribonucleic Acid Synthesis

The cellular anvil upon which proteins are fabricated, the ribosome, consists of two loosely associated particles that contain ribonucleic acid (RNA) and proteins. In prokaryotes, as well as chloroplasts and mitochondria, the monomeric particle that sediments in the 70 S range, though can be dissociated into a larger 50 S and a smaller 30 S particle, contains 23 S, 16 S and 5 S RNA and at least 50 proteins. Eukaryotes, in contrast, have monomers that sediment at 80 S, can be dissociated into 60 S and 40 S subparticles, and contain 28 S, 18 S and 5 S RNA plus a greater number of proteins (Rogers *et al*., 1998). My interest in research on the ribosomes of *Plasmodium* was accidental. It was piqued during a May 1969 Panel Workshop on Experimental Malaria (organized by Elvio Sadun and held at the Walter Reed Army Institute of Research (WRAIR, Washington) when Joseph Ilan, a biochemist with considerable experience working with ribosomes and ribosomal RNA (rRNA), announced that the malaria parasite was able to synthesize its proteins by 'stealing' a 60 S subparticle from the host red cell and combining this with a plasmodial-synthesized 40 S subparticle. By extension, he went on, this inability of the *Plasmodium* to fabricate a complete ribosome would be a basis for its living as an obligate intracellular parasite.

Joseph and Judith Ilan (then at Temple University, Philadelphia, Pennsylvania) and now at Case Western Reserve University (Cleveland, Ohio) were attracted to work on malaria parasites because of their biochemical expertise, the availability of funding from the United States Army and the fact that Judith's father was the eminent Israeli parasitologist Saul Adler. They based their contention on the following observations: after injecting *Plasmodium berghei*-infected mice with $NaH^{32}PO_4$ the

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25 S, 15 S and 4S rRNA, as well as the 80 S and its 40 S subparticle were radiolabeled; however, the existence of the 60S subparticle had to be inferred because no ^{32}P was associated with it. They stated, 'The source of the 60 S subparticle is a mystery ... there is the possibility that the large rRNA component of malarial ribosomes is provided in part or entirely from the host ribosomes.' Upon hearing this (and later reading it in their publication Tokuyasu *et al*., 1969), I found it difficult to conceive of a mechanism that would allow the malarial parasite to ingest host ribosomes (or RNA) *via* its cytostome, place these in food vacuoles (FVs) where the contents would be digested and then to have the residuum become the larger subparticle of the parasite ribosome. But, I did not have an opportunity to follow up on this until 1973 when I took a sabbatical leave from University of California, Riverside (UCR, Riverside, California) and received a special research fellowship from the National Institutes of Health (NIH, Bethesda, Maryland) to work at the National Institute for Medical Research (NIMR, London, United Kingdom) to learn how to isolate plasmodial ribosomes and then to use these in a cell-free protein-synthesizing system.

In the NIMR laboratory were Peter Trigg, Peter Shakespeare, Neil Brown, Iain Wilson and James Williamson. The NIMR had access to *P. knowlesi* in rhesus monkeys and in the Division of Biochemistry was R. A. Cox, an expert in ribosomeology, and his very capable technician, Betty Higginson. During the Fellowship I learned to isolate rabbit reticulocyte ribosomes, developed a Triton X-100 lysis method to release ribosomes from knowlesi-infected red cells and then was able to separate these by density-gradient centrifugation and characterize them (Sherman *et al*., 1975). With *P. knowlesi* the sedimentation constant was 80 S (which had been reported earlier by R. Cook *et al*., 1971, at the WRAIR as was that of *P. berghei* (Tokuyasu *et al*., 1969). This indicated that we were working with eukaryotic ribosomes. However, their base composition was 37% G + C—typically protozoan— and distinctly different from those of the mammalian host (which was 67% G + C). Confirmation of this was evident from the melting profiles of *P. knowlesi* rRNA from the larger subparticle that was distinctly different from the 60 S subparticle of rabbit reticulocyte rRNA. Later work by Trigg, Shakespeare and Kyd (1975) showed that *P. knowlesi* had RNAs with sedimentation coefficients of 16.6 S and 24.2 S but host RNAs of 28 S and 18 S were absent. Taken together, we concluded that it was most unlikely that the large ribosomal subunit of malarial ribosomes was provided by the host.

Returning to UCR similar studies were carried out with *P. lophurae* (Sherman and Jones, 1976, 1977). Having specific nucleotide markers for plasmodial ribosomes made it possible to study their biosynthesis. When *P. lophurae*-infected duckling red cells or 'erythrocyte-free' parasites were incubated in the presence of ^{3}H-adenosine radioactivity was recovered in both the 60 S and 40 S subparticles and the rRNA consisted of 25 S and

17 S, both of which were labeled. Radioactivity was recovered only in the adenosine monophosphate (AMP) fraction of the hydrolyzed rRNA providing evidence that these RNAs are the transcription products of parasite cistrons.

In 1978, Miller and Ilan, now aware that their 'stolen subparticle' hypothesis was without merit, found the source of their error to be 'a specifically hydrolyzed RNA species ... associated with the 60 S ribosomal subunit' and 'uncontrolled ribonuclease may ... explain why the larger rRNA was always isolated in a degraded form.' After 1984, the Ilan laboratory was no longer involved in research on the biochemistry of malaria parasites (Miller and Ilan, 1984).

Several studies have shown the rRNA genes of *Plasmodium* to be present as four to seven non-identical copies situated on different chromosomes (Li *et al*., 1997; Rogers *et al*., 1996; Waters *et al*., 1995) and not transcribed in association with a nucleolar complex (Shaw *et al*., 1996). Further, the transcription of the nuclear-encoded rRNAs were developmentally regulated in their expression during the various stages of the life cycle, and thus named A-type (asexual), O-type (oocyst) and S-type (sporozoite) (Sharma and McCutchan, 2005). In *P. vivax* and in *P. falciparum* there are also stage-specific variations in the base sequence of the 18 S, 5.8 S and the 28 S rRNAs, suggesting that these may reflect functional differences that allow the parasite to exercise novel post-transcriptional mechanisms for stage-specific gene expression (Thompson *et al*., 1999). However, in *P. berghei* the 'lack of structural differences in core regions of rRNA molecules, the lack of temporal differences in the expression of the S-type, as well as the observation that only one copy of the S-type rRNA genes is sufficient for complete development and the fact that both A and S-type ribosomes are active in the mosquito all indicate functional equivalence of the different ribosome types' (van Spaendonk *et al*., 2001).

Thomas McCutchan (1967–) graduated with a bachelor of arts (BA) from Depauw University (Greencastle, Indiana), majoring in chemistry. It was an interest in organic chemistry that led him towards the chemistry of nucleic acids at Purdue University (West Lafayette, Indiana) where he got his doctor of philosophy (PhD) in 1973. At Purdue his thesis advisor was P. T. Gilham who had worked with the Nobel laureate, Gobin Khorana, during the days when the genetic code was broken. In addition to supervising his thesis '*Innovations in oligonucleotide synthesis*', Gilham taught him two things of great value for his future career. First, know exactly what is going on in a test tube before you add the reagents together and, second, almost everything has been proposed at one time or the other but the credit goes to the

one who has enough faith in an idea to invest themselves and their time in bringing it to a logical conclusion. After a post-doctoral stint with Dieter Soll at Yale University (New Haven, Connecticut) working on transfer RNA (tRNA; 1973–1977), McCutchan joined Maxine Singer's laboratory at the National Cancer Institute, National Institutes of Health (NIH, Bethesda, Maryland) working on the defective variants of SV40 (along with about half the scientific world). Realizing that he needed to work on something that had a chance of having a direct influence on people rather than the very real pleasures of simply solving a problem in the laboratory, in 1978, he accepted a job at the NIH Laboratory of Parasitic Diseases (LPD, Bethesda, Maryland) where together with John Dame cloned the gene for the circumsporozoite protein (CSP) gene of *P. falciparum*. This was a real feat in 1983! Tom became a permanent fixture in the LPD (where he continues to work) which provided him with the opportunity to explore the backwaters of parasitology, including the influence of translational control on the development of the malaria parasite.

Andy Waters (1957–) studied biochemistry at the University of Leeds (Leeds, United Kingdom) and obtained a PhD (1983) from the University of Glasgow (Glasgow, United Kingdom) for a study of the transcriptional responses of uterine tissue to anti-estrogens. Thereafter, he wanted to work on a subject area that offered some possibility (although he had no idea how distant) of making a more direct contribution to a health problem. Sydney Cohen, an immunologist, provided the opportunity to begin to work with malaria at Guy's Hospital (London, United Kingdom) that through collaboration with McCutchan and Miller at the NIH, led to cloning a primate malaria gene encoding a leading erythrocytic vaccine candidate, AMA-1. Science works through many fortuitous circumstances and so because of the previous collaboration in 1987, McCutchan invited him to work in his group. There, he was really introduced to the fascinating biology of the malaria parasite and the possibility that it might be feasible to understand, at the molecular level, the development of the parasite and its interactions with its host and vector. There, Waters worked on the stage-specific ribosomes that McCutchan had discovered and used this to develop ideas about developmental biology, parasite evolution and diagnosis. During this period, another collaboration was initiated with Chris Janse and Barend Mons at the University of Leiden (Leiden, The Netherlands). In 1991, when Mons left, Chris invited Waters to join the group and together they attempted to develop Janse's sophisticated rodent model of malaria

further, allowing the generation of global analytical data and translation into an understanding of the interactive role of individual parasite elements in parasite survival. This is a seemingly never-ending process, but now they have begun to address specific questions about parasite protein structure and function. The Waters' group works towards providing fundamental understanding of parasite-specific processes at the molecular level with the hope that this information will provide a basis for the development of new approaches towards malaria therapy. In 2008, Waters will return to Glasgow to establish a group that will focus further on specific mechanisms of developmental regulation in the malaria parasite.

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CHAPTER 15

De Novo Synthesis of Pyrimidines and Folates

In the late 1960s, our laboratory, supported by funding from the United States Army Medical Research and Development Command (as well as a pre-doctoral fellowship from the National Institutes of Health to Charles Walsh), began to study the sources for nucleic acids in *Plasmodium lophurae*. We were fascinated by the work of Schellenberg and Coatney (1961) who found that the anti-malarials pyrimethamine (Daraprim) and proguanil (Paludrine) inhibited the incorporation of ^{32}P into the deoxyribonucleic acid (DNA) of *P. gallinaceum*, but not into ribonucleic acid (RNA). The drugs acted primarily on nuclear division rather than growth and although the action was believed to be due to a block in the production of tetrahydrofolate (THF; possibly by the inhibition of the enzyme DHFR), the lack of information about DNA synthesis of malaria parasites made it impossible to understand the precise mechanism of action of these drugs. With this as background, we proceeded to delineate the capabilities of malaria parasites for nucleic acid synthesis in the hope that such information would provide a basis for interpreting the effects of such drugs.

Using radioactive bicarbonate (^{14}C-$NaHCO_3$), we found it to be incorporated by infected red cells and free parasites into pyrimidines suggesting that *P. lophurae* synthesized cytosine, uracil and thymine *de novo* (Walsh and Sherman, 1968b). Further, evidence was found for the pyrimidine-pathway enzymes orotidine-5′-monophosphate pyrophosphorylase (OMPDC; also named orotidine-5′-decarboxylase) and thymidylate synthase (TS) in parasites but not in the red cells. The presence of TS made it possible for us to interpret the action of folic acid analogues in the same way as other microbes: inhibition of DHFR would lead to a

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depletion of THF, which in turn would prevent the synthesis of thymidylate and DNA. We went on to suggest that the insensitivity of parasite RNA synthesis to pyrimethamine and proguanil was not due to the non-oxidation of the THF (as had been shown for other organisms). Rather, because we found that malaria parasites did not synthesize purines *de novo* (i.e. formate was not incorporated) and had to rely on exogenous sources for these compounds, the only portion of the pathway for the synthesis of nucleic acids that would be sensitive to folate analogues would be the synthesis of thymidylate and its incorporation into DNA. We expected (as did George Hitchings and his group at Burroughs Wellcome) that a firmer base for malaria chemotherapy would be attained by studies of pyrimidine metabolism, and, although in subsequent years we went on to study the enzymes of the purine salvage pathway in more detail, we did nothing more with the *de novo* pyrimidine pathway and folates. Regrettably, after Charles Walsh completed his doctor of philosophy (PhD) in my laboratory and produced such ground-breaking work he never did any further research with malaria parasites.

Our finding of two pyrimidine biosynthetic enzymes (OMPDC and TS) in *P. lophurae* suggested that, unlike the duckling host, malaria parasites synthesized pyrimidines *de novo* from precursors such as CO_2, glutamine and aspartic acid (Walsh and Sherman, 1968b). Since that time, the enzymatic reactions of the *de novo* pyrimidine pathway and TS have been demonstrated in a variety of *Plasmodium* spp. and for *P. falciparum* (available online at: http://sites.huji.ac.il/malaria/maps/pyrimidinemetpath.html; last accessed 16 July 2008).

Paul F.G. Sims (1949–) was led into microbial/molecular genetics at Maidstone Grammar School for Boys (Maidstone, United Kingdom) by a chance encounter with professor Neville Symonds who had just been appointed as one of the founding fathers of the School of Biology at the University of Sussex (Brighton, United Kingdom). Neville fascinated Sims with his explanation of how microbes could be used to study genetics and was the stimulus for a successful application to carry out undergraduate study at Sussex (1968–1971). At Sussex, he met Ken Stacey who was later to supervise his PhD and who, along with Neville plus professors James Sang and John Maynard-Smith guided him towards his final year specializations of microbial genetics, biochemistry of proteins and nucleic acids, and genetics and development. After graduating from Sussex (1971) with a BSc in biological sciences, he followed Ken Stacey, who had become the first director of the Biological Laboratory at the University of Kent. Here graduate studies (from 1971 to 1974) were carried out under Ken's supervision, ultimately generating a thesis entitled

'*Trimethoprim resistance and the control of dihydrofolate reductase in* E. coli *K12*' for which he was awarded the PhD. This was his first contact with the folate biosynthetic pathway, which has become a major research focus of his to this day. The PhD work generated his first publication in the folate area (Breeze *et al*., 1975), reporting the mapping of the *E. coli DHFR* gene using P1-transduction. From 1974 to 1980, he worked as a post-doctoral research associate under the supervision of Don Wild at the Microbiology Unit, Biochemistry Department, Oxford University and whilst working there on ribosome assembly, first met John Hyde who was at a similar stage of his career working on chromatin structure with Ian Walker. Although the research at Oxford was not directly relevant to later malaria work, it was during this period that he first became familiar with the use of two-dimensional polyacrylamide gels for the analysis of complex protein mixtures, technology that he would later use in malaria proteomics studies.

From Oxford he moved to Manchester, as a founding member of Paul Broda's Department of Biochemistry and Applied Molecular Biology at UMIST. Here he was closely involved in cloning, and determining the sequence of, the *L. casei DHFR* gene which was part of a collaborative project with Gordon Roberts and Jim Feeney at Mill Hill that was to become one of the earliest large-scale protein-engineering studies carried out in the United Kingdom and for which an accurate gene sequence was essential to try and understand the molecular basis of Fansidar resistance. At about this time (1984), John Hyde was appointed to a lectureship in the UMIST department and, with their combined experience in molecular biology and folate metabolism, they were well placed to collaborate on the cloning of the *DHFR* gene from pyrimethamine-sensitive and -resistant parasites. In October 2004, UMIST merged with the Victoria University of Manchester to form The University of Manchester. Shortly afterwards, the new Manchester Interdisciplinary Biocentre (MIB, Manchester, United Kingdom) was opened allowing increased scope to pursue a long-standing interest in scientific instrumentation, in particular, the application of mass spectrometry to molecular biology.

Over the last 20 years, the majority of Hyde and Sims' studies have focused on folate biosynthesis in some way or another: they have cloned and reported the sequence of all but one (DHNA) of the genes originally believed to make up the parasite's endogenous biosynthesis pathway, showed that the parasite can and does utilize exogenous (host-derived) folate thus posing additional questions about why and how anti-folates are successful in the treatment of malaria, and demonstrated the existence of a previously unrecognized gene likely to be involved in pterin metabolism that may provide an

alternative route for folate biosynthesis and thus explain the apparent absence of a DHNA from the parasite.

With the completion of the *P. falciparum* genome-sequencing project (Gardner *et al*., 2002), his current focus is on using this information to carry out proteomic analysis of parasite biochemistry. To this end the first procedure for carrying out quantitative proteomics experiments with *P. falciparum* and methods to look at the extent and variation of post-translational modification across the parasite's proteome were developed by the Hyde and Sims group.

During the 1970s and early 1980s, pyrimidine biosynthesis was investigated at the Wellcome Research Laboratories (Beckenham, United Kingdom). The earliest reports concerned the pyrimidine biosynthetic pathway enzymes of *P. berghei* (Hill *et al*., 1981a,b) and identified five enzymes: carbamoyl phosphate transcarbamylase (CPTC), aspartate transcarbamylase (ATC), dihydroorotatase (DHO), orotate phosphoribosyl-transferase (OPRT) and OMPDC. A collaborative research project between Wellcome and the University of Kent (Gutteridge, United Kingdom) resulted in a seminal discovery: the pyrimidine enzyme dihydroorotate dehydrogenase (DHOD) was intimately connected to the electron transport chain through ubiquinone and, although the enzyme was similar to the iso-functional mammalian enzyme, it was predicted to be a useful chemotherapeutic target because the malarial ubiquinones were different from those of mammals (Gutteridge *et al*., 1979). Subsequently, the pyrimidine project became associated with the University of New South Wales (Sydney, NSW, Australia), when Annette Gero, a student of William (Bill) O'Sullivan, who had worked with him on a radioassay for DHODH, began to work in Gutteridge's laboratory. Years earlier, a connection between O'Sullivan and Wellcome had been developed when he worked on the effects of allopurinol on pyrimidine metabolism (Fox and O'Sullivan, 1971), developed assays for the pyrimidine enzymes OPRT and OMPC in human erythrocytes, and later worked in Beckenham (United Kingdom) with Harry Richards on the OPRT and OMPC of *P. berghei* and inhibition by 5-azauracil and 6-azauracil (O'Sullivan and Ketley, 1980).

With the availability of *in vitro* cultured *P. falciparum*, Gero *et al*. (1981) went on to characterize three of its pyrimidine enzymes: OPRT, DHODH and OMPC. On her return to Australia she assayed all six enzymes of pyrimidine biosynthesis from *in vitro*-grown *P. falciparum* through a single asexual cycle and showed activity to be highest at the late trophozoite stage when nucleic acid synthesis was occurring. In addition, when, after finding inhibition of *P. falciparum* DHODH by a range of electron transport inhibitors, she concluded that the enzymes were intimately linked to the electron transport chain (Gero *et al*., 1984). Later, Gero and O'Sullivan

demonstrated that pyrazofurin, a pyrimidine nucleoside analogue that usually inhibits uridine-5′-phosphate synthetase, inhibited both OPRT and OMPC from *P. falciparum* (Scott *et al*., 1986). Reyes *et al*. (1982) and Rathod and Reyes (1983) at the School of Medicine, University of New Mexico (Albuquerque, New Mexico), also studied these two enzymes in *P. falciparum* and found evidence of activity for uridine monophosphate-cytidine monophosphate (UMP-CMP) kinase and deoxythymidine monophosphate (dTMP) kinase. However, those kinases associated with pyrimidine salvage (i.e. uridine-cytidine kinase, deoxycytidine kinase and thymdine kinase) were not found. O'Sullivan continued to work on pyrimidine enzymes cloning carbamoyl phosphate synthase (CPS; Flores *et al*., 1994) -work that led to the development of 'ribozymes' targeting unique regions of the parasite *CPS* gene (Flores *et al*., 1997). In addition, cytidine triphosphate synthetase (CTS), cloned and expressed in *Escherichia coli*, showed the plasmodial isoenzyme to differ from those of mammals (Hendriks *et al*., 1994, 1998; Yuan *et al*., 2005). However, by the 1990s, Gero had moved away from research on pyrimidine enzymes and focused instead on purine membrane transport (see p. 156).

John Hyde (1950–) received his bachelor of science (BSc) in chemistry at Oxford University (Oxford, United Kingdom), continued as a doctoral student there working on chromatin and protein–DNA interactions, then followed those investigations at University of California, Berkeley (Berkeley, California) (1975–1977) and the University of Munich (Munich, Germany) (1977–1979) as a post-doctoral associate with fellowships from the Harkness Commonwealth Fund, the Alexander von Humboldt Stiftung and EMBO. In 1980, while visiting with a German friend, Werner Zolg, who had been at the Munich laboratory and who had joined Geoffrey Beale's group at Edinburgh University (Edinburgh, United Kingdom) a few months before, Hyde learned that John Scaife (see earlier) was initiating a collaborative project with Beale to look at *P. falciparum* using the new tools of molecular biology with the hope that antigens suitable for a vaccine could be found. (Malaria research in the Division of Biological Sciences at Edinburgh University was started by Beale whose classical studies on the genetics of *Paramecium aurelia* had come to the attention of P. C. C. Garnham, at the time professor of medical parasitology at the London School of Hygiene and Tropical Medicine (LSHTM, London, United Kingdom), who suggested he should turn his attention to a more 'important' protozoan—malaria! With funding from the MRC, work on the genetics of malaria parasites began.) Scaife was looking for a researcher who knew something about DNA, RNA and the like, and Hyde, anxious to return to the United Kingdom, gambled on a

change of research focus and joined the Scaife group as a post-doctoral associate. He remained at Edinburgh with Scaife for the next 4 years working mostly on establishing parasite gene libraries, studying messenger ribonucleic acid (mRNA) and ribosomal ribonucleic acid (rRNA), and investigating cloned genes of potentially important antigens and other proteins.

In 1984, he received an appointment to the University of Manchester Institute of Science and Technology (UMIST, Manchester, United Kingdom) where he was able to set up his own group and where he quickly forged a link with Paul Sims, already at UMIST. It was immediately obvious that Hyde's background in the molecular biology of malaria together with Sims' experience with the folate biosynthetic pathway put them in an ideal position to initiate a project to clone the *P. falciparum DHFR* gene, and to begin to try and understand the molecular basis of Fansidar resistance. That initial focus expanded to include the *DHPS* gene and eventually the other components of folate metabolism, their roles and properties. This productive collaboration has continued to the present time. After working through the ranks of lecturer, senior lecturer and reader at UMIST, Hyde was awarded a personal chair in molecular parasitology in 2000. His 1990 landmark book '*Molecular parasitology*', though now regrettably out of print and dated, was his attempt to draw together a decade of molecular biology approaches to parasitic systems and was aimed at PhD students and above. It continues to serve as a primer for those interested in molecular approaches to malaria and other parasites.

Through recombinant DNA technology and the availability of laboratory-grown *P. falciparum*, cloning and characterization of all the pyrimidine biosynthetic enzymes has been accomplished largely by Krungkrai's group (2003) at Chulalongkorn University in Bangkok (Bangkok, Thailand) and Christopherson's group (2004) at the University of New South Wales. The first six enzymes catalyzing the conversion of HCO_3^-, adenosine triphosphate (ATP), *L*-aspartate and *L*-glutamine to uridine 5′-monophosphate (UMP) viz. *P. falciparum* carbamoyl phosphate transcarbamylase (PfCPSII), *P. falciparum* aspartate transcarbamylase (PfATC), *P. falciparum* dihydroorotatase (PfDHO), *P. falciparum* dihydroorotate dehydrogenase (PfDHODH), *P. falciparum* orotate phosphribosyl transferase (PfOPRT) and *P. falciparum* OMP decarboxylase (PfOMPDC) have been cloned and sequenced (Christopherson *et al.*, 2004; Krungkrai *et al.*, 2004). In *Plasmodium* each is carried on a separate protein whereas in the human a single gene encodes CPSII-ATC-DHO and another gene encodes OPRT-OMPDC. The genes for PfCPSII and PfATC are on chromosome 13 and that for PfDHO on chromosome 14.

The fourth enzyme in the pathway, PfDHODH has two homologues. The gene for one is on chromosome 7 and codes for the mitochondrial-associated enzyme and the second homologue is on chromosome 9 and encodes the cytosolic form (Krungkrai *et al*., 2003). Recombinant PfDHODH contained flavin mononucleotide (FMN) as the prosthetic group and required both *L*-dihydroorotate and ubiquinone for maximal activity. The kinetic properties of the recombinant enzyme were similar to the native enzyme isolated from *P. falciparum* (Krungkrai, 1995) and *P. berghei* (Krungkrai *et al*., 1990, 1991); atovaquone (see p. 101) inhibited both the human and malarial enzymes, however, the IC_{50} values for the human enzyme were more than 1000-fold greater.

The structures of human and falciparum DHODH with a bound inhibitor have been studied by x-ray crystallography (Hurt *et al*., 2006) and the two enzymes have completely different binding modes for the same inhibitor (i.e. A77-1726, atovaquone and brequinar). Although early experiments using an indirect measure of inhibition of radiolabeled parasites with atovaquone suggested it was active against *de novo* pyrimidine biosynthesis (Hammond *et al*., 1985), later studies found it disrupted the electron transport chain through inhibition of the cytochrome bc_1 complex (Srivastava *et al*., 1999). Atovaquone does not inhibit PfDHODH *in vivo* and a truncated form of the enzyme expressed in *E. coli* was poorly inhibited by potent human DHODH inhibitors such as redoxal, dichloroallyl lawsone and A177-1726 analogues (Baldwin *et al*., 2002). Baldwin *et al*. (2005) screened a chemical library that contained 220,000 compounds and found the most active compound (compound 6) was a competitive inhibitor of coenzyme Q with an IC_{50} of 16 nM against PfDHODH. Compound 6 was among seven of the most potent inhibitors all of which were 2-nitro-3-methyl benzamide derivatives. Unfortunately these compounds had weak anti-malarial activity in cell-based assays, probably due to inefficient uptake.

The fifth and sixth enzymes, PfOPRT and PfOMPDC, catalyzing the conversion of orotate to OMP and OMP to UMP exist as a multi-enzyme complex of 140 kDa containing two OPRT of 33 kDa each and two OMPDC of 38 kDa each (Krungkrai *et al*., 2004, 2005). The PfOPRT and PfOMPDC genes have been cloned and expressed in *E. coli* (Krungkrai *et al*., 2005; Menz *et al*., 2002). The PfOPRT and PfOMPDC had 28% and less than 20% similarity to the human enzyme, respectively. The falciparum OMPDC was highly similar to the enzyme from *P. knowlesi*, *P. yoelii* and *P. berghei*. As with PfDHOD, PfORT had two homologues that mapped to different chromosomes (5 and 7) whereas the gene for PfOMPDC was on chromosome 10. The function of the OMPDC homologues is not known. In humans, unlike plasmodia, the genes for PfOPRT and PfOMPDC produce a bifunctional enzyme with UMP synthase activity (Krungkrai *et al*., 2003, 2004). Although pyrazofurin

(3-ribofuranyosyl-4-hydroxypyrazole-5-carboxamide) and its 5′-monophosphate metabolite inhibit the activity of PfOPRT it cannot be used as an anti-malarial because of low solubility and high toxicity.

Jerapan Krungkrai (1958–) is a contributor to an understanding of the *Plasmodium* pyrimidine pathway. He received his PhD in 1986 in the Department of Biochemistry at Mahidol University (Bangkok, Thailand), where he investigated the folate biosynthetic pathway and from 1988 to 1991 was a biomedical fellow at the Rockefeller University (New York, New York) with Anthony Cerami, studying the pyrimidine biosynthetic pathway. He was appointed to the Department of Biochemistry Faculty of Medicine, Chulalongkorn University in 1987 and rose to the rank of professor in 2001. His interest in the field of malaria biochemistry was inspired by professor Yongyuth Yuthavong and by lectures given by Irwin Sherman, who, 26 years ago, was a visiting professor in the Faculty of Science at Mahidol University. From 1980 to 1989, Krungkrai investigated enzymes in the malaria parasite: superoxide dismutase (SOD), catalase, Ca-pump, GTP cyclohydrolase, B_{12}-dependent methionine synthase (of folate and C1 transferring pathway) and from 1990 to 1999 the biochemistry of the mitochondrion and *de novo* pyrimidine biosynthesis. His more recent work (2000–) has focused on the use of recombinant DNA technology for studying the enzymes of cellular pH regulation and the pyrimidine pathway, especially carbonic anhydrase, dihydrooroatate dehydrogenase, orotate phosphoribosyl transferase and OMPDC.

Malaria parasites, unlike their hosts, synthesize folates *de novo*, using a pathway that in other organisms is mediated by five enzymes: GTP cyclohydrolase (GTPCH), dihydroneopterin aldolase (DHNA), hydroxymethyl dihydropterin pyrophosphokinase (PPPK), dihydropteroate synthase (DHPS) and dihydrofolate synthase (DHFS). The interconversion amongst the various forms involves four other enzymes: DHFR, TS, serine hydroxymethyl transferase (SHMT) and folylpolyglutamate synthase (FPGS) (reviewed in Hyde, 2005; Nzila *et al*., 2005). Several approaches have been undertaken to characterize the pathway enzymes. Initially, several enzymes were identified using classical biochemistry; this required growing large numbers of parasites *in vivo* so that there would be ample material for isolation and characterization.

Employing such methods my colleague, Edward Platzer (1972), working first in Trager's Rockefeller laboratory and later at University of California, Riverside (UCR, Riverside, California), reported that SHMT was increased in *P. lophurae*-infected red cells, however, the activities

of 10-formyl-tetrahydrofolate synthetase (FTHFS) and MTHFDH (also required for the formation of the co-factors involved in the *de novo* biosynthesis of purines in host cells) were decreased, and enzyme activity was not found in parasite extracts. The SHMT of *P. lophurae* was an isozyme, distinct from that of the host cell enzyme in molecular weight, pH optimum and thermostability. In 2000, Alfadhli and Rathod (2000) cloned, sequenced and expressed SHMT from *P. falciparum*. The gene coded for a 442 amino acid protein with 38–47% identity in amino acid sequences of SHMT from the human, rabbit and yeast. There was no obvious mitochondrial targeting sequence in the *falciparum* enzyme confirming Platzer's cytoplasmic location in lophurae SHMT (Platzer, 1977). Using homology modeling and molecular dynamics, Franca *et al*. (2005) proposed a three-dimensional model for PfSHMT that revealed differences in the binding of glycine and 5-THF from that of the human and suggested that the enzyme could be a dimer in solution.

Edward G. Platzer was born in Vancouver, Canada in 1938 and received his bachelor of science (BS) and master of science (M Sc) degrees from the University of British Columbia (Vancouver, BC Canada) in 1961 and 1964, respectively, then his PhD from the University of Massachusetts, Amherst (Boston, Mass, 1968) on vitamin requirements of the tapeworm *Hymenolepis diminuta* with Larry S. Roberts. Platzer's continuing interest in vitamin metabolism was inspired after reading '*The biochemistry of intracellular parasitism*' by James Moulder. Shortly thereafter he received notification of a National Institutes of Health (NIH, Bethesda, Maryland) research fellowship to work with William Trager at Rockefeller University on '*Folate biosynthesis in malarial parasites*'. By September 1968 he started in Trager's laboratory where there were great interactions with Philip D'Alesandro and George Jackson who were part of Trager's Parasitology Department, as well as post-doctoral fellows Dickson Despommier, Susan Langreth, Abraham Held, Curtis Patton and Earl Weidner. Initially, Platzer's studies were focused on DHFR in *P. lophurae*, the primary model system in Trager's laboratory. Unlike most previous studies on malaria biochemistry that used saponin lysis for isolation of malaria parasites they elected to use an antibody lysis technique that effectively produced malaria free of host erythrocyte contamination. Additionally, Trager had just acquired a Gilford® automatic cuvette changer, which facilitated spectrophotometric enzyme studies. The DHFR from *P. lophurae* had a specific activity higher than that of rodent and primate malarias, but the pH optima and affinity for the dihydrofolate (DHF) were similar (Platzer, 1974). The *P. lophurae* DHFR exhibited less sensitivity to pyrimethamine inhibition than the

rodent and primate malaria enzymes, which correlated nicely with the whole animal studies in which avian enzymes were less sensitive to pyrimethamine than the rodent and primate malarial enzymes. The molecular mass of *P. lophurae* DHFR was significantly lower than that determined for DHFR from rodent and primate malaria but several fold greater than that for microbes and vertebrates. It remained for later investigators to realize the significance of the high molecular mass observations. Platzer was interested in correlating folate metabolism with that of pyrimidines and purines as demonstrated by Walsh and Sherman (Platzer, 1972; Walsh and Sherman, 1968b). However, Platzer was unable to demonstrate significant activity of FTHFS or 5,10-methylene tetrahydrofolate dehydrogenase (MTHFDH) in *P. lophurae* using the spectrophotometric techniques of that era. In contrast, *P. lophurae* exhibited high specific activity of SHMT that correlated nicely with the findings of Walsh and Sherman. Borrowing the concept of the 'thymidylate synthesis cycle' from current cancer chemotherapy research he applied it to the folate metabolism situation in *P. lophurae* (Platzer, 1972). In this proposed cycle for malaria parasites, inhibition of DHFR by anti-folates (e.g. pyrimethamine) blocked the regeneration of THF resulting in the cessation of deoxythymidylate synthesis and thereby stopped DNA synthesis and reproduction. Subsequently, Platzer joined the faculty at UCR, and received an NIH grant to characterize and purify SHMT from *P. lophurae*. Fortunately, Irwin Sherman (a colleague at UCR) had an established *P. lophurae*/duck system and was amenable to sharing the facility. The purified SHMT was significantly smaller than the host enzyme (Platzer and Campuzano, 1976) and, using an Aminco® French Pressure Cell Press which facilitated the selective rupture of the cell membrane of malaria parasites prepared by antibody lysis, the SHMT was shown to be primarily cytoplasmic (Platzer, 1977). The affinity of SHMT for serine and THF was lower that that known for microbial and vertebrate enzymes. The requirement of pyridoxal phosphate (PLP) as a co-factor for SHMT spurred the investigation of the metabolism of pyridoxine by an undergraduate student (Judith Kassis, a student from University of California at Santa Barbara (California), who in 1977 received a summer National Science Foundation Undergraduate Research Participation Grant). They found that pyridoxine kinase (PK) was greatly increased in infected duckling erythrocytes and this was related to the presence of the parasite PK. *P. lophurae* PK had a higher affinity for the substrate and the specific inhibitor, deoxypyridoxine. These results suggested a potential role for anti-vitamin B_6 compounds as anti-malarials. (Kassis eventually joined the faculty at University of California, San Francisco (San Francisco, California). Previously, Trager (1977) had proposed, on the basis of his

extracellular culture system, that malaria parasites might have a biosynthetic lesion in vitamin B_6 metabolism similar to that identified for pantothenate metabolism. Clearly, this was not the case and Platzer has always wondered whether Trager was annoyed with these findings since the issue of metabolic lesions in the flow of nutrient materials from the host cell was a keystone in his research on the host–parasite interface. This was Platzer's swan song for further metabolic research in malaria since the chair of his department (nematology) indicated he would prefer to see a research program focused on nematodes. The switch was made to a nematode parasitic on mosquitoes that had relevance to malaria vector control and this has proved to be another exciting adventure in parasitology.

The presence of TS as well as DHFR (i.e. *P. berghei* (Reid and Friedkin, 1973), *P. chabaudi* (Walter and Konigk, 1971)) and *P. lophurae* led Platzer to suggest there was a thymidylate synthesis cycle: DHF- → THF- → 5,10 MTHF- → DHF-. Such a cycle has been confirmed for all *Plasmodium* spp. and conveniently accounts for the *de novo* synthesis of pyrimidines by plasmodia as well as the absence of *de novo* purine biosynthesis. Indeed, *P. berghei* was unable to reduce exogenously supplied folate to DHF suggesting that the parasites lack DHF reductase (Ferone and Hitchings, 1966). However, when *P. berghei*-infected cells are supplied with DHF they do form THF. This being so, how might one explain the beneficial effects of added folic and folinic acid (leucovorin) on *P. lophurae* growing intracellularly and extracellularly (Siddiqui and Trager, 1966), and the finding that *p*-aminobenzoic acid (pABA) reversed the inhibition of DNA synthesis by sulphalene and not pyrimethamine? A simple explanation may be that the samples of folic and folinic acid contained 4-aminobenzoyl glutamate or that the host or the red cell extract contained enzymes that were able to breakdown the added folates into pABA and then this was converted to THF (Ferone, 1977; Platzer, 1972). Alternatively, if malaria parasites truly utilize folic acid it would require folate reductase activity and, although this activity has not been reported for *P. falciparum*, it is possible, as Wang *et al*. (2004a) suggest, that DHFR-TS can convert folic acid in an enzyme- and co-factor-dependent fashion to a compound behaving as THF. In *P. falciparum* it has been shown that radiolabeled folinic acid is taken up and processed efficiently by the parasite providing a much better source of exogenous folates in culture than the usual supplementation with folic acid in RPMI 1640 (Nzila *et al*., 2005; Wang *et al*., 2004a) and also promoting more robust and reliable growth. However, how folinic acid is used so efficiently by the parasite remains to be determined.

Although it has been possible to isolate and characterize folate enzymes from several species of *Plasmodium* by standard biochemical techniques, with *P. falciparum* this is made more difficult because of the limited amounts of material obtained from *in vitro* cultures as well as the instability of the enzymes. In recent years, the easiest route has been to employ recombinant DNA technology to identify the relevant genes and to express and characterize the gene products. In addition, through metabolic labelling, genetic manipulation and inhibitor studies a more complete picture of the folate pathway has emerged (see http://sites.huji.ac.il/malaria/maps/folatebiopath.html; last accessed 16 July 2008). Starting with the cloning of the *DHFR-TS* gene in 1987 (Bzik *et al.*, 1987) all of the genes encoding the isoenzymes of the pathway have been identified in the *P. falciparum* genome save for the gene encoding DHNA (Gardner *et al.*, 2002); all have been cloned and sequenced and the protein product characterized (Brooks *et al.*, 1994; Triglia and Cowman, 1994; as reviewed in Cowman, 1998; Lee *et al.*, 2001; Uhlemann *et al.*, 2005). Lack of identification of DHNA may be due to the protein being so divergent that it is unrecognizable using present day bioinformatics tools, or that the gene is fragmented into many small exons or that it is genuinely absent. It remains to be determined whether DHNA activity is carried out by a non-orthologous protein, or a non-protein-based mechanism although recent data indicate that an unusual variant of a pterin-metabolizing enzyme can provide the 6-hydroxymethyl dihydropterin required by the next enzyme in the pathway PPPK (Hyde *et al.*, 2007). Of some interest is that the PPPK of *P. falciparum* includes two long insertions not found in bacteria or other non-apicomplexan eukaryotes. PPPK catalyzes the dephosphorylation of 2-amino-4-hydroxy-6-hydroxymethyldihydropterin, and the resulting compound, under the action of DHPS, is condensed with pABA to generate dihydropteroate (DHP). Hydroxymethyl dihydropterin pyrophosphokinase (PPPK) and dihydropteroate synthase (DHPS) occur as a bifunctional enzyme in *Plasmodium* spp., other protozoa and plants. The next enzyme in the pathway, DHFS, catalyzes the conversion of DHP to DHF by addition of a single *L*-glutamate moiety. The gene encoding DHFS has been characterized and is expressed as a bifunctional enzyme that also has FPGS activity, which adds further glutamate residues (Lee *et al.*, 2001; Salcedo *et al.*, 2001).

Activity for the first enzyme in the folate pathway, GTP cyclohydrolase (GTPC), catalyzing the conversion of GTP to dihydroneopterin triphosphate (DHNP) has been measured in several malaria parasites (Krungkrai *et al.*, 1985) and the gene has been characterized in *P. falciparum* (Lee *et al.*, 2001). Consistent with its role in folate biosynthesis, GTPC activity peaks during the early trophozoite stage (Nirmalan *et al.*, 2002). Of some interest is the finding of amplification that could contribute to an increase in flux through the pathway and may facilitate parasite resistance to anti-folate

drugs (Kidgell and Winzeler, 2006). The essential role of GTPC, however, remains uncertain since there are no specific inhibitors and attempts to knockout the gene in *P. falciparum* have not been reported.

As noted above, in malaria parasites the synthesis of thymidylate (thymidine-5′-phosphate, TMP) from UMP requires the enzyme TS because plasmodia (as has been demonstrated in *P. lophurae*, *P. falciparum* and *P. berghei*) lack the enzyme thymidine kinase and they cannot salvage pre-formed thymidine from the host. (It is of interest to note that before it was recognized that malaria parasites lacked thymidine kinase investigators were perplexed as to why they were unable to label the parasite nucleus with tritiated thymidine, as had been the standard practice with other eukaryotes!) Although thymidine cannot be salvaged, recent studies have shown that UMP (which can be produced *de novo*) may be formed from uracil *via* salvage by the action of UPRT or by the sequential action of uridine phosphorylase (UP) and uridine kinase. The PfUP homologue on chromosome 5 has been cloned and sequenced and shows similarity to the bacterial enzyme but only 37% similarity to the human enzyme (Krungkrai *et al.*, 2003). The TS of malaria parasites differs from that of other organisms (Hall *et al.*, 1991) where it is a distinct dimeric enzyme with a native relative molecular mass (M_r) of about 75 kDa. However, in plasmodia it exists as a bifunctional protein in combination with DHFR with dimeric falciparum DHFR-TS, located on chromosome 4, having an M_r of about 144 kDa; the individual domains are DHFR around 27 kDa, TS around 34 kDa and a junction region at around 11 kDa (Bzik *et al.*, 1987; Snewin *et al.*, 1989).

Incubation of *P. falciparum* with radiolabeled 5-fluoroorotate resulted in a significant incorporation into parasite RNA. Both 5-fluororotate and 5-fluoro-2′-deoxyuridylate (FdUMP) inhibited the TS of *P. falciparum* with a K_i of 2 nM (Hekmat-Nejad and Rathod, 1996) and the former compound at 70 nM was effective in reducing parasite proliferation by 50% *in vitro*. TS has been suggested to be the target of 5-fluoroorotate since it inhibits TS activity, whereas DHFR activity is unaffected (Rathod *et al.*, 1992). Seymour *et al.* (1994) counter the proposal that TS is the primary target and suggest that accumulation of 5-fluor-uridine triphosphate (FUTP) may be responsible for blocking pyrimidine biosynthesis and cytotoxicity could result either from misincorporation of FUTP into RNA or of dFUTP into DNA or from inhibition of TS by FdUMP. Seymour *et al.* suggest a direct proof could be obtained by measuring cellular levels of FdUMP, deoxythymidylate triphosphate (dTTP) and related metabolites. Notwithstanding this controversy, 5-fluoroorotate would not be a suitable anti-malarial since *in vivo* it would be necessary to achieve a serum concentration of 1–10 μM and at such concentrations it is toxic to mammalian cells (Jiang *et al.*, 2000).

Pradipsinh K. Rathod (1956–) was born in Dar es Salaam, Tanzania and received his PhD in biochemistry with Jack Fellman at the Oregon Health & Science University (Portland, Oregon) (1981). He did post-doctoral research at the University of New Mexico (Albuquerque, New Mexico) (1981–1982), and at Brandeis University (Waltham, Massachusetts) (1982–1985). Pradip then joined the Department of Biology at The Catholic University of America (Washington, DC) (1986–2000), was a visiting professor of biochemistry at Stanford University (Palo Alto, California) (1995–1996), and currently is professor of chemistry at the University of Washington (Seattle, Washington) (2001–).

Given his upbringing in Africa, and interests in bioorganic chemistry, Pradip made an early and strong commitment to study drug development for treating malaria. In 1980, after reading a review article on malaria biochemistry by Irwin Sherman, Pradip went to the laboratory of Phil Reyes at the University of New Mexico School of Medicine, where he began to survey the enzymes of purine and pyrimidine metabolism using *in vitro* grown *P. falciparum*. (The Trager-Jensen culture system had come to New Mexico due to the presence of Karl Rieckmann, who was then head of United States Agency for International Development (USAID) Vaccine Program.) In the Reyes laboratory, Pradip also examined the effects of about 50 inhibitors of purine and pyrimidine metabolism on the proliferation of malaria parasites. The potency and selectivity of compounds like 5-fluoroorotate served as a basis for his future research. However, the poor cellular activity of most potent inhibitors of pyrimidine metabolism, an essential pathway in malaria parasites, raised questions about the dominant paradigms for knowledge-based drug discovery. For his second post-doctoral position, Pradip went to Robert H. Abeles at Brandeis University to strengthen his knowledge of bioorganic chemistry and to study the mechanism of β-fluoroasparagine toxicity against leukemia cells. Contact with Abeles, Jencks and their students reinforced some fundamental values about the importance of technical and intellectual risk-taking in science, and the long, painful paths to indisputable truths.

At the Catholic University, Pradip and his students (Ashok Khatri and Zaida Gomez) demonstrated that 5-fluoroorotate was a potent and selective anti-malarial. Toxicity at very high doses could be avoided by supplementing the pro-drug with nucleosides that only the host could use. Later, together with graduate students (Nilima Pai Leffers, Mohammed Hekmat Nejad and Kai Zhang), the target of 5-fluoroorotate was determined to be DHFR-TS and the exquisite vulnerability of malaria parasites to 5-fluoroorotate, and to anti-folates,

was discovered to result in part from the very low levels of the target in parasites and an unusual form of gene regulation that prevented the parasite from overproducing the target. Rathod's laboratory has shown that this privileged target can be attacked successfully in many different ways, including folate-based TS inhibitors. Graduate students Safwat Gassis and Becky Lee helped demonstrate that highly drug-resistant parasites from Southeast Asia could acquire resistance to new pyrimidine biosynthesis inhibitors at 1,000 times higher frequency than parasites from Africa and South America. This was one of the earliest hints in microbiology that pathogens of the same species could show large variations in mutability to gain selective advantages.

At the turn of the century, the landscape for malaria research and 'big biology' changed considerably. Inspired by a sabbatical at Stanford University (Palo Alto, California), Pradip and his students, (John White, Karthik Ganessan and Lei Jiang) moved to the University of Washington. Together with Joseph DeRisi (University of California, San Francisco, (San Francisco, California) the team helped develop a rapidly disseminated spotted microarray technology and with Mike Ferdig (University of Notre Dame, (Notre Dame, Indiana), quantitative trait loci (QTL) approaches are used in combination with DNA microarrays to describe networks that govern gene regulation in malaria parasites. The Rathod laboratory is developing a general, genome-wide approach to produce malaria proteins in functional form using the technical expertise of Devaraja G. Mudeppa, a post-doctoral student with excellent training in cell-free protein production. Pradip's engineering students, Thurston Herricks and Meher Antia, are introducing microfluidic technologies to study malaria pathogenesis, with the long-term goal of treating severe disease, as opposed to parasite proliferation. With Meg Phillips (University of Texas Southwestern School of Medicine, (Dallas, Texas) a 200,000 molecule screen has been set up and a post-doctoral student Ramesh Gujjar is developing novel inhibitors directed at *Plasmodium* DHODH.

The bifunctional DHFR-TS complex has been purified and the gene (on chromosome 4 in *P. falciparum*) has been cloned and expressed in *E. coli* (Hall *et al.*, 1991). This expression system provided small amounts of enzyme presumably either because of the high A + T content of the plasmodial gene or toxicity of the product. To express the gene more efficiently, synthetic oligonucleotides that changed the gene's codon usage have been used to increase the yield 10-fold, with the product constituting approximately 30% of the total bacterial protein. Cell-free protein synthesis of DHFR-TS from *P. falciparum* has recently been

reported (Mudeppa *et al*., 2007) and this may prove to be of great use for studies of potential anti-malarials.

The DHFR-TS from *P. vivax* has been cloned and sequenced (de Pecoulas *et al*., 1998). It codes for a protein with 623 amino acids, 286 representing the TS domain, 237 the DHFR domain and a 100 amino acid linker. Little similarity to other *Plasmodium* spp. was found in the DHFR and linker domains, however, the TS domain was conserved. *P. vivax* has a highly biased GC codon usage. Reflecting this, the TS and junctional domains are both GC rich (40% and 58%, respectively) as are other vivax genes. Within the DHFR domain, which is also rich in GC (50.8%), there are short repetitive arrays that are absent from the *P. falciparum* gene. In pyrimethamine-resistant *P. vivax* the DHFR has two residue changes: Ser → Arg (58) and Ser → Asn (117), corresponding to the substitutions Cys → Arg (59) and Ser → Asn (108) in *P. falciparum*.

George Hitchings (1908–1998) received the Nobel Prize in 1988 'for ... discoveries of important principles for drug treatment.' The discoveries were built upon the work of those who preceded him ... and they were inspired by basic questions concerning the biochemistry of cellular reproduction. Indeed, the biochemistry of folates and pyrimidine biosynthesis is a continuation of Paul Ehrlich's studies on the selective staining of dyes and anti-microbial activity. In addition, as with Ehrlich's discovery of 'magic bullets', the development of sulphonamides as anti-parasitic agents begins with the synthesis of a group of azo dyes containing a sulphonamide group that included the red-coloured prontosil (see Hitchings, 1978). The dyes were of interest to I. G. Farben (Bayer) for their ability to contribute to fastness for acid wool dyes not for their anti-microbial activity! Indeed, it was fortuitous that in 1933–1935 Gerhard Domagk, in trying to understand the lack of correlation between *in vitro* and *in vivo* antibacterial tests, resorted to *in vivo* testing of prontosil; this led to his discovery that it protected mice against a streptococcal infection, but was without effect on bacteria *in vitro*. Dyes similar to prontosil had been synthesized almost a decade earlier (1919) by Heidelberger and Jacobs but they were discarded after they were tested *in vitro* and showed poor anti-microbial activity. Later, it would be shown that in animals the prontosil had been converted to the active anti-microbial, *p*-aminobenzenesulphonamide (= sulphanilamide) and subsequently it was found that sulphonamides on their own were as effective as the parent dyestuff in protecting mice infected with streptococci.

Almost at the same time that sulphanilamide was identified as the active principle of prontosil, Coggeshall (1938a, 1940) found it to be an effective anti-malarial in monkeys infected with *P. knowlesi*. Shortly thereafter sulphadiazine and diamino-diphenyl sulphone derivative (Promin) were also found to be effective against *P. vivax* and, in 1941, Maier and Riley showed that the activity of sulphanilamide against *P. gallinaceum* could be reversed by pABA. Shortly thereafter it was shown that sulphadiazine was active

against *P. falciparum* and *P. vivax* in humans (Coggeshall *et al.*, 1941). In addition, the Harvard group (Ball *et al.*, 1945) showed that not only could pABA reverse the action of sulphadiazine (Ball, 1945), but it was a requirement for the *in vitro* growth of *P. knowlesi* (Ball *et al.*, 1945; Ferone, 1977). Later studies demonstrated that depletion of host pABA (milk diet) inhibited plasmodial infections in mice and monkeys and supplementation of the diet with pABA resulted in a resurgence of the infection (Hawking, 1954; Kicska *et al.*, 2003; Maegraith *et al.*, 1952). Thus, although plasmodia can synthesize pABA *de novo* (Hyde, 2005; McConkey *et al.*, 1994) *via* the shikimate pathway utilizing phosphoenolpyruvate **(**PEP) (from glycolysis) and erythrose-4-phosphate (from the pentose phosphate pathway, PPP) and the genes for the enzymes of the pathway are present in the genomes of *P. falciparum* and *P. yoelii*, the parasites appear to be unable to synthesize sufficient quantities to survive *in vivo* (Hyde, 2005; Nzila, 2006).

Yongyuth Yuthavong (1944–) was awarded a Thai Government scholarship to study in the United Kingdom, where he obtained a BSc in chemistry with first-class honours from the University of London, and a D.Phil. from Oxford University (1969), based on research on enzyme mechanisms using chemical kinetics. Returning to Thailand, he joined the faculty of Mahidol University, and faced with the country's problems on malaria, particularly the ever-increasing resistance to antimalarials (especially Fansidar) began to concentrate his research on malaria biochemistry and chemotherapy with emphasis on antifolates, a topic he continued to pursue at the National Center for Genetic Engineering and Biotechnology (Pathumthani, Thailand). He became the first director of the National Science and Technology Development Agency (NSTDA; Pathumthani, Thailand) in 1992 and Minister of Science and Technology of Thailand in 2006. He was given the 'Outstanding Scientist of Thailand' award in 1984 and the Nikkei Asia Award for Science, Technology and Innovation in 2004, for 'resolving the three-dimensional structure of a particular protein the parasite produces, thus helping efficient development of a new generation of drugs able to overcome resistant strains of malaria.'

Hitchings' interest in nucleic acids stemmed from his work at Harvard with Cyrus J. Fiske who had discovered ATP in muscle. The development of micro-analytical methods for the purine bases to follow the metabolism of ATP was the theme of Hitchings' doctoral thesis. After completion of his PhD in 1933, during the depths of the depression, he experienced a nine-year period of impermanence both financial and intellectual with short appointments at Harvard's C. P. Huntington Laboratories in cancer

research, the Harvard School of Public Health (Boston, Massachusetts) in nutrition research and at Western Reserve University in electrolyte research. In 1942, after joining Burroughs Wellcome Company (a subsidiary of the British company founded by Sir Henry Wellcome) as the sole member of the Biochemistry Department, which was housed in a converted rubber factory in Tuckahoe, NY, he began to explore synthetic analogues of pyrimidine and purine bases as inhibitors of nucleic acid biosynthesis. He theorized (based in part on the anti-metabolite principle expressed by Woods, 1940 and Fildes, 1940) that it should be possible to alter the way cells grow and develop by substituting slightly different compounds for those occurring naturally. 'The reasons for choosing pyrimidine derivatives as a basis for molecular modification turned out, however, to be largely fallacious. Resemblance was sought with earlier anti-malarial compounds ... based on the rather far-fetched analogy of sulphonamides as derivatives of aniline, with a side chain performing the same function as the basic side chain of older anti-malarials. Pyrimidines were looked upon favorably because they showed the characteristic resonance of quinacrine ... and because there was a chance that they could interfere with ... pyrimidine metabolism' (Rollo, 1975).

Hitchings, working in concert with Elvira Falco and Barbara Roth, used a folate-dependent strain of *Lactobacillus casei*, and found several inhibitory analogues. The most active ones were 2,4-diaminopyrimidines. This, as well as other work with *Streptococcus faecalis*, which showed that folinic acid was about 500 times more potent in reversing the inhibition than was folic acid, suggested that the pyrimidines were interfering somewhere in the biosynthesis of folinic acid from folic acid. At the Wellcome Laboratories in the United Kingdom, Len Goodwin (who had joined the company in 1939) had set up a screen using *P. gallinaceum* in chicks to test promising compounds to replace quinine. Of the 300 substituted 2,4-diaminopyrimidines sent by Hitchings, Roth and Falco the most active ones were in the series substituted with a phenyl in the 5 position and the highest activity was found with 2,4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine (later named pyrimethamine). A single large dose of pyrimethamine in humans produced peak serum concentrations in the first 2h and declined thereafter. The anti-folic acid activity of the serum was confirmed using *L. casei*, and subsequently it was found that the duration of anti-malarial activity follows the anti-folic acid activity of the blood. In the years since these fundamental discoveries were made, the genes encoding all the folate pathway enzymes have been cloned and characterized in the laboratories of David Bzik, Tom Wellems, Alan Cowman, John Hyde, Paul Sims and Yongyuth Yuthavong. Elucidation of the gene structure has provided the impetus for developing new therapeutics (see Nzila, 2006), as well as a better understanding of the genetic basis for anti-folate drug resistance.

David Bzik (1955–) began his scientific career as an undergraduate at Lehigh University (Bethlehem, Pennsylvania) studying bacteriophage and for his graduate work studied membrane fusion in herpes simplex virus in the laboratory of Stanley Person at the Pennsylvania State University (University Park, Pennsylvania) from which he received his PhD in 1983. He was an EMBO fellow at the Medical Research Council (MRC) Virology Unit in Glasgow, Scotland, United Kingdom, studying immediate early gene activation induced by herpes simplex virus, VP16, when it became clear to him that although herpes simplex viruses were fascinating, and contained only a few tens of genes, the field already had several hundred investigators, and this prompted a decision to change fields. Encouraged by his wife, Barbara A. Fox, he began to think about the global problem of malaria. In the spring of 1985 during a visit to his former mentor at Pennsylvania State University, he attended an invited lecture given by David Kemp on malaria and its (repetitive) antigens. After a chat with Kemp, he was 'hooked' on malaria. In early December of 1985, Bzik began his first investigations on malaria as a research associate in the laboratory of Joseph Inselburg at Dartmouth College (Hanover, New Hampshire). At the time, Inselburg had two research associates, one who later went onto medical school, was trying to clone DHFR-TS using immunological methods, and the other, Toshihiro Horii, who after fundamental work on recA protein of *E. coli*, was working on cloning malaria genes based on monoclonal antibodies that blocked parasite growth. Bzik's role was to collaborate with Horii and move this project forwards faster. Since the research associate working on DHFR-TS was leaving shortly, Bzik pleaded with Inselburg for 1 month of time to try to clone malaria DHFR-TS. (The sequence of DHFR-TS had just been published from another protozoan parasite.) In early 1986, Dartmouth had received its first DNA synthesizer machine, and with the help of Horii and Fox, a single degenerate DNA primer based on a very crude alignment of bacterial and protozoan TS was designed by Bzik. In early December, the same replica filter of lambda phage DNA libraries that had been used to clone the blood-stage malaria vaccine candidate gene *SERA* was screened. One week before Christmas 1986, the films from screening about 100,000 individual clones showed a handful of very bright, and a few brighter spots, and these hopeful 'putatives' were isolated and grown. Individuals derived from these clones were sequenced manually using home-made reagents. On development, the films were too faint to see anything so they were left to over-develop in the freezer for 1 week. Returning on Christmas morning, a single film was developed and it revealed faint but clear

spots. With the publication of the other protozoan DHFR-TS amino acid sequence in-hand, and the genetic code well imprinted in his brain, the short DNA sequences from the putative clones were manually read and two clones revealed amazing and contiguous amino acid identity to other TS genes. In less than 1 month of effort, the elusive *DHFR-TS* gene had been cloned. This finding led to more than 21 years of research that has exposed a now virtually complete understanding of pyrimethamine resistance at the molecular level and has also led to the development of vastly improved inhibitors of the key DHFR-TS enzyme functions. The cloning of malaria DHFR-TS also launched the first viable genetic selections in malaria based on pyrimethamine resistance, and this new knowledge of pyrimethamine resistance was also adapted and used to establish the first robust genetic selections in the related parasite *Toxoplasma gondii*. In the mid-1990s, with a reduction in NIH funding for research in tropical medicine and malaria, Bzik's career took a turn: he began research using *T. gondii* as a genetic model to discover biochemical pathways relevant to malaria more rapidly. He remains confident that research on this amenable model and sister parasite will intimately mesh with key research on *Plasmodium* and will contribute significantly to the eventual control of malaria.

The potency of different DHFR inhibitors against *P. falciparum* parasites varies widely. WR99210 (4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(2,4,5-trichlorophenoxy) propyloxy)-1,3,5-triazine hydrobromide) is the most potent, whereas cycloguanil (a metabolite of proguanil) and chlorcycloguanil (the active metabolite of chlorproguanil) are more potent than pyrimethamine (Gregson and Plowe, 2005). Because parasite resistance to anti-folates was found shortly after their introduction, attempts were made to increase their schizonticidal activity and to reduce the emergence of resistance. A 1959 study found that sulphadoxine potentiated pyrimethamine in human falciparum infections. Presently, attempts to delay resistance involve the use of synergistic combinations such as: chlorproguanil-dapsone (Lap-Dap), sulphadoxine-pyrimethamine (Fansidar) and proguanil-atovaquone (Malarone). Most likely this synergism is due to the fact that these drugs act on different enzymes in a common biosynthetic pathway (Cowman, 1998). To further counteract the development of resistance a triple combination (chlorproguanil-dapsone-artesunate) is being developed. However, a major disadvantage of these combinations is their high cost.

Sulphadoxine, the most commonly used sulpha drug is a structural analogue of pABA and acts as a competitive inhibitor of plasmodial DHPS. Resistance to sulphadoxine in *P. falciparum* involves amino acid substitutions in DHPS that alter enzyme function. Resistance to

sulphamethaxozole and sulphathiazole, as well as sulphones, also involve amino acid substitutions in DHPS—especially in residues Ser→Ala 436, Ala→Gly 437, Lys→Glu-540, Ala→Gly581 and Ala→Ser 613—and as such there is cross resistance to these drugs. The Ala→Gly 437 and Lys→Glu 540 mutations appear to be the initial and most important for resistance and higher levels require multiple mutations in addition to that at codon 437 (Triglia *et al*., 1998; Wang *et al*., 1997).

In *P. falciparum*, pyrimethamine resistance is due to decreased affinity in the binding of the drug to DHFR owing to mutations in the codons specifying amino acid residues: Ser→Asn 108, Asn→Ile 51, and Cys→Arg 59, Ile→Leu 164; and resistance to cycloguanil was found to be linked to a pair of mutations in DHFR: Ala→Val 16 and Ser→Thr 108. Evidence that DHFR mutations gave rise to resistance was strengthened by the fact that recombinant mutant enzyme showed decreased drug binding and transfection of wild-type parasites with constructs bearing mutant forms gave rise to resistant phenotypes. Interestingly the mutations appear to have arisen in Asia and then spread to Africa (Roper *et al*., 2004). Although the mechanism for resistance usually involves mutations that alter drug binding in some strains, increased expression of DHFR, as well as the ability to salvage folates (i.e. Dd2) bypassing the *de novo* pathway, can also contribute to the level of drug resistance. In gene knockout experiments designed to evaluate the relative importance of *de novo* versus salvage of folates, it was found that complete disablement of the *DHPS* gene product could not be tolerated and DHPS did not play a major role in salvage. Thus, it appears that biosynthetic capability is essential in spite of the parasite's ability to salvage folate (Wang *et al*., 2004a).

The crystallization of DHFR-TS and DHPS of *P. falciparum* has allowed for a better appreciation of the action of folate inhibitors. With DHFR-TS, most inhibitors fit between residues 108 and 54 within the active site (Yuvaniyama *et al*., 2003). WR99210 most closely resembles the flexibility seen in the natural substrate, DHF, and perhaps this is the reason for its greater potency and reduced susceptibility to point mutations. Neither *P. falciparum* hydroxymethyl dihydropterin pyrophosphokinase (PfPPPK)-DHPS nor the DHPS domain itself have yet been crystallized, however, inferences about their structure, made from homology modeling of the falciparum sequences to bacterial x-ray structures, show that codons 436, 437 and 540 all line the channel of the active site where both substrate and inhibitor binding occurs; codons 581 and 613 are only one to three positions away from the channel suggesting these two mutations may be compensatory in function, and explaining why the two residues are not seen in isolation but only in association with mutations at 436, 437 or 540 (Gregson and Plowe, 2005). Differences in cross-reaction patterns and point mutations have raised the hope that new anti-folates can be developed against resistant strains providing they are able to retain

binding affinity to the mutant forms (Yuthavong, 2002; Yuthavong *et al*., 2005). In addition, there may be another possibility for retarding the emergence of resistance. It has been found that point mutations in the *DHFR* gene also result in resistance to pyrimethamine in *P. vivax*, however, the mutations that confer resistance to pyrimethamine also render the DHFR of *P. vivax* exquisitely sensitive to WR99210. Hastings and Sibley (2002) suggest that pyrimethamine and WR99210 could 'exert opposing selective forces on the *P. vivax* population, and if used in combination ... could force the selection by some mechanism other than simple point mutations and greatly slow the selection of parasites resistant to both drugs.' Indeed, it is possible that by combining them with other drugs having an entirely different mechanism of action the selection of resistance could be greatly retarded (Hastings and Sibley, 2002; Hawkins *et al*., 2007).

'The history of malaria control has shown that ... chemotherapy strategies need to be evidence based and tailored to the epidemiological, as well as economic circumstances of a given setting. Anti-folate drugs have had a mixed record as prophylactic anti-malarials and since the 1960s no new anti-malarial anti-folate drug has entered into clinical trials. Yet they can still hold considerable promise if deployed thoughtfully in combination with each other and with other suitable pharmacokinetic and pharmacodynamic considerations' (Gregson and Plowe, 2005).

CHAPTER 16

Salvage of Purines

The earliest (and perhaps most unappreciated) clue to a purine salvage pathway in malaria parasites was the observation by Anfinsen *et al*. (1946) that a mixture of 'purines and pyrimidines' in the Harvard medium favored *in vitro* growth of *Plasmodium knowlesi*. Whether both purines and pyrimidines were required was not determined at that time, however, 25 years later Trigg and Gutteridge (1971) reported that addition of pyrimidines was unnecessary! In 1968, Büngener and Nielsen, working with the rodent malarias *P. berghei* and *P. vinckei*, found that infected red cells incorporated exogenously supplied purines (hypoxanthine and adenosine) but not pyrimidines (Büngener and Nielsen, 1968). That same year we hypothesized, '*Plasmodium* (*lophurae*) synthesizes purines *de novo* to a limited extent and therefore must rely on exogenous sources . . . the most obvious being the host erythrocyte' (Walsh and Sherman, 1968b). Substantiating this hypothesis was an additional finding of Büngener and Nielsen (1968): when red cells pre-labeled with radioactive adenosine were transfused into mice infected with *P. vinckei*, radioactivity was incorporated into the parasite's nucleic acids. Taken together, such observations (i.e. that malarial parasites utilize exogenously supplied purines but not pyrimidines) led us to study the character of the purines, the manner by which they are obtained, as well as the identification, purification and characterization of the salvage pathway enzymes of *Plasmodium*.

Approximately 80% of red blood cell purines are in the form of adenosine triphosphate (ATP) with an intracellular concentration estimated to be 2–3 mM. In glucose-deprived or aged red cells there is a progressive decline in the ATP content of the erythrocyte leading to the formation of adenosine diphosphate (ADP) and adenosine monophosphate (AMP); AMP is then dephosphorylated (*via* 5′-nucleotidase) to

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adenosine, which forms inosine *via* adenosine deaminase (ADA), and then to hypoxanthine. In the presence of glucose the pathway is ATP → ADP → AMP → inosine 5′ monophosphate (IMP) (*via* AMP deaminase) followed by dephosphorylation of IMP to inosine (*via* 5′-nucleotidase) and then to hypoxanthine (Roth *et al*., 1989). Although the mature red blood cell has a requirement for ATP this cannot be derived from IMP since erythrocytes lack the enzymes adenylosuccinate synthase and adenylosuccinate lysase, and the end product of both pathways, hypoxanthine, which cannot be utilized for the formation of ATP, plays no role in red blood cell metabolism; it diffuses out of the cell to be metabolized further in the gastrointestinal tract or liver by xanthine oxidase (absent in red cells) to uric acid.

Using the highly synchronously developing avian malaria *P. lophurae*, we found a dramatic decline in the level of ATP in parasitized erythrocytes (Yamada and Sherman, 1981a). In spite of the observed decline in the level of ATP, the adenylate charge ratio (calculated: ATP + 1/2ADP/ATP + ADP + AMP) was maintained at 0.93 during most of the schizogonic cycle. However, just prior to merozoite release the energy charge ratio fell to about 0.6. We speculated that the relative nucleotide levels were maintained by adenylate deaminase converting AMP to IMP, and by adenylate kinase elevating the ATP:ADP ratio, and as a consequence the parasitized cell would be buffered against a drop in energy charge during early development of the parasite and the host cell's metabolic integrity would provide for its survival and the parasite contained therein. Such a stabilizing process would also contribute to an enhanced production of hypoxanthine. *In vitro* studies with a variety of *Plasmodium* spp. have shown that the parasites are unable to directly utilize nucleotides (ATP, ADP, AMP, IMP) but are able to incorporate nucleosides (Booden and Hull, 1973; Hansen *et al*., 1980; Tracy and Sherman, 1972). With *P. lophurae*, we estimated that about 25% of its purine requirement could be satisfied by red cell ATP, and although Berman *et al*. (1991) calculated that in *P. falciparum* the ATP of the red cell could theoretically provide only 2% of the parasite-required purine, the validity of this calculation may be questionable.

Intraerythrocytically, adenosine, inosine and hypoxanthine are the purines available for salvage by the *Plasmodium*, and these may be supplemented by that present in plasma. However, because the plasma levels of adenosine (as well as inosine and adenine) are quite low (at around 0.1 μM) whereas hypoxanthine levels are much higher (1–10 μM) it appears that *in vivo* the plasma-supplied nucleoside for the parasites is hypoxanthine. Support for the critical role of hypoxanthine in purine salvage can be found in the reports of increased levels (0.3 to about 4 μM) in the plasma of ducklings infected with *P. lophurae*, the increased severity of *P. chabaudi*, *P. berghei* and *P. vinckei* infections by adding allopurinol

(an inhibitor of xanthine oxidase) to the drinking water of infected rats and mice, the inhibition of *P. falciparum* growth when xanthine oxidase was added to *in vitro* cultures (Berman *et al*., 1991) and its favoring effect when present in the culture media for *P. lophurae* (Walsh and Sherman, 1968a), *P. knowlesi* (Trigg and Gutteridge, 1971) and *P. falciparum* (Asahi and Kanazawa, 1994; Ifediba and Vanderberg, 1981; Zolg *et al*., 1982). The exceptional finding by Siddiqui *et al*. (1969) that exogenous purines were not required for *in vitro* growth of *P. knowlesi* may be explained by the fact that aged uninfected red cells in the culture provided supplemental hypoxanthine.

Red blood cells take up adenine from the plasma and are able to synthesize AMP *via* adenine phosphoribosyl transferase (APRT); however, activity for this enzyme in extracts of *P. falciparum* (Queen *et al*., 1989) and *P. chabaudi* (Walter *et al*., 1970) was quite low suggesting that in malaria parasites synthesis of AMP *via* adenine is a minor pathway. More likely, the pathway for AMP synthesis by *Plasmodium* involves adenosine (see http://sites.huji.ac.il/malaria; last accessed 16 July 2008). Adenosine can be metabolized *via* adenosine kinase (AK) or ADA to yield AMP or inosine, respectively. Although low activity for AK was found in extracts of *P. lophurae* (Yamada and Sherman, 1981b) and *P. falciparum* (Reyes *et al*., 1982), the gene for this enzyme was not found in the *P. falciparum* genome (Gardner *et al*., 2002). This suggests that the observed activity was an artefact or due to red cell contamination. Indeed, the genome of *P. falciparum* lacks APRT, AK, methylthioadenosine nucleoside (MTAN) and methylthioadenosine phosphorylase (MTAP), as well as methylthioribose kinase (MTRK) (Ting *et al*., 2005). In contrast, high activity for ADA has been demonstrated in extracts from several malaria species and, using conventional biochemical methods, the enzyme has been purified and characterized in *P. lophurae* (Schimandle and Sherman, 1983) and *P.falciparum* (Daddona *et al*., 1984). In these studies, although striking differences were not found in molecular weight (34 kDa) of host and parasite enzyme, they were unique in their binding properties to an adenosine affinity column, sensitivity to deoxycoformycin and insensitivity to eythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride (EHNA). *P. falciparum* adenosine deaminase (PfADA), expressed in *Escherichia coli*, had less than 13% amino acid homology with human and *E. coli* ADA. The PfADA was inhibited by deoxycoformycin and coformycin, however, EHNA was a poor inhibitor. In this regard, PfADA is similar to the ADA activity obtained in lysates of *P. falciparum*-infected ADA-deficient cells (Daddona *et al*., 1984). A single dose of deoxycoformycin reduced the parasitemia in *P. knowlesi*-infected monkeys (Webster *et al*., 1984b), however, the compound has not been used clinically because of its high toxicity and its lack of specificity for

PfADA. 'Nonetheless ... inhibition profiles of PfADA and mammalian ADA suggest that there are sufficient differences in catalytic function that can be exploited for the synthesis of malaria-specific ADA inhibitors' (Ting *et al*., 2005).

The next enzyme in the pathway, purine nucleoside phosphorylase (PNP), catalyses the phosphorylysis of inosine (formed by deamination of adenosine) into ribose phosphate and hypoxanthine. Although PNP was isolated and reasonably well-characterized from *P. lophurae* (Schimandle *et al*., 1985) and *P. falciparum* (Daddona *et al*., 1986) more than 20 years ago, and some would have expected little else could be learned from further investigation, this has turned out not to be the case. The *P. falciparum* purine nucleoside phosphorylase (PfPNP) cloned, expressed and crystallized (Schnick *et al*., 2005) has been shown to be a hexamer (relative molecular mass (M_r) 260 kDa) with inosine and guanosine, but not adenosine, as its substrates. Although active with the pyrimidine, uridine purines are the preferred substrates. As shown earlier (Schimandle *et al*., 1985), the *P. lophurae* enzyme (with a subunit M_r of around 24 kDa and an apparent M_r of 125 kDa) is similar to the PNP of *Salmonella typhimurium*, and was inhibited by formycin B but not by formycin A, azaguanine or 8-aminoguanosine, whereas PfPNP was inhibited by 8-amino-5'-deoxy-5'-chloroguanosine and 8-amino-9-benzylguanine (Daddona *et al*., 1986).

For more than a decade little was done with PfPNP inhibitors until it was found by Vern Schram's laboratory that exposure to Immucillin H, a C-nucleosidic iminoribitol transition state analogue, inhibited the enzyme from *P. falciparum* and, when added to cultures, parasites were killed through purine starvation when both the host and parasite PNPs were inhibited (Kicska *et al*., 2002). (The strong inhibition of PNP activity is due to the parasites lacking AK.) In addition, when it was discovered that PfPNP was also active with methylthioinosine (MTI) it was hypothesized that a 5'-methylthioino-substituted Immucillin would bind preferentially to it. Indeed, MT-Immucillin H killed parasites in culture (Kicska *et al*., 2002; Ting *et al*., 2005). Further, finding that 5'-methylthioadenosine (MTA) was formed from MTI by the action of PfADA led to the suspicion that a link might exist between purine salvage and polyamine metabolism (Shi *et al*., 2004). This was confirmed when PfADA was overexpressed in *E. coli* and both adenosine and MTA were found to be equivalent substrates. (MTI is formed from MTA because *falciparum* lacks MTA phosphorylase.) Thus, PfPNP functions at the intersection of polyamine metabolism and purine salvage, and it has been suggested that its activity is crucial for the *in vitro* growth of *P. falciparum* without supplemental hypoxanthine. (An interesting and valuable biochemical comparison of the PNP of *Toxoplasma gondii* with *P. falciparum* as well as purine salvage pathways in *T. gondii* and other apicomplexans is found in Chaudhary *et al*., 2004, 2006.)

Vern Schram (1941–) was an undergraduate in chemistry and bacteriology at South Dakota State College (Brookings, South Dakota) when he was awarded a scholarship to study for a masters degree (1965) in nutrition and biochemistry at the Harvard School of Public Health (Boston, Massachusetts). His doctor of philosophy (PhD, 1968) studies were on the mechanism of enzyme action with John F. Morrison at the Australian National University (ANU, Canberra, ACT, Australia) where he began a life-long interest in purine nucleoside chemistry and enzymology. During his time as a faculty member at the School of Medicine, Temple University (Philadelphia, Pennsylvania, 1971–1987) he began studies on enzymatic transition state structure through kinetic isotope effects. In 1987, he moved to the Albert Einstein College of Medicine in New York applying transition state theory to the design of inhibitors. Based on the transition state structures of bovine, human and malaria PNPs, powerful and specific inhibitors were designed to match each specific transition state. Several of these inhibitors are now in clinical trials for T-cell leukemia and autoimmune disease. Specific inhibitors of malaria PNP are being proposed for trials leading to therapy for malaria.

Salvage of hypoxanthine through hypoxanthine-guanine phosphoribosyl transferase (HGPRT) is essential for satisfying the purine requirement of *Plasmodium*. This enzyme, catalyzing the formation of IMP from hypoxanthine, was purified and characterized by classical biochemical methods from *P. lophurae* (Schimandle *et al*., 1987) and the rodent malarias *P. berghei* and *P. chabaudi* (Walter and Konigk, 1974b). With the advent of recombinant deoxyribonucleic acid (DNA) techniques the HGPRT of *P. falciparum* (PfHGPRT) was cloned and expressed in *E. coli* (Vasanthakumar *et al*., 1990) and *S. typhimurium* (Shahabuddin and Scaife, 1990), and the recombinant enzyme has been purified, crystallized and characterized (Keough *et al*., 1999; Mbewe *et al*., 2007; Sarkar *et al*., 2004; Subbayya and Balaram, 2000). Whereas the red cell enzyme can efficiently convert hypoxanthine and guanine into their respective monophosphates, PfHGPRT can use xanthine as well. Since site-directed mutagensis of the histidine at position 196 abrogated xanthine and guanine utilization without affecting conversion of hypoxanthine to IMP it suggests the possibility for design of a plasmodial-specific inhibitor (Sarkar *et al*., 2004). The pronounced inhibition of the guanine forwards reaction and the high Michaelis constant (K_m) for xanthine suggests that guanine and xanthine are not the biologically relevant purine bases, at least not for

the parasite stages growing in red cells; however, it is entirely possible that activity with xanthine may play a role in insect stages.

Immucillin GP and HP were reported to inhibit both malarial and human HGPRT (Li *et al*., 1999; Wang *et al*., 2001). Recent studies have shown that 6-chloroguanine, 8-azaguanine and 8-azahypoxanthine are more effective substrates for PfHGPRT than the human enzyme and these compounds also inhibit *P. falciparum* growth *in vitro* at micromolar concentrations (Keough *et al*., 2006). In addition, 6-mercaptopurine and 6-thioguanine, shown to be effective as anti-parasitic agents (Queen *et al*., 1990), were found to be inhibitors of *P. lophurae* HGPRT (Schimandle *et al*., 1987). Although 6-mercaptopurine and 6-thioguanine are currently in clinical use in maintenance therapy for acute lymphoblastic leukemia, these compounds produce cytotoxicity because of their incorporation into DNA. Similarly, 8-azaguanine is too toxic to be used though it might have potential as a lead compound for anti-malarials.

Parenthetically it should be noted that PfHGPRT activated T cells *in vitro* and immunization of mice with recombinant HGPRT reduced parasite growth in mice after challenge with *P. yoelii* 17XNL (Makobongo *et al*., 2003). Potentially, T cell epitopes from HGPRT could serve as a part of a protective immunogen.

IMP dehydrogenase (IMPDH), which converts IMP to xanthosine 5′-monophosphate with the concomitant reduction of nicotinamide adenine dinucleotide (NAD), is a critical rate-limiting step in the biosynthesis of guanine nucleotides and is an important regulator of cell proliferation. Significant differences in IMPDH enzyme kinetics and inhibitor sensitivities between that from humans and microbes suggest that it could serve as a chemotherapeutic target (Sullivan *et al*., 2005). In support of this, bredinin, an inhibitor of IMPDH, inhibited the growth of *P. falciparum* in culture (Webster and Whaun, 1982). Recombinant human IMPDH was inhibited by mycophenolic acid, a fermentation product of *Penicillium brevicompactum* and related species (Hariharan *et al*., 1999) as was the enzyme from other apicomplexans (Sullivan *et al*., 2005). Mycophenolic acid is a reversible non-competitive inhibitor of IMPDH that depletes GTP and also induces a striking conformational change in IMPDH such that the protein aggregates and enzyme activities are inhibited; the aggregates are reversible by addition of GTP (Ji *et al*., 2006). As of 2008, the falciparum IMPDH has not been cloned neither have the inhibitory properties of mycophenolic acid for it been evaluated.

Up until 2003, evidence for the presence of adenylosuccinate synthetase, which catalyses the condensation of IMP with aspartate to form adenylosuccinate and is the first committed step in the synthesis of AMP or GMP from IMP, has relied on the inhibitory action of hadacidin, an aspartate analogue (Webster *et al*., 1984a). But, in 2002, the dimeric

enzyme for *P. falciparum* was cloned, expressed, purified and later crystallized (Eaazhisai *et al*., 2004; Jayalakshmi *et al*., 2002). Its unique properties may provide insight into the development of novel anti-malarials. GMP formation is mediated by IMPDH and GMP synthase. The gene encoding GMP synthase has been isolated and expressed (McConkey, 2000) and the GMP synthase inhibitor, psicofuranine, was found to inhibit the *in vitro* growth of *P. falciparum*.

Salvage of hypoxanthine is essential for satisfying the purine requirements of *Plasmodium* spp. (Sherman, 1998b). Because isoenzymes of the purine salvage pathway—especially ADA, PNP and HGPRT—allow the malaria parasite to form hypoxanthine (and IMP) from erythrocytes and plasma, they will continue to provide grist for the drug designers' mills (el Kouni, 2003; Hyde, 2007).

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CHAPTER 17

Polyamines

Polyamines are ubiquitous cell components that play roles in regulation of gene expression, macromolecular syntheses, differentiation and proliferation. Eukaryotes contain the polyamines putrescine (PU), spermidine (SPD) and mostly spermine (SM). Human red cells, devoid of ornithine decarboxylase (ODC) and *S*-adenosyl methionine decarboxylase (AdoMetDC) activity, lack the capacity for the synthesis of polyamines and, as a result, intracellular levels are low, principally obtained by uptake. In uninfected human red cells only traces of PU, SPD and SM are found. However, upon infection with *Plasmodium falciparum* a strong increase and maximal levels of polyamines were attained at the schizont stage; the increased amount of polyamines was attributed to synthesis by the parasite (Das Gupta *et al*., 2005). In the host organism, uptake of PU by erythrocytes also occurs and a novel transporter was identified in *P. knowlesi*-infected erythrocytes. PU influx was inhibited by N1,N4-bis-(7-chloroquinoline-4-yl) butane-1,4-diamine (BCBD) and mice were cured of their *P. yoelii* infection after its administration (Singh *et al*., 1997).

The precursor for polyamine synthesis in mammalian cells as well as in *P. falciparum* is arginine, which is converted by arginase to urea and ornithine (Müller *et al*., 2005) the latter is subsequently decarboxylated by ODC to PU. However, there is evidence for an alternative pathway in the related apicomplexan *Cryptosporidium*, where arginine is decarboxylated to agmatine and subsequently converted to PU (Keithly *et al*., 1997). Thereafter in mammals SPD and SM are formed by the catalytic action of SPD synthase and SM synthase. AdoMetDC and ODC are the rate-limiting enzymes for polyamine synthesis.

In 1978, Metcalf and colleagues developed α-difluoromethylornithine (DFMO), a derivative of the amino acid ornithine, that leads to irreversible inhibition of the enzyme ODC by alkylation of its active site and

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depletes the cell of intracellular polyamines, which in turn results in a slowing down of proliferation of many cell types. Since DFMO was shown to eliminate *Trypanosoma brucei* infections in mice (Bacchi *et al*., 1980) it was natural to examine its effect on malaria parasites. *In vitro* DFMO is cytostatic rather than cytotoxic for *P. falciparum*, although it could be shown that erythrocytic schizogony of *P. falciparum* in culture could be inhibited by 5 mM DFMO, a concentration unlikely to be achieved *in vivo* (Gillet *et al*., 1983). DFMO inhibited the ODC of liver-stage *P. berghei* parasites, blocked the exo-erythrocytic schizogony in mice as well as in human hepatoma cells and had a curative effect. However, the erythrocytic stages were hardly affected *in vivo* (Assaraf *et al*., 1984; Bitonti *et al*., 1987; Hollingdale *et al*., 1985b). The effects of DFMO on the survival of *P. falciparum* in culture and on the erythrocytic development of *P. berghei* are modest, possibly because of poor uptake of DFMO and/or the existence of alternate pathways and/or the presence of exogenous sources of polyamines that supply PU (Müller *et al*., 2001).

Much of what we know about the enzymes involved in polyamine synthesis in malaria parasites has come from **Rolf Walter (1940–)** who received his doctor of philosophy (PhD) in 1973. Working with colleagues in the Bernhard-Nocht Institute for Tropical Medicine (BNI) in Hamburg, Germany, Walter became interested in the malaria field during his undergraduate studies, when he learned about malaria and the problems of malaria especially in Africa. At that time, difficulties in the treatment of malaria were exacerbated by the spread of chloroquine resistance. Furthermore, there was the war in Vietnam and the problems associated with drug resistance had forced the United States to invest in a huge effort synthesizing and screening new compounds for anti-malarial activity. In parallel, strategies of rational drug development emerged. Biochemistry became a tool to study the metabolic peculiarities of pathogenic agents that could be exploited for rational drug design. Theodor von Brand (1891–1959), a founder of parasite biochemistry who had been a researcher at the BNI and emigrated from Germany to the United States in 1934, encouraged such research strategies by those at his former institute, and, in 1971, a Department of Biochemistry was installed and headed by Eberhard Königk (1921–). Walter joined the group and the department worked for many years on purine salvage and folate biosynthesis in the rodent malaria parasite *P. chabaudi*. The 7,8-dihydropteroate-synthesizing enzyme complex was identified and nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) production by glutamate dehydrogenase investigated (Walter and Königk, 1974a; Walter *et al*., 1974). In 1985, Walter followed as head and continued malaria studies on cultured *P.*

falciparum. As a cause of anti-folate resistance, an altered dihydrofolate reductase (DHFR) was found (Walter, 1986; Walter *et al*., 1991). Further, techniques of gene cloning and manipulation, recombinant protein expression and analysis of protein structure became available and were added to the former biochemical methods. Research focus was on glutathione (GSH) and thioredoxin metabolism (Gilberger *et al*., 1997; Krnajski *et al*., 2001, 2002; Luersen *et al*., 2000; Müller *et al*., 1996; Perbandt *et al*., 2004) and polyamine synthesis as described below. More recently the department returned to vitamin research, now focussing on pyridoxal and thiamin. The biosyntheses of both vitamin B_1 and B_6 were identified in *P. falciparum*, the proteins involved were characterized and the pathways shown to have potential as targets for chemotherapy due to their absence in the host (Wrenger *et al*., 2005, 2006).

In all organisms investigated thus far, ODC and AdoMetDC are usually derived from separate genes and act individually as highly regulated monofunctional enzymes. However, in *P. falciparum*, there is only one gene coding for an unique polypeptide that possesses both ODC and AdoMetDC activity. The derived protein consists of the *N*-terminal AdoMetDC domain and the *C*-terminal ODC domain linked by a hinge region. The protein forms a heterotetrameric complex consisting of two bifunctional polypeptides post-translationally cleaved in the AdoMetDC domain (Müller *et al*., 2000). The bifunctional nature of AdoMetDC/ODC is unique to plasmodia and is shared by at least three species. The bifunctional protein contains parasite-specific regions, and these inserts are important for activity and protein–protein interactions. Intermolecular interactions between AdoMetDC and ODC appear to be vital for optimal ODC activity (Birkholtz *et al*., 2004). The bifunctional organization does not allow substrate channelling, however, the biological advantage might be that the control of polyamine synthesis is achieved by having to regulate the abundance and activity of only one protein. The parasite enzyme complex differs from its mammalian counterparts not only in structure and half-life, but also in response to regulation of the activities of the domains. Unlike its mammalian counterpart, the PfAdoMetDC is not stimulated by PU, thus plasmodia lack the regulatory mechanism proposed for mammalian cells to relate PU abundance to the synthesis of SPD. However, the plasmodial ODC activity is inhibited by its reaction product, PU, a regulatory step unique to the parasite that allows feedback control of SPD synthesis (Wrenger *et al*., 2001).

The diamines 3-amino-oxy-1-aminopropane and its derivatives CGP52622A and CGP54619A, reversible inhibitors of PfODC, act by depleting the cell of polyamines. Designed as ODC inhibitors for tumour

therapy they were found to be potent inhibitors of the plasmodial enzyme and showed anti-proliferative activity against cultured malaria parasites (Das Gupta *et al*., 2005). Furthermore, CGP 40215A and CGP 48664A, synthesized by Ciba-Geigy (now Novartis Pharma), as inhibitors of AdoMetDC, were active against *P. falciparum* in culture and the latter against *P. berghei* in mice (Brun and Walter, personal communication; Das Gupta *et al*., 2005). These may serve as lead compounds for the development of anti-malarials.

The *P. falciparum* genome contains only one aminopropyl transferase, and this gene encodes a SPD synthase, whereas a gene for SM synthase is lacking. The cytoplasmic enzyme, which is more closely related to that from plants, catalyzes the transfer of an aminopropyl group from decarboxylated *S*-adenosyl methionine onto PU. However, in contrast to mammalian SPD synthase, SPD can partially replace PU, whereby the plasmodial enzyme has the capacity to catalyze the formation of small amounts of SM found in the erythrocytic stages of *P. falciparum*. The SPD synthase inhibitor, trans-4-methycyclohexylamine (MCHA), was a potent inhibitor of the enzyme; it also inhibited the growth of *P. falciparum in vitro* and its effects could not be reversed by SPD suggesting an insufficient uptake of SPD (Haider *et al*., 2005). Very recently the three-dimensional structures of complexes of the plasmodial SPD synthase with substrates and inhibitors have been solved, which should allow the future design of more potent inhibitors (Dufe *et al*., 2007).

Interference with the functions of polyamines has been a strategy in the search for novel anti-malarials for more than 20 years, yet not a single therapeutic drug has emerged. The reasons may be that the putative targets are often involved in complex regulatory pathways that allow the parasite to adapt to changes in polyamine concentrations, or that the inhibitors do not permeate the infected red cell. In spite of such disappointments, there is continued optimism that the unique properties of the *Plasmodium* isoenzymes involved in polyamine synthesis will lead to an effective drug with severe consequences for the parasite without harming the host (Walter, personal communication).

CHAPTER 18

New Permeability Pathways and Transport

In 1948, Overman provided 'inferential evidence of altered permeability . . . in malaria', however, it would take another 20 years before the first study on the mechanism of solute transport into malaria-infected red blood cells would be conducted (Sherman *et al*., 1967). The impetus for our work on transport was a test of a hypothesis formulated by Clark P. Read of Rice University (Houston, Texas): parasites having low homeostatic capacity must rely on their host to provide physiologic balance. My familiarity with the work of Read stemmed from several summers (beginning in 1964) when I was an instructor in the invertebrate zoology course at the Marine Biological Laboratory (MBL) in Woods Hole (Massachusetts) and he was the course director. Read was one of the giants of parasitology and he and his students were actively testing the hypothesis that tapeworms had low regulatory capacities and so a balance of amino acids in the gastrointestinal tract of the host would be necessary for balanced parasite protein synthesis. Stimulated by their findings, we set out to examine the applicability of this hypothesis for malaria parasites by studying membrane transport (beginning in 1967 with Ragunath Virkar, a student of Grover Stephens and another instructor/colleague in the MBL course).

Most of the work on membrane transport with malaria parasites prior to 1990 concerned itself with studies of bird, murine and monkey plasmodia (*Plasmodium lophurae*, *P. berghei* and *P. knowlesi*) and this was summarized some 20 years ago (Sherman, 1979, 1988). With the successful *in vitro* culture of *P. falciparum*, membrane-transport phenomena of malaria-infected red cells and free parasites have concerned themselves principally with this species and this too has been the subject of periodic review (e.g. see Kirk's tour de force, 2001).

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Since a malaria-infected red cell contains three membrane interfaces: the plasma membrane of the red cell, the parasitophorous vacuolar membrane (PVM) and the parasite plasma membrane (PPM) studying membrane transport in such a system is not straightforward. Substrates have to traverse multiple membrane systems and a single membrane may contain multiple transporters for a particular substrate. To complicate the matter further, when we (and others) embarked on such studies not only were we usually unaware of the complexities of the transport systems that might be encountered, we were also unappreciative of the technical difficulties as well as possible artefacts that might result from isolating parasites and using heavy infections in unnatural hosts or from *in vitro* cultures.

To those uninitiated with membrane transport a word about it may be in order. In cells, transport is mediated by integral membrane proteins that facilitate the translocation of molecules and ions across the membrane. There are two classes of membrane transporters—channels and carriers (also known as permeases)—that differ from one another in their functional characteristics as well as in their mechanism of action. A channel, when open, typically shows high throughput by means of a diffusion pathway and the substrate moves down the electrochemical gradient. Carriers, like enzymes have specific substrate binding sites, do not provide an open diffusion pathway and, for the substrate to cross the membrane, the protein must undergo a conformational change. As a result, there is usually a much lower throughput rate than through channels and, unlike channels, they are capable of active transport of solutes against an electrochemical gradient. Channels and carriers can be susceptible to inhibition by pharmacological agents and these drugs may assist in discrimination between the types of transport.

As noted above, the earliest studies of membrane transport in malaria began with *P. knowlesi* growing in monkey red cells, *P. berghei* in mouse red cells and *P. lophurae*-infected duckling erythrocytes (Sherman, 1988). The studies with *P. lophurae* were designed to examine the validity of Read's hypothesis as well as to answer the following question: by what mechanisms do malaria-infected red cells and malaria parasites accumulate amino acids? Duckling red cells were suspended in an artificial medium containing a 1-mM concentration of amino acid and a radioactive tracer. Five of the 10 amino acids shown to enter the uninfected duck red cell by a saturable, non-uphill transport system (i.e. alanine, leucine, histidine, methionine and cysteine) entered infected cells *via* a mechanism that, unlike the system of uninfected cells, showed diffusion kinetics. The five others (i.e. glycine, serine, threonine, lysine and arginine) had an increase in the transport constant, K_t (Sherman and Tanigoshi, 1974;

Sherman and Mudd, 1966). No evidence was found to support Read's hypothesis; however, what was discovered was that the *Plasmodium* could change the permeability properties of its host cell rendering it highly permeable to most amino acids.

In the early 1970s, McCormick, working at the Walter Reed Army Institute of Research (WRAIR), showed that the rate of isoleucine transport into monkey red cells infected with *P. knowlesi* was increased relative to the uninfected red cell and accumulation could be over 20 times that of the normal red cell; the highest distribution ratios and the highest levels of incorporation were with isoleucine and methionine (McCormick, 1970). With *P. lophurae*-infected red cells suspended in a medium devoid of other amino acids but containing glucose, the infected red cells had a higher distribution ratio than did normal cells for proline alone, but when suspended in a medium similar in free amino acid composition to plasma, proline and methionine were both accumulated to higher distribution ratios. When studies of transport were conducted with this avian malaria parasite two patterns were evident: (1) mediated transport in the uninfected duckling red cell and diffusion entry or an elevated K_t (roughly equivalent to the apparent Michaelis constant, K_m) in the parasitized red cell or (2) diffusion entry in both kinds of cells. Thus, it appeared that the *Plasmodium* (*P. knowlesi* and *P. lophurae*) had induced alterations in permeability in the red cell for several amino acids; this increased 'leakiness' of malaria-infected red cells would be later attributed to transport pathways termed new permeability pathways (NPPs; see below).

After 1976, with the availability of *P. falciparum* in continuous *in vitro* culture, it became possible to study the transport properties of a human malaria parasite. Beginning in 1982, a group at the Hebrew University of Jerusalem (Jerusalem, Israel) led by Hagai Ginsburg and Ioav Cabantchik reported that the permeability of *P. falciparum*-infected red cell to a range of amino acids, including tryptophan and isoleucine was increased (Kutner *et al*., 1982). However, Elford *et al*. (1985) found no effect on isoleucine transport when human red cells were infected with *P. falciparum*. However, a recent report (Martin and Kirk, 2007) showed that isoleucine uptake by uninfected human red cells was rapid and non-concentrative (reaching a distribution ratio of about 0.9) and infection with *P. falciparum* resulted in a five-fold increase in the rate of influx; the parasite-induced transport component was wholly blocked by the NPP inhibitor, furosemide, consistent with it being *via* the NPP (see below). It was claimed that the NPP served as the major route for the uptake of isoleucine into *P. falciparum*-infected red cells and presumably for other species as well.

Hagai Ginsburg (1937–) received his early education in Israel and joined the Hebrew University of Jerusalem in 1971, as lecturer in physiology and biophysics and was promoted to full professor in physiology and biochemistry in the same institution in 1983. He became interested in malaria research around 1980 when he responded to the call of the World Health Organization/Special Program for Research and Training in Tropical Diseases (WHO/TDR, Geneva, Switzerland) for basic scientists to join the efforts in malaria research. Prior to that, he was working on the kinetic characterization of transporters of normal red blood cells and thought that examining these topics in malaria-infected red cells could be interesting. Since then he has published more than 150 articles on malaria-related topics, including: reconstitution of membrane transporters in artificial membranes, kinetic analysis of carriers in the erythrocyte membrane, the mode of action of anti-malarial drugs and mechanisms of drug resistance, the fate of heme in malaria-infected erythrocytes, redox metabolism in infected cells, characterization of transport systems in *Plasmodium*-infected erythrocytes, and mathematical modeling of malaria chemotherapy. Ginsburg developed and curates a website dedicated to the biology, physiology and biochemistry of the malaria parasite *Plasmodium falciparum* (http://sites.huji.ac.il/malaria; last accessed 16 July 2008).

Glucose is rapidly consumed by malaria parasites and in some instances the rate has been calculated to be as much as 100 times greater than the uninfected red cell. Herman *et al*. (1966) postulated that the rate of entry of glucose into an uninfected red cell could not support the rate of glucose consumption by *P. gallinaceum*-infected red cells and, to permit increased entry, malaria parasites would either have to change the permeability of the red cell or alter the metabolic pathway by removing an inhibitor or enhancing active transport of glucose across the red cell membrane. Because Herman *et al*. had been unable to discriminate between transport and metabolism, we used radiolabeled glucose as well as a non-metabolizable analogue (3-*O*-^{14}C) methyl glucose (3-OMG) and rapid centrifugation techniques to study glucose transport into *P. lophurae*-infected red cells. Using (*D*-^{14}C) glucose (UL) as well as 3-OMG we found increased entry to be due to the infected red cell becoming 'leaky' with entry rates directly related to parasite size (Sherman and Tanigoshi, 1974). Although the mechanism for the induced change was unclear, it was obvious (from the very rapid separation of cells from medium and the uptake of 3-OMG) that increased uptake was

separate from glucose catabolism. This work was confirmed by Homewood and Neame (1974, 1975) who showed that *L*-glucose entered *P. berghei*-parasitized cells but not normal mouse red cells by a mechanism that appeared to be 'diffusion-like'.

Because the transport constants for glucose entry into erythrocyte-free *P. lophurae* differed from that of the uninfected and infected duckling red cell (i.e. the maximum velocity (V_{max}) was twice that of the infected red cell and 15 times greater than the uninfected red cell and the K_t was less than half), it suggested that the parasite differed from the host cell in the number and nature of specific glucose transporters. *P. falciparum* glucose uptake into the parasite was shown to be a saturable, equilibrative process attributed primarily to a *P. falciparum* hexose transporter, PfHT, on the PPM (Joet *et al*., 2003a). The PfHT, a single-copy gene located on chromosome 2, has been cloned, sequenced and localized to the PPM and, when expressed in *Xenopus* oocytes, transports both glucose and fructose, unlike its mammalian orthologues GLUT1 and GLUT5, which transport glucose and fructose, respectively. The compound 3361 (3-*O*-(undec-10-en)-yl) *D*-glucose) is a selective inhibitor of PfHT and addition of this compound to parasites results in a rapid decline in adenosine triphosphate (ATP) levels and a loss of pH control in both the parasite cytosol and the food vacuole (FV). As a consequence, the *in vitro* growth of *P. falciparum* is blocked and the *in vivo* multiplication of *P. berghei* is limited (Joet *et al*., 2003a; Saliba *et al*., 2004). In addition, the hexose transporter from *P. vivax* has also been cloned, expressed in oocytes and is also inhibited by compound 3361 (Joet *et al*., 2004).

Work in our laboratory, establishing that the parasite has to salvage purines from the host, led quite naturally to studies of purine transport. A graduate student, Susan Tracy, and I found infected red cells had a high uptake (= transport plus incorporation) of adenosine, inosine and hypoxanthine and the degree of accumulation related directly to the size of the parasite (Tracy and Sherman, 1972). However, for adenine the reverse was true; adenosine monophosphate (AMP), adenosine diphosphate (ADP) and ATP were taken up to a very limited degree. Adenosine transport was inhibited by the presence of hypoxanthine and inosine but such mutual inhibition was not seen in uninfected red cells. Although adenine was transported, it was not incorporated into nucleic acids by either 'free' *P. lophurae* or infected duckling red cells. We suggested that adenosine was deaminated to inosine shortly before or during uptake and concluded that the infected red cell membrane had a common 6-oxopurine transport site that transported adenosine-inosine and hypoxanthine. Hansen, Sleeman and Pappas (1980) found three transport loci in uninfected rat red cells (adenosine-inosine, hypoxanthine and adenine)

whereas in *P. berghei*-infected red cells and 'free' parasites there were two loci (adenosine-inosine-hypoxanthine and adenine).

Studies by Gero and colleagues at the University of New South Wales (Sydney, NSW, Australia) showed that *P. falciparum*-infected red cells induced NPP for nucleosides (Upston and Gero, 1995). Permeation of *L*-adenosine, the non-physiological isomer of *D*-adenosine that is not transported into normal human erythrocytes, was inhibited by phlorizin, and furosemide but not nitrobenzylthioinosine (NBMPR), nitrobenzylthioguanosine (NBTGR), dipyridamole or dialzep. Similar findings were found for mouse erythrocytes infected with *P. yoelii* (Gati *et al*., 1987). The altered transport characteristics of malaria-infected red cells have suggested two possible approaches for drug development. The first involves the simultaneous administration of two compounds—a toxic nucleoside that kills the parasite and a nucleoside transport inhibitor that protects normal host cells from the toxic compound. Indeed, when tubercidin in combination with NBMPR was administered over four consecutive days to *P. berghei*- or *P. yoelii*-infected animals parasitemias decreased and survival times doubled (Gati *et al*., 1987; Gero *et al*., 1999). The second approach utilizes cytotoxic nucleosides unable to enter normal cells through the endogenous nucleoside transporter but able to enter infected red cells through the NPP. When tested it was found that although *L*-enantiomers of physiological *D*-nucleosides did enter infected red cells and parasites there was no anti-malarial effect; however, *L*-enantiomers of *D*-nucleosides such as 6-thio-*L*-guanosine, 6-thio-*L*-inosine and *L*-sangivamycin inhibited wild-type and resistant strains of *P. falciparum* with IC_{50} values of 160, 200 and 25 μM, respectively. None of these compounds entered normal cells. The *D*-nucleoside analogues of these compounds eliminated *P. knowlesi* infections in monkeys, however, their major disadvantage remains toxicity, a property that would not occur using the *L*-enantiomers that would enter only parasite-infected red cells (Gero *et al*., 1999). Another possibility would be to use dinucleotide monophosphate dimers that are *L*-nucleosides to selectively deliver a toxic *D*-nucleoside (tide) (Gero *et al*., 2003).

Annette Gero (1944–) received her bachelor of science (BSc) from the University of Sydney (Sydney, NSW, Australia) (1966), a master of science (MSc) from Macquarie University (NSW, Australia, 1977) and a doctor of philosophy (PhD, 1982) from the University of New South Wales. She first became involved with malaria while working with Bill O'Sullivan at the University of New South Wales. Bill, who specialized in the pyrimidine pathway and was working on human spleen enzymes, became her PhD supervisor. While doing her PhD on the fourth enzyme of the pathway, dihydroorotate dehydrogenase

(DHOD), Win Gutteridge from Burroughs Welcome contacted Bill and asked whether they might collaborate and do a comparative project between the human and the malaria enzymes. The collaboration worked and Annette completed her PhD on '*Dihydroorotate dehydrogenase*: *in mammalian and parasitic protozoa*'. After a short stint at Walter and Eliza Hall Institute (WEHI) with Graham Brown and Graham Mitchell to learn how to culture *P. falciparum*, the human malaria DHODH project began at University of New South Wales. There was a great collaboration with Burroughs Welcome as these scientists produced many pyrimidine analogues that reacted with ubiquinone, the co-factor of DHODH. The ubiquinone co-factors in malaria and humans were so different there was specific inhibition of the malaria enzyme. The best was BW 566C and its derivatives, which eventually became atovaquone (see Chapter 12, p. 101).

After the completion of her PhD, Annette was awarded an National Health and Medical Research Council (NHMRC, Australia) fellowship that entitled her to set up her own research laboratory, which specialized in purine transport in a variety of parasites (*Plasmodium*, *Giardia*, *Leishmania*, *Trichomonas*) and to work on the altered permeability of the membrane of the malaria-infected erythrocyte and those changes that occur when the parasite invaded the red cell (i.e. transport of nutrients such as purine nucleosides). The major thrust of the work was to take advantage of the altered membrane permeability of the infected red cell to selectively deliver inhibitors of the purine salvage pathway, on which the parasite depends for survival. By chance, at a conference in Sydney, she met Alan Paterson from the University of Alberta in Edmonton (Edmonton, AB, Canada), who talked about NBMPR, which inhibited the transport of purines into normal cells, and about optical isomers of purine nucleosides, which inhibited the growth of *Giardia*. She asked him, 'would this work in malaria?' 'Let's try,' he said. So she went to Edmonton with a visiting professorship award, to set up malaria research there and to learn how to do transport studies across membranes in parasites. A career was born. NBMPR did not inhibit the transport of purines across the infected red-cell membrane but did allow toxic purines to enter. It was a selective system permitting drugs to enter the parasite but not normal human cells. However, since using NBMPR involved a two-drug system, a better approach would be one that used a single compound that could selectively enter only the malaria-infected cells. This turned out to be optical isomers of purine nucleosides, with *L*-adenosine as the model. *L*-Adenosine could not enter normal cells but was easily transported into malaria-infected red cells as was *L*-thymidine, *L*-guanosine and most optical isomers of nucleosides. However, the optical isomer of the natural nucleoside was not toxic.

She was awarded the first of many World Health Organization (WHO, Geneva, Switzerland) grants to look at this. Then she asked, could one modify *L*-adenosine to an analogue toxic to malaria? Or could one complex *L*-adenosine to another drug to carry the drug into the malaria-infected cell? Again fortuitously (as so often happens in science) she gave the plenary lecture at a conference on purines and pyrimidines in Bloomington, Indiana on the transport of *L*-adenosine and sat down to breakfast the next day and asked, 'What do you do?' 'We have a company which produces analogues of *L*-adenosine!' Another collaboration was born. Alex Weis, of Lipitek International, Texas produced 20–30 analogues of *L*-adenosine with 5-fluorodeoxyuridine attached at many different positions. The United States Department of Defense awarded a $1 million grant to determine the most potent *L*-adenosine analogue. This led to the development of 'carrier molecules', which could selectively deliver a toxic compound into the malarial-infected cell with no deleterious effects on the host. A unique group of *L*-purine nucleoside compounds were developed that could selectively carry a toxic drug into the malarial-infected cell. These 'carrier' compounds were dimers of two nucleosides linked *via* a phosphate group, in which the carrier part of the molecule was an optical isomer (an *L*-nucleoside). The most successful compound was a dimer of 2-deoxy-*L*-adenosine and 5-fluoro-*D*-2-deoxy uridine, metabolized within the malaria parasite to 2-deoxy-*L*-adenosine and the toxic 5-fluoro-*D*-2 deoxy uridine monophosphate. The carrier *L*-nucleoside did not enter normal cells but when linked to other toxic molecules could deliver the toxic agent into the plasmodia at their site of action. These compounds were excellent inhibitors of malaria parasite growth in culture.

Human red cells express high-affinity equilibrative nucleoside transporters that mediate the sodium-independent uptake of a broad spectrum of purine and pyrimidine nucleosides. A purine transporter of *P. falciparum*, PfNT1, located on chromosome 14, was cloned, sequenced, localized to the PPM and expressed in *Xenopus* oocytes (Carter *et al*., 2000; Rager *et al*., 2000). In a subsequent study, using isolated *P. falciparum* adenosine, inosine and hypoxanthine were found to equilibrate rapidly between the extracellular and intracellular compartments, reaching a distribution ratio of 1, via the low-affinity transporter, PfNT1, 'before being accumulated to higher levels via one or more high-affinity mechanisms (Downie, Saliba, Broer, Howitt and Kirk, 2008). Adenine uptake by isolated parasites resulted from a low-affinity, ATP-independent, as well as an ATP-dependent high- affinity component that also involved PfNT1. PfNT1 was also involved in the transport and utilization of xanthine, guanine and guanosine (El Bissati, Downie, Kim, Horowitz, Carter, Ullman

and Ben Mamoun, 2008). However, another investigation (Quashie *et al.*, 2008) claimed the existence of four separate transport activities. Hypoxanthine, the primary purine source for the parasite, was taken up with a 12-fold higher efficiency than adenosine. A knockout of PfNT1 abolished the high-affinity transport of hypoxanthine but did not affect the transport of adenosine; separate high-affinity and low-affinity/high capacity carriers for adenine were also found. The discrepancies could be due to differences in parasite preparations, as well as ''differences in experimental design (and a lack of) consideration of the rapid metabolism of purines'' (Downie, Kirk and Ben Mamoun, 2008). PfNT1 had a broad specificity for the nucleosides adenosine and inosine, but no affinity for nucleobases (Carter *et al.*, 2000). Another study identified a gene identical to PfNT1 (called PfENT1) that was localized to chromosome 13; it too was cloned, sequenced and expressed in oocytes (Parker *et al.*, 2000). PfENT1 had a low affinity for adenosine, adenine and hypoxanthine (Downie *et al.*, 2006). PfNT1 had also been described as a low-affinity transporter based on expression in *Xenopus* oocytes, however, a recent study (Quashie *et al.*, 2008) that directly measured transport into the *Plasmodium* suggested the uptake of hypoxanthine and adenine was comprised of two components: rapid equilibration *via* a low-affinity transport mechanism and accumulation *via* a higher-affinity process.

The importance of PfNT1 was established using genetically disrupted (i.e. 'knockouts') of *P. falciparum* (El Bissati *et al.*, 2006). The knockout of PfNT1 affected only the high-affinity hypoxanthine/nucleoside transporter, whereas the low-affinity transport of adenosine and adenine was unchanged. Further, parasites lacking PfNT1 were unable to use hypoxanthine, inosine or adenosine and *in vitro* growth was impaired but could be restored by provision of high concentrations of these nucleosides in the medium. Additionally, because *P. falciparum*-infected red cells were able to convert radiolabeled adenosine into hypoxanthine and the hypoxanthine was found in the parasite cytoplasm, it suggested that the parasite exploits the host enzymes for conversion of adenosine and inosine into hypoxanthine before translocation across the PPM and that hypoxanthine uptake was the principal purine salvaged (El Bissati *et al.*, 2006). The identification and cloning of PfENT1 suggests there may be potential to exploit nucleoside transport for chemotherapy (Baldwin *et al.*, 2007).

These findings support a sequential pathway for purine acquisition (i.e. they are first transported across the red cell membrane into the red cell cytoplasm, after crossing the PVM they are then translocated into the parasite by specific transporters). PfNT1 mediates the highly efficient uptake of hypoxanthine (and adenosine) and guanine as well as adenine; however, the adenine is not metabolized. In large part, these studies confirm the findings by Tracy and Sherman (1972) and Yamada and Sherman (1981a) using *P. lophurae*.

Buddy Ullman (1950–) received a bachelor of arts (BA) (1970) in chemistry from Oberlin College (Oberlin, Ohio) and a PhD (1975) from the Harvard Medical School (Boston, Massachusetts). His doctoral work, under the mentorship of Robert L. Perlman, focused on the purification and characterization of mammalian phosphoprotein phosphatases. Post-doctoral studies (1975–1981) in the Department of Biochemistry and Biophysics at the University of California, San Francisco (San Francisco, California) under the direction of David W. Martin, Jr centred upon two inborn errors of purine metabolism associated with immunological abnormalities. In that work, he exploited somatic cell genetic approaches to dissect the lymphospecific toxicities of these two genetic diseases in the S49 cell culture model, discovering in the process that the enzyme ribonucleotide reductase was adversely targeted by both genetic deficiencies. In 1981, he took a faculty position in the Department of Biochemistry at the University of Kentucky College of Medicine (Lexington, Kentucky), where he began to investigate nucleoside transporters of the mammalian variety. An array of mutant lines with genetically altered nucleoside transporter activities creating loss-of-function, gain-of-function, substrate specificity and inhibitor-binding mutants were found and then characterized. During this time he began to dabble in protozoan parasites, specifically *Leishmania donovani*. In 1985, after moving to the Department of Biochemistry and Molecular Biology at the Oregon Health & Science University (Portland, Oregon), he concentrated on genetic approaches to dissect and characterize purine transporters and purine salvage enzymes of *Leishmania*. In the late 1980s, he discovered that *Leishmania* possessed two nucleoside transporters of non-overlapping ligand specificities, but it took an additional decade to clone the genes encoding these transporters. The two leishmanial genes were cloned by functional complementation of *L. donovani* lines deficient in each of the two nucleoside transporters, and isolated in collaboration with Scott Landfear. Because the *L. donovani* nucleoside transporter genes were the first parasite nucleoside transporter genes to be cloned, a logical and natural extension of this work was to examine other members of the nucleoside transporter family in other genera of parasites. As genome-sequencing projects began to reveal the identities of other parasite nucleoside transporters, Ullman's laboratory began to investigate these other transporters, including those from *P. falciparum*. In collaboration with Choukri Ben Mamoun of the University of Connecticut Health Center (Farmington, Connecticut), all four members of the nucleoside transporter family from *P. falciparum*, PfNT1, were cloned, functionally characterized and localized to the parasite cell

surface. A PfNTL knockout was developed that exhibits a conditionally lethal phenotype and its survival and growth are dependent upon the presence of excessive, non-physiological concentrations of purines in the culture medium. Further, characterization of this PfNTL null mutant demonstrated that all host purines are funneled to hypoxanthine in the erythrocyte cytosol and then hypoxanthine is transported into the parasite through PfNT1 and incorporated into the parasite nucleotide pool *via* the *P. falciparum* hypoxanthine-guanine phosphoribosyl transferase.

For more than 50 years it has been recognized that erythrocytes infected with the asexual stages of malaria parasites have an altered permeability to a wide variety of low-molecular solutes (Elford *et al*., 1985; Kirk, 2001; Kirk and Saliba, 2007; Sherman, 1988). This increase, attributable in the main to what has been called the NPP provides a route for the uptake of metabolites and also allows for the efflux of metabolic wastes such as lactic acid (from glycolysis) and amino acids (from proteolysis of hemoglobin) (Ginsburg and Kirk, 1998). The NPP also changes the ionic environment of the red cell from being one that is low in Na^+/high in K^+ to one that is high in Na^+/low in K^+ (Lee *et al*., 1988; Sherman and Tanigoshi, 1971; Staines *et al*., 2001). In *P. falciparum*-infected erythrocytes, glucose permeates through the NPP but the rate is much lower than the rate at which it is transported *via* the red cell glucose transporter, GLUT1. As with glucose, the endogenous nucleoside transporter is fast, however, the flux of nucleosides *via* the NPP is not crucial for serving the parasite's nucleoside needs. Isoleucine (Martin and Kirk, 2007), as well as choline (Biagini *et al*., 2004; Kirk *et al*., 2001) and the water-soluble vitamin, pantothenate (Saliba *et al*., 1998), also enter *via* the NPP. Since the continuous *in vitro* growth of the blood stages of *P. falciparum* requires the presence of these substances (and others) it has been contended that they must be acquired in increased amounts from exogenous sources such as the blood plasma or the red-cell cytosol. However it should be noted that for many, perhaps most, of the nutrients required by the parasite, the endogenous transport activity of the red blood cell is (more than) sufficient to meet the parasite's requirements (Kirk and Saliba, 2007). Indeed, there are very few nutrients for which this is not the case. In falciparum-infected human red cells pantothenate is one and isoleucine another, but for other species of malaria parasites and/or their host cells, the situation is likely to be different (Kirk, 2004). If the assumption is correct, that the survival of the parasite requires the up-regulation of existing transporters and/or creates new permeation pathways, then compounds that would prevent such acquisition might be effective anti-malarials (Biagini, 2005; Kirk, 2004; Kirk and Saliba, 2007). Regrettably, despite intensive research

over the past three decades with *in vitro* grown *P. falciparum*, questions remain regarding the number and kind of channels that make up the NPP and whether they originate from parasite-encoded proteins or from modifications of host proteins.

During the intraerythrocytic growth of *P. falciparum* there is a four-fold increase in the phospholipid (PL) content of the infected red cell and approximately 45% of this is in the form of phosphatidylcholine synthesized by the parasite *via* the Kennedy pathway or from serine *via* pathways involving methylation of phosphoethanolamine (Membrane Lipids, Chapter 22). In the uninfected red cells, choline is taken up *via* a saturable carrier, whereas in *P. vinckei*- and *P. falciparum*-infected red cells there is an additional (non-saturable) component (i.e. the NPP; Biagini *et al*., 2004; Kirk *et al*., 1991; Lehane *et al*., 2004a; Staines and Kirk, 1998). (Parasitized red cells from animals infected with *P. vinckei* (Staines and Kirk, 1998) and *P. knowlesi* (Ancelin *et al*., 1991; Vial *et al*., 1999) also show an increase in the rate of influx of choline *via* the saturable component but precisely how this occurs is still undetermined.) Once choline is inside the infected red cell it crosses the PVM and is transported across the PPM. Using saponin-freed *P. falciparum*, the choline carrier was localized to the PPM. The transporter has a much higher transport capacity than the NPP (Biagini *et al*., 2004), is sodium independent and appears to be energized by a proton pump. The effects of pharmacological agents support the notion of a parasite membrane proton pump, that is, furosemide was without effect on this in 'free' parasites and bafilomycin A1, an inhibitor of vacuolar H^+-ATPase (V-ATPase) proton pumps and the protonophore, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), reduced choline transport in 'free' parasites (Biagini *et al*., 2004; Lehane *et al*., 2004a). Although PL synthesis is sensitive to *bis*-quaternary ammonium compounds such as T16 and G25, it appears that this not due to a direct inhibition of choline transport. Significantly, choline influx was inhibited competitively by quinine (Lehane *et al*., 2004a).

Pantothenate, a precursor of coenzyme A, is an essential nutrient for the growth of several species of *Plasmodium*. A dietary deficiency of pantothenate in chickens, as well as inhibitors of pantothenate, reduced parasitemia in chickens infected with *P. gallinaceum* (Brackett *et al*., 1946) and when pantothenate was added to *in vitro* cultures of *P. lophurae*, infectivity was demonstrated after 8 days *in vitro* and the presence of male gametocytes capable of exflagellation after 16 days. In the absence of pantothenate, both infectivity and exflagellation persisted for only 5 days (summarized in Trager, 1977). In short-term *in vitro* experiments with *P. coatneyi* and *P. falciparum*, anti-metabolites of pantothenate inhibited parasite development (Trager, 1971). Similarly, pantothenate analogues and pantothenol inhibited the *in vitro* growth of *P. falciparum* (Saliba *et al*., 2005; Spry *et al*., 2005). Pantothenate is impermeant to normal red blood

cells; however, it does enter *P. falciparum*-infected red cells *via* the NPP as attested to by uptake being sensitive to furosemide and non-saturability (Saliba *et al*., 1998). Unlike the situation in *P. lophurae* where Trager and co-workers (Bennett and Trager, 1967; Brohn and Trager, 1975) were able to show that all five enzymes involved in the conversion of pantothenate to coenzyme A were in the cytosol of duck red cells, in *P. falciparum* the phosphorylation of pantothenate takes place in the parasite cytosol (Saliba *et al*., 1998). It is not known whether this inconsistency reflects species-specific differences.

As noted earlier (see p. 10) the earliest biochemical studies of Fulton and co-workers indicated that glycerol could serve as an energy source, but it would take almost 50 years to show that the pathway for entry of glycerol into the *Plasmodium*-infected red cell was *via* an orthologue of the *P. falciparum* aquaglyceroporin (Promeneur *et al*., 2007). In *P. berghei*, there is only a single gene encoding the aquaglyceroporin and the protein shows 62% amino acid homology to that of the falciparum transporter; when the gene was expressed in *Xenopus* oocytes the transporters became permeable to water and glycerol. The aquaglyceroporin was localized to the PPM. Null parasites were viable but grew more slowly presumably because glycerol, which is used by the parasite for its lipid backbone, can be obtained *via* glycolysis. Null parasites were also less virulent (Liu *et al*., 2007). Since the proliferation of *P. falciparum* in culture was inhibited by methylglyoxal (IC_{50} 200 μM) and dihydroxyacetone, which permeate the infected red cell *via* the aquaglyceroporin transporter (Pavlovic-Djuranovic *et al*., 2006), there may be a possibility for design of novel anti-malarials.

One of the earliest observations on transport was the movement of Na^+ into the erythrocyte cytosol of malaria-infected red cells (Dunn, 1969; Overman, 1948; Overman *et al*., 1949, 1950) yet until recently little was known about whether this was of any value to the intra-erythrocytic *Plasmodium*. In 2006, it was discovered that the parasite exploits not an H^+-coupled transporter but a Na^+ electrochemical gradient to energize the transporter for inorganic phosphate, an essential nutrient. The transporter was cloned, localized to the parasite surface and, when expressed in *Xenopus* oocytes, resulted in uptake properties similar to those of the parasite (Saliba *et al*., 2006).

Transporter genes have been attractive candidates for understanding the mechanisms of drug resistance. Although well over 100 genes encoding transporters were found in the *P. falciparum* genome and polymorphisms have been found in 11 that display *in vitro* drug resistance to chloroquine and/or quinine, only two of the four known drug-resistance loci in *P. falciparum* are well characterized as transporters (Martin *et al*., 2005). Wellems *et al*. (1991) first identified a locus on chromosome 7 involved in chloroquine resistance and later a 3.1-kb gene, named *pfpcrt*,

was discovered and characterized as being the transporter responsible for chloroquine resistance (Fidock *et al*., 2000; Valderramos and Fidock, 2006). The gene product of *pfcrt* is localized to the FV membrane (see pp. 60–61).

Thomas E. Wellems (1951–) was born in Anaconda, Montana and received undergraduate degrees in physics and chemistry at the New Mexico Institute of Mining and Technology (Socorro, New Mexico) (1973). His graduate years were spent in the Medical Scientist Training Program at the University of Chicago (Chicago, Illinois), where he earned his PhD in biophysics (1980) and his doctor of medicine (MD) in 1981. In the course of his PhD studies, Wellems' work on sickle-cell anemia brought him into contact with literature on malaria and its enormous selective force in the evolution of erythrocyte and hemoglobin polymorphisms. This sparked an interest in malaria that gelled during his internal medicine residency (1981–1983) at the Hospital of the University of Pennsylvania (Philadelphia, Pennsylvania). In 1984, he joined the Laboratory of Parasitic Diseases in the Division of Intramural Research of the National Institute of Allergy and Infectious Diseases (NIAID, Bethesda, Maryland). Since that time, he has focused on *P. falciparum* malaria. From this research, he and his collaborators published the first descriptions of the transporter molecule responsible for chloroquine resistance (2000), of the *var* gene family responsible for antigenic variation and immune evasion by *P. falciparum* parasites (1995) and of a molecular mechanism for malaria protection by hemoglobin C (2005). Other primary reports from his work provide original descriptions of a protein sequence used in rapid diagnostic test kits for the detection of *P. falciparum* infections, that is, PfHRP II (1986), of dihydrofolate reductase (DHFR) mutations responsible for pyrimethamine resistance in malaria (1998, 1990), of deoxyribonucleic acid (DNA) transfection methods for *P. falciparum*-infected erythrocytes (1995, 1996) and of a high-resolution genetic map of *P. falciparum* (1999). Among his greatest career satisfactions have been results of field programs with his collaborators in Africa and his publications with scientists he has mentored who now have their own faculty appointments and independent programs.

Multi-drug resistance (MDR) in tumor cells (i.e. reduced drug accumulation and reversal by verapamil) has some features in common with drug resistance by malarial parasites. A parasite homologue of the mammalian MDR gene, *pfmdr*, was cloned, sequenced and characterized (Foote *et al*., 1989). The gene is located on chromosome 5 and its product, a P-glycoprotein, PfPgh-1, with an relative molecular mass (M_r) of

162 kDa, is a member of the ATP-binding cassette transporters, located on the membrane of the FV, and is expressed during intraerythrocytic development (Rohrbach *et al*., 2006). There is a strong association between *pfmdr* amplification (first described in the laboratory of Dyann Wirth (Wilson *et al*., 1989) and mefloquine treatment failure and *in vitro* resistance, as well with resistance *in vitro* to halofantrine and quinine. Decreasing the copy number of *pfmdr* heightens susceptibility to lumefantrine (LUM), halofantrine, quinine, artemisinin and mefloquine (Sidhu *et al*., 2006). Polymorphisms at amino acid residues 86, 184, 1034, 1042 and 1246 have been associated with altered susceptibility to chloroquine, quinine, mefloquine and artemisinin (Sidhu *et al*., 2005). PfPgh-1 may affect resistance by directly transporting drugs into or out of the FV or it may influence partitioning as a result of altered transport of other substrates (Duraisingh and Cowman, 2005). Inwardly directed transport is consistent with the predicted topology of PfPgh-1 with its ATP-binding domain facing the cytoplasm. Recently it was shown that the pattern of staining with fluor-4 provided for a rapid cell-based diagnostic assay for Pfmdr-1 with mefloquine, halofantrine, quinine and artemisinin (Rohrbach *et al*., 2006).

Dyann Wirth (1951–) received her BA from the University of Wisconsin (Madison, Wisconsin) in 1973, spent a year as a Fullbright Fellow in Freiberg, Germany, and was awarded a PhD (1978) from the Massachusetts Institute of Technology (Cambridge, Massachusetts) with a thesis on '*The synthesis of Sindbis virus integral membrane proteins*' under the supervision of Harvey Lodish and Philip Robbins. She was an National Institutes of Health (NIH, Bethesda, Maryland) post-doctoral fellow in molecular biology at Harvard University (Cambridge, Massachusetts; 1978–1981) with the 1980 Nobel laureate Walter Gilbert. In the summer of 1980, she became an instructor in the biology of parasitism course at MBL directed by John David where she became acutely aware of the power of molecular biology to solve problems associated with parasitic diseases. In 1981, Dyann joined the faculty of Harvard School of Public Health (Boston, Massachusetts) as an assistant professor. She has remained at Harvard rising rapidly through the ranks (associate professor 1986–1990; professor 1990; director of the Division of Biological Sciences 1996; director of the Malaria Initiative 1997). Presently she is Richard Pearson Strong professor and chairman, Department of Immunology and Infectious Diseases. In 1997, she was elected as president of the American Society of Tropical Medicine and Hygiene and in 2004 elected to the Institute of Medicine, National Academy of Sciences.

Dyann has developed many of the molecular genetic tools used in the investigation of malaria and leishmania. The focus of her research has been on the mechanisms of drug resistance and developing new drugs

for MDR parasites. Her group was the first to discover MDR mechanisms in *Leishmania* and *Plasmodium*. She is particularly interested in the application of genomics to drug and vaccine development. Her early research involved identification of visceral *Leishmania* spp. using cloned sequences of kinetoplast DNA, transcriptional mapping of *L. enrietti* tubulin messenger ribonucleic acids (mRNAs), detection of *Leishmania* within sand flies by kinetoplast DNA hybridization, and characterization of the MDR gene from *L. donovani*. Her work on malaria has contributed to an understanding of the sexual-stage antigens, analysed gene expression using chip technology, identified mutations in DHFR and dihydropteroate genes in African populations, analyzed *pfcrt* and *pfmdr1* polymorphisms in *P. falciparum* isolates, cloned the ribosomal ribonucleic acid (rRNA) genes of *P. lophurae*, characterized population structure and transmission dynamics of *P. vivax* in rural Amazonia, developed a high-throughput *P. falciparum* growth assay for malaria drug discovery, and characterized the *pfmdr2* gene for *P. falciparum*. Her present research involves an examination of genomic diversity in *P. falciparum*, characterization of the mechanisms by which the ATP-binding cassette transporters modulate drug resistance, as well as identification and characterization of hepatocyte molecules interacting with thrombospondin-related adhesive protein (TRAP).

In 1982, Kutner and Ginsburg working at the Hebrew University of Jerusalem began using an amino reactive reagent 4,4′-diisothiocyano 2,2′-dinitrostilbene sulphonate (DIDS; Cabantchik and Rothstein, 1972). DIDS is impermeant to normal red cells and specifically labels exofacial lysine residues of the anion transporter band 3 protein; however, Kutner, Ginsburg and co-workers found that *P. falciparum*-infected red cells (at the time when ring stages were becoming trophozoites) were highly permeable to this reagent and a variety of otherwise impermeant substances (Ginsburg *et al*., 1983, 1985; Kutner *et al*., 1982, 1983). The authors claimed the increased permselectivity to be the result of a new pathway. (In fact, the 'new pathway' was already known and had been referred to as 'leakiness'; Sherman and Tanigoshi, 1974). When the Israeli group discovered that the efflux of NBD-taurine (a substrate of the anion transporter) was very rapid in infected red cells and that this efflux was not blocked by DIDS, it was concluded that the increased permeability had a pore-like quality and that band 3 protein was probably not a constituent of the pore (Ginsburg *et al*., 1983). The positively charged pore was estimated to have a radius of 0.7 nm and as such facilitated the movement of small anions and excluded cations (Ginsburg *et al*., 1985). Selectivity by the pore was unchanged with parasite growth (Ginsburg *et al*., 1983) and pore development was asynchronous (Ginsburg *et al*., 1983; Krugliak and Ginsburg,

2006). They found other peculiarities when studying the transport of *L*-tryptophan: in human freshly sampled red cells uptake could be resolved into linear and saturable components but upon infection or storage of red cells the linear component was substantially increased whereas the K_t and maximum velocity (V_{max}) remained constant. (They also contended that the presence of parasitized red cells altered the perms-electivity of uninfected red cells.) Further, the changes in the perm-selectivity of the *P. falciparum*-infected red cells was unaffected by p-chloromercuribenzoate (PCMB) and cytochalasin B, inhibitors for glucose transport, as well as DIDS and DNDS for anion transport, but was inhibited by phloretin, a modifier of the membrane dipole potential shown to block a variety of mediated and non-mediated transport mechanisms. Phloretin also inhibited the *in vitro* growth of *P. falciparum*. This work is reviewed in Ginsburg and Kirk, 1998.

Based on the forgoing, it is apparent that at the same time the intraerythrocytic parasite begins to grow in size and its metabolic activity increases there is an increase in the permeability of the host cell. This increase in permeability has been demonstrated and in some cases characterized for several species of *Plasmodium* most notably in recent years for *in vitro* grown *P. falciparum* using a wide variety of techniques. The inhibitory effects of a range of pharmacological agents have been characterized. These include broad-specificity inhibitors of anion transport as well as quaternary ammonium compounds and a biotin derivative. The pathways have been studied using the influx and efflux of radiotracers, hemolysis of parasitized red cell suspended in iso-osmotic solutions of solutes to which the uninfected red cell is impermeable or of low permeability, and, more recently, electrophysiological (patch clamp) techniques. It has generally been assumed that these techniques, though quite different in methodology, would provide a similar determination of the character of the 'pore' and its origin. Unfortunately, this has not been the case.

Hagai Ginsburg and Ioav Cabantchik at the Hebrew University of Jerusalem and Kiaran Kirk of the Australian National University (ANU, Canberra, ACT, Australia) have characterized the NPP as a poorly selective anion channel with many characteristics of volume-sensitive anion channels. Kirk *et al*. (1994) postulated the NPP to be a single channel type. However, a recent analysis of tracer flux and iso-osmotic lysis suggests the NPP may consist of two kinds of channels: one present in low copy number per cell (around 4 copies/cell) is selective for charge and size whereas the other is more abundant (around 400 copies/cell) and allows the movement of anions (Cl^- and lactate) and nucleosides (thymidine and adenosine) (Ginsburg and Stein, 2004).

Electrophysiological evidence for the NPP is contradictory and some of the discordant conclusions certainly could stem from differences in techniques and methods used by the different groups involved in such

studies (i.e. degree and type of cell washing, presence or absence of serum, cell–cell contact, age of cells, ionic composition of the medium, type of solute, parasitemia and cell lysis; Decherf *et al*., 2004; Staines *et al*., 2007; Thomas and Lew, 2004). Desai and co-workers at the NIH contend the NPP consists of around 1000 parasite-encoded anion-selective channels that are of a single type (Alkhalil *et al*., 2004; Desai *et al*., 2000). They have named the channel the *Plasmodium* surface anion channel (PSAC) and claim that it is specifically inhibited by dantrolene (Kang *et al*., 2005; Lisk *et al*., 2006). Although no suitable candidates have turned up in the *P. falciparum* genome or in the permeome (see below), Hill *et al*. (2007) suggest that because resistance to blasticidin S was correlated with a reduction in permeability to multiple solutes, was tightly linked to defects in PSAC activity and underwent rapid reversion, there is evidence for 'changes in the expression of genes, one or more mutation hotspots or transcriptional switches'.

In contrast, the experiments from several groups support an endogenous channel. The Thomas group at the Station Biologique in Roscoff, France, using the patch clamp technique has shown three anion channels with at least two being endogenous (Bouyer *et al*., 2006). The channels could be activated in uninfected red cells by a combination of protein kinase A and ATP (Decherf *et al*., 2004; Egee *et al*., 2002) or by membrane stretch (Egee *et al*., 2002). The Huber-Lang group in Tübingen, Department of Physiology, University of Tübingen, Germany, using similar patch clamp techniques as Desai and co-workers as well as inhibitor studies, claimed there are two anion-selective channels and that these could be induced by oxidation (Duranton *et al*., 2004; Huber *et al*., 2002). Another study that did not support the hypothesis of Desai and co-workers was that of Verloo *et al*. (2004) where it was shown that *P. falciparum* developed normally in red cells from cystic fibrosis patients in which an inwardly rectifying conductance was lacking, and yet such a conductance (as measured by patch clamping of ring-infected red cells) is activated long before the appearance of the NPP.

In a recent publication, Staines *et al*. (2006) have stated that the findings of Deasai and co-workers 'are not consistent with an electroneutral solute transport occurring solely *via* a channel with low open-state probability at positive peak velocity (V_{max}) values unless the channel has an unusual gating system, whereby the channel is closed to anions but not to electroneutral solutes at high positive V_{max} values. The conductance of anions *via* either of the single-channel types proposed to form the NPP is also at odds with the total movement of anions (as measured by radiotracer techniques)…'

In an attempt to determine whether the NPP is endogenous or parasite-encoded Baumeister *et al*. (2006) studied the effects of proteases. Pre-treatment of *P. falciparum*-infected red cells with chymotrypsin (but

not trypsin) abolished NPP activity and the increased anion conductance. Although it would appear that band 3 protein (which is cleaved by chymotrypsin) might be implicated in the NPP, these investigators discounted involvement of band 3 for the following reasons: (1) pre-treatment of intact infected red cells with chymotrypsin cleaved band 3 within 2 min yet there was no effect on NPP activity until more than 10 min and (2) when chymotrypsin treatment was carried out at 22 °C band 3 was cleaved as it was at 37 °C, however, at the lower temperature the NPP and the parasite-induced conductance were unaffected. Further, cycloheximide and brefeldin inhibited the appearance of NPP in the hours following chymotrypsinization indicating that parasite viability and secretion are involved in NPP formation. Possibly, they suggest, the channels are endogenous to the red cell and are either activated and maintained by parasite-derived, chymotrypsin-sensitive auxiliary proteins exposed at the erythrocyte surface or they become chymotrypsin sensitive only once they have been activated by parasite-encoded proteins. Although these experiments tend to provide tantalizing evidence for the involvement of a parasite-encoded protein it is noteworthy that on exposure to oxidative stress uninfected red cells show properties similar to the NPP (Huber *et al*., 2002) and were the NPP to be formed in part by modified band 3 protein only a small number of molecules would need to be involved but their recruitment would still require a growing parasite to incur oxidant stress (Becker *et al*., 2004).

Kirk and co-workers, using the *P. falciparum* genome and a combination of bioinformatic approaches, have attempted to describe the full range of channels and transporters encoded by the parasite, referring to them as the parasite 'permeome' (Kirk *et al*., 2005; Martin *et al*., 2005). In this approach more than 100 putative transport proteins were identified. For the majority of these, substrate specificity and sub-cellular location remain unknown. But, as Kirk and Saliba (2007) have noted, this base can be valuable for 'as we learn more about the pathways involved in the uptake and metabolism of key nutrients by the parasite it may be possible to identify/develop combinations of inhibitors that attack these pathways at multiple sites. The rational design of such combinations will be dependent on our having a detailed knowledge of the mechanisms involved . . .'

In 1999, Bernhard Deuticke reviewed the experimental strategies that had been used in the past to form aqueous leaks and enhance bilayer PL (phospholipid) mobility in the erythrocyte and concluded that such alterations were not a model for the pathophysiological state of the -malaria-infected red cell. However, I believe, because some of these treatments (but not all) mimic the observed changes in the malaria-infected red cell they do provide clues to the dynamic changes that are probably occurring during the intraerythrocytic growth of the *Plasmodium*. True, no single perturbation has produced all of the permeability properties of a

malaria-infected red cell, but this is understandable when one considers that the NPP of the infected cell is a product of a cascade of subtle changes that influence the anion transporter (and perhaps chloride channels) such as changes in PL asymmetry, altered levels of membrane cholesterol, oxidation, changes in intracellular pH as well as levels of calcium and sodium ions and so on. Indeed, I would venture to guess that the NPP results from the modification of endogenous transporters and not by insertion of a parasite-encoded protein.

Kiaran Kirk (1961–) did his PhD in the Department of Biochemistry at the University of Sydney (1985–1988) with Philip Kuchel using nuclear magnetic resonance (NMR) spectroscopy to study the physiological properties of erythrocytes. Upon completion of the doctorate he took up an Oxford Nuffield Medical Fellowship and in January 1989 joined Clive Ellory's laboratory in the Oxford University Laboratory of Physiology (Oxford, United Kingdom) as a post-doctoral fellow. He spent 2 years studying the physiology of erythrocytes from the lamprey (an ancient, jawless, blood-sucking fish) before a catastrophic power cut resulted in the demise of his lampreys and left him without a project.

Aware of the work that Barry Elford and others had done on the altered transport properties of *P. falciparum*-infected human erythrocytes he cycled up the hill from the Physiology Department to the Institute of Molecular Medicine (IMM, Oxford, United Kingdom) to begin a collaboration with Elford and Chris Newbold. For the next few years Elford, Newbold, Ellory and Kirk collaborated: Elford grew the parasites and each week, with characteristic generosity, delivered a few hundred microliters of parasitized cells to the physiology laboratory, Kirk would do the experiments, then cycle up the hill to the IMM to do the subsequent analyses. They published a number of papers on different aspects of the increased membrane permeability of falciparum-infected erythrocytes. In 1993, Kirk was awarded a Lister Institute Research Fellowship and was joined first by a research assistant, Heather Horner who set up and oversaw a small malaria parasite culture laboratory in the Oxford Physiology Department, and then by Henry Staines who carried out his PhD on the increased cation permeability of infected erythrocytes. In 1996, Kirk took up the chair of biochemistry at the Australian National University (ANU, Canberra, ACT, Australia). There he established a new laboratory focused primarily on the cell and molecular physiology of the intraerythrocytic parasite itself.

CHAPTER 19

Hemoglobinases

Members of the genus *Plasmodium* are obligate intra-erythrocytic parasites. Hemoglobin, the predominant soluble cytoplasmic protein of the erythrocyte, can be an abundant and vital nutrient source for the plasmodia when they are able to degrade and assimilate the resultant peptides and amino acids. This being so it would seem, a priori, that plasmodial hemoglobinases would be suitable targets for potential anti-malarials.

Electron microscopic studies have shown that the organelle responsible for the ingestion of host cell cytoplasm is the cytostome. Cytostomal feeding has been established in all malarias studied and a single parasite may have several cytostomes. The process is: a bolus of engulfed erythrocyte cytoplasm containing a portion of the parasitophorous vacuolar membrane (PVM) is budded off from the wall of the enlarged cytostomal cavity and the cytostomal opening is sealed off by a membrane, together with the cytostomal wall so that 'transport vesicles' with a double membrane are formed. Fusion of these vesicles with the parasite food vacuole (FV) results in an enlarged digestive vacuole (Aikawa, 1988; Aikawa *et al*., 1966; Slomianny, 1990). Malaria pigment can be seen in the transport vesicles coincident with the disappearance of one of the membranes as well as in the FV. The lysosomal markers, acid phosphatase, aminopeptidase and arylamidase, were found in the FVs of *Plasmodium chabaudi* (Slomianny *et al*., 1983) but not in the isolated FVs of *P. falciparum* (Goldberg *et al*., 1990). A highly acidic pH will denature hemoglobin and release heme, however, at the pH of the FV this is a very slow process (Gabay and Ginsburg, 1993; Gamboa de Dominguez and Rosenthal, 1996) and it appears that protease activity is required to separate the heme from globin in the FV. It has been suggested that proteolysis may begin prior to heme release (Kamchonwongpaisan *et al*., 1997).

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Although Brown (1911) suggested that the parasites degrade hemoglobin by the action of proteolytic enzymes to produce malaria pigment it was not until 35 years later that Moulder and Evans (1946) found such an enzyme in extracts of *P. gallinaceum*: an acid protease. Later, Cook, Grant and Kermack (1961) found evidence for two proteases in *P. berghei* and *P. knowlesi* that degraded denatured hemoglobin and the work on *P. knowlesi* was subsequently confirmed by Cook *et al*. (1969) at the Walter Reed Army Institute of Research (WRAIR). Levy and co-workers (Levy and Chou, 1973, 1975; Levy *et al*., 1974) found a pepstatin-inhibitable acid protease in *P. berghei*, *P. knowlesi* and *P. falciparum*. A 43-kDa protease from *P. lophurae*, inhibited by pepstatin, was purified by Sherman and Tanigoshi (1981, 1983a) and this cathepsin D-like protease degraded denatured hemoglobin as well as high-molecular-weight membrane proteins. After 1976, and with the successful continuous *in vitro* culture of *P. falciparum* (Trager and Jensen, 1976) it became possible to study its proteases. In 1982, Gyang, Poole and Trager described an acid protease that degraded hemoglobin optimally at pH 3.5, was inhibited by pepstatin and had an exceptionally high molecular weight (148,000). Two other peptidases had optimal activity at neutral and alkaline pH; the former was suggested to be an aminopeptidase and the latter an endopeptidase. Although these workers claimed that 'inhibition of host cell hemoglobin may explain, in part, the action of anti-malarial drugs' it is unlikely that this is so since the concentrations of amodiaquine, primaquine and chloroquine were non-physiological and greater than 25 mM. During the 1980s, David VanderJagt's group at the School of Medicine, University of New Mexico (Albuquerque, New Mexico) using conventional biochemical methods, purified and characterized a pepstatin-inhibitable, protease of less than 10 kDa that degraded denatured hemoglobin optimally at pH 4.5 and was inhibited by ferriprotoporphyrin (FP) and a chloroquine-ferriprotoporphyrin (CQ-FP) (Vander Jagt *et al*., 1986), three hemoglobin-degrading aspartic proteases (Vander Jagt *et al*., 1992), a 100-kDa aminopeptidase inhibited by bestatin, chloroquine, mefloquine and quinacrine, but not leupeptin, pepstatin, quinine or primaquine (Vander Jagt *et al*., 1984) and compared the proteases from chloroquine-resistant and -sensitive strains concluding that resistance was not the result of changes in parasite proteases (Vander Jagt *et al*., 1987).

David Vander Jagt (1942–) received his doctor of philosophy (PhD) in 1967 in synthetic/mechanistic organic chemistry at Purdue University (West Lafayette, Indiana), in the laboratory of the Nobel Laureate H. C. Brown. After Purdue, he took a post-doctoral position at Northwestern University (Chicago, Illinois) in the laboratory of M. L. Bender in bioorganic chemistry/enzymology (1967–1969). When the opportunity came for a joint appointment in the Department of Biochemistry,

University of New Mexico School of Medicine (Albuquerque, New Mexico), and the Department of Chemistry, University of New Mexico (Albuquerque, New Mexico), he seized it not knowing that he would be teaching metabolism to medical students for the next 37 years.

Though the 1970s, Vander Jagt's research was at the chemistry/biochemistry interface and involved synthetic chemistry and enzymology, with a focus on detoxification enzymes and chemical carcinogens. He fully expected to continue with the same research themes, however in 1980, quite by accident, Phil Reyes, also an enzymologist in the Department of Biochemistry, and Vander Jagt began to attend seminars at the United States Agency for International Development (USAID)-funded malaria vaccine project at the university, mainly because they knew some of the people, including Karl Rieckmann, the director. One day, Reyes and Vander Jagt were given a minute pellet of isolated trophozoites of *P. falciparum* to see what enzymology they could carry out using such a tiny sample, at least they were tiny compared with the tissue samples normally used by biochemists for isolation of enzymes of interest! Soon they were hooked on studying the biochemistry of *P. falciparum*, a project that continued for more than 15 years, becoming an important part of their academic lives. Vander Jagt still studies the enzymology and drug-binding properties of parasite lactate dehydrogenase (LDH), in collaboration with Michael Makler, Rob Piper and John Dame.

In 1990, Schrevel *et al*. were able to record the presence of a variety of acid aspartic and cysteine proteases and metalloproteases, as well as alkaline proteases and aminopeptidases in the erythrocytic stages of a variety of *Plasmodium* spp. Unfortunately, since many of the enzymes catalogued were only partially purified, and sometimes appeared aggregated, their biochemical characteristics remain ill defined at best. More recent findings for plasmodial enzymes—principally cysteine, aspartic and metalloproteases—can be found in Rosenthal (2005) and in the *Plasmodium* database PlasmoDB (http://plasmodb.org) as well as in the website developed and curated by Hagai Ginsburg (http://sites.huji.ac.il/malaria).

Analysis of the *P. falciparum* genome suggests the number of putative proteases to be as high as 92 (Rosenthal, 2005). Most of these protease genes are transcribed, however, in many cases not only have the biochemical properties of the gene products not been characterized, but it is becoming clear that not all are hemoglobinases. Of the 10 aspartic proteases, called plasmepsins, the best-characterized plasmepsins (I, II and IV as well as histoaspartic proteinase (HAP)) are optimally active at acid pH, work best against denatured hemoglobin, are inhibited by pepstatin and are made as proenzymes, requiring cleavage (by a convertase) to produce an active

enzyme (Goldberg, 2005). (Plasmepsin V may remain unprocessed.) Hemoglobin digestion may occur *via* a semi-ordered process beginning with plasmepsins I and II that produce the first cleavage of the Phe^{33}–Leu^{34} bond in the hinge region of the α chain of globin causing the molecule to unravel. Genome sequencing and sub-cellular localization to the digestive FV have identified HAP, which is also believed to play a role in hemoglobin degradation (Klemba *et al*., 2004). The HAP has been expressed and partially characterized: it is inhibited by pepstatin A and able to hydrolyze acid-denatured globin (Xiao *et al*., 2006). The genes encoding the four FV plasmepsins (I, II, IV and HAP) occur in a tandem array on chromosome 14 and plasmepsins I and II show approximately 60% sequence identity to IV (Rosenthal, 2005 and http://www.plasmodb.org; last accessed 16 July 2008). Recent gene disruption studies showed that a knockout of any single plasmepsin gene was not lethal although some knockouts had diminished growth rates. With a knockout of plasmepsin II there was abnormal morphology of the mitochondrion (Omara-Opyene *et al*., 2004). John Dame's laboratory has shown that knockout of plasmepsins I, II and III leaves the parasites unaffected. In addition, although a knockout of all four plasmepsins resulted in abnormal parasites they did not die. The knockout data suggest redundancy in plasmepsin function in the digestive FV, however, since not all functions of plasmepsin IV could be compensated by other plasmepsins or proteinases this plasmepsin may be a critical one (Bonilla *et al*., 2007a,b). Since pepstatin gets into the FV and inhibits all four FV plasmepsins at picomolar concentrations, and kills falcipain-2 knockout parasites but not wild-type parasites, it suggests that inhibitors of FV plasmepsins are unlikely to be useful on their own (Goldberg personal communication; Liu *et al*., 2006). Despite this, some investigators continue to be optimistic that plasmepsins could become successful anti-malarial targets when increased selectivity over human cathepsin D as well as increased activity in infected red cells can be achieved (Esmark *et al*., 2006).

John B. Dame (1949–) was born in Winterhaven (Florida), he joined the faculty at the University of Florida (Gainesville, Florida), College of Veterinary Medicine in 1986, and is currently professor and chairman. Although his bachelor of science (BS) (1971) at Florida State University (Tallahassee, Florida) was in chemistry he found he liked biology better. Enrolling at the University of Washington (Seattle, Washington) he trained as a biochemist with an emphasis on membrane biochemistry and *Escherichia coli* genetics receiving his PhD (1977) for research on '*Membrane-Altered Mutants of* E. coli.' After a very challenging, but ultimately highly successful, time as a post-doctoral student at the University of North Carolina (Chapel Hill, North Carolina) in the

Department of Pharmacology (1977–1980) where he characterized the first proton-pumping ATPase with a high-energy phosphate intermediate, he determined his career would be more rewarding working on a problem with a more direct application to the health and welfare of humankind. An opportunity came in the form of an advertisement in *Science* magazine. Louis H. Miller, head of the Malaria Section of the Laboratory of Parasitic Diseases at National Institutes of Health (NIH, Bethesda, Maryland), was looking for a membrane biochemist to work as a staff fellow to characterize the membrane proteins recognized as antigens of the human malaria parasite. Joining Miller's NIH group in 1980, he found a new opportunity to bring to bear earlier training in *E. coli* genetics by working on the molecular biology of *Plasmodium* with another new hire from the National Cancer Institute (Bethesda, Maryland), Tom McCutchan. During their time together at NIH (1980–1984) they were among the first to prepare deoxyribonucleic acid (DNA) libraries from *Plasmodium* and described a novel cloning method, the mung bean nuclease digestion method, to prepare intact genes for expression cloning (and that worked well for those free of introns). At the NIH, the gene encoding the circumsporozoite protein from *P. falciparum* was cloned and stage-specific ribosomes were discovered. With only a few temporary diversions, none of which ultimately ever had the same appeal, he has tried to contribute to pre-clinical vaccine or pre-clinical drug development for malaria since 1980. In 1991, he embarked on a 'genes-first' approach to large-scale sequencing of the *P. falciparum* genome to identify genes encoding potential vaccine and drug targets based upon DNA sequence tags. Utilizing the mung bean nuclease cloning method, clones were prepared for random sequencing and catalogued and their putative identities based upon sequence comparisons with known sequences in GenBank. Although investigators at The Institute for Genomic Research (TIGR) and Sanger Centre (Cambridge, United Kingdom) came to be recognized as better prepared to complete the genome sequence, that did not occur before Dame had identified several putative drug targets. The seventy-fifth clone analyzed in the project was what is now known as plasmepsin 2. Since 1993, he has been working on this gene and other members of this gene family.The *P. falciparum* genome sequencing project uncovered genes encoding 10 plasmepsins, four of which were localized to the digestive vacuole, including plasmepsin 2. Dame and co-workers have now simultaneously knocked out all four genes encoding the plasmepsins and found that the parasite is attenuated with impaired vacuole function, and requires exogenous amino acids for growth. These findings suggest a new role for these 'hemoglobinases' in digestive vacuole function and provide new insights on the functional properties of plasmepsins.

A total of 33 cysteine protease genes were identified in the *P. falciparum* genome (Rosenthal, 2005). Four, called falcipains, are typical members of the papain family and are inhibited by E-64 and other cysteine protease inhibitors. The genes for falcipain-2 and -3 are found on chromosome 11 whereas falcipain-1 is on chromosome 14. The discovery of falcipain activity in *P. falciparum* was serendipitous. Philip Rosenthal, within the first month of being in James Leech's laboratory at the University of California, San Francisco (San Francisco, California), made a dramatic finding while trying to show the effects of leupeptin on erythrocyte rupture and invasion. He observed the trophozoites to be odd-looking with FVs swollen and filled with undegraded hemoglobin. Indeed, it has since been shown that when falcipain activity is blocked by cysteine protease inhibitors (such as leupeptin) swollen FVs with undegraded hemoglobin result; however, in spite of this clear block in hemoglobin degradation, *P. falciparum* survive if the inhibitor is subsequently removed from the culture medium.

Philip Rosenthal (1956–) was born in Poughkeepsie, NY, received undergraduate training in biochemistry at the State University of New York (SUNY, Stony Brook, New York) (BS 1978), training in Medicine at New York University (NYU, New York, New York) (doctor of medicine, MD in 1982) and clinical training in Internal Medicine at the University of Michigan (Ann Arbor, Michigan). In 1985, he went to the University of California, San Francisco for a fellowship in infectious diseases, and soon thereafter began working with James Leech, one of the first scientists to begin to unravel the mechanism of cytoadherence by *P. falciparum*-infected erythrocytes. Leech suggested a new project, to study proteases of malaria parasites, based in part on work he had heard presented at University of California, San Francisco by James McKerrow, who continues to study proteases of a number of protozoans and helminths. Studies of proteases were initially directed towards dissecting mechanisms of erythrocyte rupture and invasion (which remain rather poorly described to this day), but a seminal observation after 1 month in the laboratory was that cysteine protease inhibitors caused trophozoites to develop an abnormal morphology, with a swollen, dark-staining FV, indicating a block in hemoglobin hydrolysis. Work since those early days has involved the characterization of a family of cysteine protease hemoglobinases and, in collaboration with chemistry groups, attempts to develop cysteine protease inhibitors as anti-malarial drugs. More recently, another direction has been the conduct of clinical trials of anti-malarial efficacy of leading new drugs in Africa and extensive translational studies utilizing samples from the clinical trials to characterize the molecular epidemiology of malaria better.

The dominant cysteine protease in trophozoites, the stage at which most hemoglobin degradation takes place, is falcipain-2 (Shenai *et al*., 2000). Falcipain-3 is biochemically very similar to falcipain-2, and is expressed later in the life cycle. Falcipain-1, the first falcipain discovered, is also expressed in erythrocytic parasites, but has an uncertain role. Falcipain-2′ is a near identical copy of falcipain-2. Gene disruption studies have been very informative regarding the roles of falcipains. A knockout of falcipain-1 has no apparent phenotype in erythrocytic parasites, although in sexual-stage parasites oocyst production was decreased (Eksi *et al*., 2004; Sijwali *et al*., 2004). A knockout of falcipain-2 caused trophozoites to develop swollen, hemoglobin-filled FVs, as seen with cysteine protease inhibitors, confirming a role for falcipain-2 in hemoglobin hydrolysis (Sijwali and Rosenthal, 2004). However, falcipain-2 knockout parasites regain normal morphology later in the life cycle, and replicate at normal levels. The gene for falcipain-3 could not be disrupted, although it could be readily replaced by a tagged copy, strongly suggesting that it has an essential function (Sijwali *et al*., 2006). A knockout of falcipain-2′ had no obvious phenotype. Taking the available data together, it appears that falcipain-2 and falcipain-3 play key roles in hemoglobin degradation by erythrocytic parasites. As these enzymes are biochemically very similar, inhibition of both enzymes offers a potential strategy for anti-malarial chemotherapy. Indeed, a major project to develop falcipain inhibitors as anti-malarial drugs is underway through collaboration with GlaxoSmithKline and funded by Medicines for Malaria Venture (MMV, Geneva, Switzerland) (see p. 19).

Falcilysin, a zinc metalloprotease, does not cleave native or denatured hemoglobin but is active against different peptides at acidic and neutral pH, and has an additional distribution outside the FV (Murata and Goldberg, 2003a,b). The inability to knockout falcilysin (which accumulates in the apicoplast and mitochondrion where it is presumed to destroy targeting peptides) suggests it is important for parasite growth (Ponpuak *et al*., 2007; Ralph, 2007). Aminopeptidases are responsible for the additional cleavages of globin peptides: a methionine metalloaminopeptidase (PfMetAP1b) and a dipeptidylaminopeptidase (DPAP). Knockouts of the DPAP were unsuccessful (Klemba *et al*., 2004). In screening 175,000 compounds, several inhibitors of the PFMetAP1b were found, suggesting that DPAP may be a suitable chemotherapeutic target (Chen *et al*., 2006).

Daniel Goldberg (1957–) did his undergraduate training at Harvard University (Cambridge, Massachusetts) where he studied biochemistry with Eugene Kennedy. He then performed doctoral work with Stuart Kornfeld and received an doctor of medicine (MD)/PhD degree from

Washington University in St. Louis in 1985. After a residency in internal medicine at the Brigham and Women's Hospital in Boston (Massachusetts; 1985–1987) and an infectious diseases fellowship at Washington University (1987–1988), he did post-doctoral research with Anthony Cerami at Rockefeller University (New York, New York) (1988–1990) before joining the faculty at Washington University in 1990. He is presently professor in the Departments of Medicine and Molecular Microbiology as well as a Howard Hughes Medical Institute (Chevy Chase, Maryland) investigator. He is co-chief of the Division of Infectious Diseases and director of the Medical Scientist Training Program. Goldberg first became interested in malaria when, as a graduate student working on glycoproteins, he read an article by Margaret Perkins (who was then in the laboratory of William Trager) on the recognition of glycophorins, the major erythrocyte sialoglycoproteins, by malaria parasites during invasion. Intrigued by this (and it continues to be a very interesting interaction) he read more about malaria and realized that although this was one of the world's most important diseases, there was remarkably little biochemistry being done on the subject. Beginning in 1988, he focused his attention on malaria pigment, its mode of formation and the enzymes involved in the degradation of hemoglobin (Goldberg *et al*., 1990).

Although knockouts of the plasmepsins and falcipain suggest considerable redundancy in hemoglobinase activity, both aspartic and cysteine protease inhibitors do kill parasites. The aspartic protease inhibitors may be effective as anti-malarials by inhibiting processes other than the degradation of hemoglobin. Cysteine protease inhibitors, proven blockers of hemoglobin hydrolysis, can be inhibited by an array of low-molecular-weight molecules that are effective at the nanomolar range in a mouse model for *P. falciparum*. Molecules with the ability to inhibit both plasmepsins and falcipains might be ideal as anti-malarials since, in addition to their synergistic action, the probability of developing resistance would be reduced. Hundreds of compounds targeting plasmepsins and falcipains have been synthesized (Deasai *et al*., 2006; Esmark *et al*., 2006; Micale *et al*., 2006) and since some have shown efficacy there is hope that in the near term a selective chemotherapeutic will emerge.

It is generally accepted that as much as 75% of the hemoglobin in a malaria parasitized red cell may be degraded. Therefore, it is reasonable to ask the question: why do malaria parasites degrade hemoglobin? Three (non-mutually exclusive) hypotheses have been proposed to: (1) obtain amino acids for protein synthesis (Divo *et al*., 1985; Liu *et al*., 2006);

(2) provide sufficient space for the growing *Plasmodium* (Krugliak *et al.*, 2002) and (3) maintain osmotic balance and prevent lysis of infected red blood cells (Lew *et al.*, 2004). My inclination is to be skeptical of the last two and to favor the first hypothesis for which there is ample experimental evidence.

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CHAPTER 20

Erythrocyte Surface Membrane Proteins

Contents

1. THE ROAD TO PfEMP1

During the 1940s and 1950s the focus of malaria biochemistry was on the 'bag of enzymes' of *Plasmodium*. Characterizing the metabolic pathways—energy yielding, synthetic, and degradative—and how these might be affected by anti-malarials was under active investigation (see Chapter 3, p. 9). The findings, in general, conformed to the 'Einheit' of biochemistry that was being described for free-living organisms. As a consequence, there was little grist for the drug designers' mill(s) and the *raison d' être* for intracellular parasitism remained obscure. The 1960s ushered in a new biochemical era. The structure of deoxyribonucleic acid (DNA), its mode of replication, transcription and translation were now known, specific metabolites—labeled with radioisotopes—could be obtained from commercial sources, and most laboratories were able to use separation methods such as ultracentrifugation, ion-exchange chromatography and gel electrophoresis; there were sensitive ultraviolet spectrophotometers and fluorimeters and

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many laboratories had access to transmission and scanning-electron microscopes. Enzyme heterogeneity was routinely demonstrable in the tissues of an organism as well as between species and the genetic differences between and among species could be described in molecular terms. In concert, the new analytical techniques and changes in the concept of cell structure and function would open up the possibility for rational drug design and evaluation of novel chemotherapeutic targets.

By the 1970s, biochemical investigations on a variety of eukaryotic cells began to focus on the 'bag' itself—the plasma membrane—that contained the unique matrix and sub-cellular organelles. The Singer-Nicholson fluid mosaic model for membrane structure (proposed in 1972), which visualized protein icebergs floating in a lipid bilayer sea and in which the inner and outer layers differed from one another, held sway. Radiolabeled molecules and techniques for tagging specific surface components were at hand. Proteins could be solubilized in ionic or non-ionic detergents and then separated from one another by polyacrylamide gel electrophoresis (PAGE); sub-cellular fractions could be isolated by density-gradient centrifugation and the purity of each fraction could be monitored visually using electron microscopy, enzyme markers, or reactivity with antibody or chemical reagents. The elucidation of the molecular architecture of membranes was advancing and the findings were beginning to be applied to *Plasmodium*. In 1979, Wallach summarized a Special Program for Research and Training in Tropical Diseases (TDR, Geneva, Switzerland) sponsored workshop 'The Membrane Pathobiology of Tropical Diseases' noting that membrane biologists, biophysicists, biochemists and immunologists had been brought together so that the mode of action of anti-parasitic drugs and membrane-bound antigens could be better understood and contribute to serodiagnosis and immunoprophylaxis. He wrote, 'all this is possible because the major steps in the fractionation of the host cell (erythrocyte) and parasite antigens in a model monkey malaria have been achieved; parasite modification of host cell membranes can be analyzed both biochemically and immunochemically and the system allows rigorous biochemical analysis of parasite, parasite induced and host cell modified membrane lipids and proteins.' Indeed, with the possibility for the isolation and characterization of antigens came the prospect for the development of a vaccine, and as a consequence support from a variety of funding agencies soon became available. This investment in funding studies for protective vaccines continues into the present (see Chapter 4). Early work on membrane transport in *Plasmodium*-infected erythrocytes (see Chapter 18, p. 151) had clearly demonstrated that the surface of the host cell could be altered functionally. Therefore, by the 1970s, it became important to know more about the composition and architecture of this interface, the site through which nutrients and drugs first pass and the contact point between the immune

system and the invasive stages (merozoite, sporozoite). What was of particular interest was: (1) how did protein organization of the red cell plasma membrane change upon infection, (2) how might this contribute to the altered functional properties of the parasitized cell and (3) how were the membrane components of the red cell and the parasite related to one another? One of the earliest biochemical characterizations of plasmodial membranes was that of the *Plasmodium berghei*-infected red blood cell membrane by D.H. Wallach (1926–) and colleagues at the Tufts Medical Center (Boston, Massachusetts) (Weidekamm *et al*., 1973) and later the group would extend their studies to *P. knowlesi* (Schmidt-Ullrich and Wallach, 1978).

In 1976, my own laboratory embarked on biochemical studies of plasmodial and red cell membranes. Together with Yuzo Takahashi, an electron microscopist from Japan, and a post-doctoral student in the laboratory, we were able to differentiate *Plasmodium* and host cell membranes. Using cationized ferritin and ferritin-conjugated lectins distinct differences between the *P. lophurae*-infected red cell membrane, the parasite plasma membrane (PPM) and the parasitophorous vacuolar membrane (PVM) were observed using the transmission electron microscope (Takahashi and Sherman, 1978). These studies followed earlier electron microscopic studies (Aikawa *et al*., 1969; Seed and Kreier, 1976; Seed *et al*., 1973) some of which were carried out at the Walter Reed Army Institute of Research (WRAIR) and supported by the United States Army funds. Although the morphological studies indicated that there were architectural/compositional differences in these membranes, there remained a need to actually isolate the membranes in quantities such that they could be subjected to biochemical analysis.

In reviews written by Sherman (1979, 1985) and Trigg (1988) attempts were made to summarize the confusing and contradictory literature on the surface membranes of *Plasmodium*-infected red cells, most of which concerned themselves with the popular simian and rodent model malarias. In brief, some studies with *P. berghei*, *P. chabaudi*, *P. yoelii* and *P. knowlesi* showed degradation of the cytoskeletal protein spectrin (bands 1 and 2 on sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE)), and others showed modifications of glycoproteins through periodic acid-Schiff stain (PAS)-stained gels as well as by surface labelling using radioisotopes (summarized by Sherman (1985) and Trigg (1988). Some biosynthetic labelling studies showed incorporation of radiolabeled glucosamine into *P. knowlesi*-infected red cells, however, because of increased permeability and high metabolic rate, normally impermeant probes and radiotracers entered infected cells and radioactivity tended to be associated with the parasite and not the surface membrane of the red cell alone. One of the best-characterized surface glycoproteins of *P. knowlesi*-infected red cells (i.e. a 74 kDa

antigen; Schmidt-Ullrich and Wallach, 1978; Schmidt-Ullrich *et al*., 1983) was dismissed by David *et al*. (1984) as being due to contamination or uncontrolled proteolysis. Some studies using radioiodination showed an overall similarity in surface protein profiles whereas others did not. For example, when Shakespeare *et al*. (1979b) compared the patterns obtained by metabolic labelling or iodination they could find no major differences between normal and *P. knowlesi*-infected red cells, whereas Wallach and Conley (1977) and Howard (1984) found at least eight proteins, some of which they considered to be new surface antigens and some of which appeared to be related to the anion transporter band 3 protein. Russell J. Howard, working at Walter and Eliza Hall Institute (WEHI) and using several protein and sugar-labelling probes (e.g. lactoperoxidase catalysed radioiodination of tyrosyl residues, periodate oxidation and tritiated sodium borohydride reduction of sialic acid and oxidation of galactosyl/*N*-acetylgalactosaminyl residues by galactose oxidase with subsequent tritiated borohydride reduction) was able to identify several new proteins and glycoproteins on the surface of *P. berghei*- and *P. yoelii*-infected mouse red cells (Howard *et al*., 1980). In contrast, using similar labelling techniques, no changes were found in the membranes of *P. lophurae*-infected red cells (Sherman and Jones, 1979). The inconsistencies could be due to differences in species but more likely were the result of differing experimental conditions. These contradictory findings have never been adequately resolved.

Plasmodia were found to neither contain sialic acid nor were they capable of its synthesis (Schauer *et al*., 1984). Hence, when declines in sialic acid were found in *P. berghei*, *P. knowlesi* and *P. yoelii* it was assumed they were due to changes either in the quality or quantity of the host sialic acids (Howard and Day, 1981; Howard *et al*., 1980, 1986). It was claimed that the decreased reactivity of surface sialic acid for neuraminidase in murine and simian malaria-infected red cells was due to *O*-acetylation. However, they found little or no decrease in *P. falciparum*-infected red cells (Howard *et al*., 1981) and concluded that extensive removal or modification of sialic acid did not occur with human malaria, in contrast to the murine malaria. I believe their results can be explained quite simply: the samples contained too few parasitized erythrocytes (i.e. parasitemias were low and most of the parasites were small, immature trophozoites). Indeed, when we re-investigated the sialic acids in *P. lophurae*-infected red cells where the parasitemias were above 80% (Sherman and Jones, 1979) there was a significant reduction (from 79 nmol/mg protein to 36 nmol/mg protein) and this was also found in *in vitro* grown *P. falciparum* (Sherman, personal communication) and *P. vivax* (reference in Sherman *et al*., 2004). Although we still do not understand the mechanism for loss of sialic acid from malaria-infected red cells I am of the opinion that the lowered amount of surface sialic acid is both real and significant.

A reduction in surface sialic acid would lower the overall surface charge on the infected red cell thereby decreasing the repulsive forces between cells and promoting adhesion (i.e. between trophozoite/schizont-infected red cells and endothelial cells). In addition, for those malarias in which glycophorin is a receptor (see p. 242) the reduced amounts of sialic acid could prevent merozoite invasion into a red cell already containing a growing parasite.

During the period 1970–1984 there were several nagging questions: were these alterations in surface membrane proteins real and where discrepancies existed to what were they due? In one review (Sherman, 1985) I sounded a cautionary note, 'in most of the reported studies there has not been a separation of host cell from parasite membranes nor has there been an attempt to control protease activity' to which I should have added: the techniques suitable for surface labelling of uninfected red cells may not be directly applicable to infected red cells because of their fragility and, if proper precautions have not been taken, the conclusions drawn may be erroneous. Further, the absence of a membrane constituent could result from insensitivity in the analytic technique as well as too low a parasitemia, whereas a striking alteration may be due (as Trigg noted in his 1988 review to 'the greater pathogenicity of laboratory infections and their rapid increase in parasitemia compared with human infections.'

After 1976, with the availability of the Trager-Jensen method for the continuous culture of *P. falciparum*, studies of the red-cell surface membrane, especially those contributing to changes in antigenicity, could be carried out on what some investigators and funding agencies referred to as 'a real malaria'. As a consequence, work on rodent, simian and avian malarias diminished significantly. Trager assembled a splendid team of co-workers at Rockefeller University (New York, New York), including the electron microscopists Maria Rudzinska, Phyllis Bradbury and Susan Langreth, biochemists such as Araxie Kilejian and Margaret Perkins, and the immunologist Robert Reese; at the National Institutes of Health (NIH, Bethesda, Maryland), Louis Miller had in his laboratory Russell J. Howard, Peter David, James Leech, Stephen Aley and John Barnwell; WRAIR had the electron microscopist Masamichi Aikawa, and in the United Kingdom there were the electron microscopists Diane McLaren, Lawrence Bannister and biochemists/immunologists Chris Newbold, Iain Wilson and Neil Brown. During the summer of 1977, I learned much about membrane isolation and characterization by sharing a laboratory at the Marine Biological Laboratory (MBL; Woods Hole, Massachusetts) with Leonard Warren (Wistar Institute) who was a bona fide membrane biochemist. The research we conducted during that summer was on isolated red cell membranes of dogfish since at the time the MBL did not permit investigators to work on terrestrial creatures such as ducks and rodents or to use rabbits to prepare antisera (Warren *et al*.,

1979). However, the methods used for characterization of the dogfish red cell membranes proved to be of value when we began to study *P. lophurae* membranes in earnest (Sherman and Jones, 1979). In addition, for the reasons given above, my own laboratory largely abandoned research on *P. lophurae* by the early 1980s and focussed on the structural and functional properties of the *P. falciparum*-infected red cell. In this work, Jean Gruenberg, David Allred, Ian Crandall, Neil Guthrie, Enrique Winograd, Patricia Maguire and Shigetoshi Eda joined my laboratory. Our early work on the red cell membrane from falciparum-infected red cells indicated a lack of insertion of plasmodial-encoded proteins (Allred and Sherman, 1983; Allred *et al*., 1986; Gruenberg and Sherman, 1983; Gruenberg *et al*., 1983; Sherman and Tanigoshi, 1983b), however, this was not the case with investigations from other laboratories. Indeed, Araxie Kilejian working in Trager's laboratory at Rockefeller University showed incorporation of radioactive glucosamine into *P. falciparum*-infected red cells bearing schizonts (Kilejian, 1980) and Margaret Perkins, also working at Rockefeller, found eight labeled proteins that were sensitive to pronase suggesting they were on the outer surface of the erythrocyte (Perkins, 1982); in contrast, Howard *et al*. (1984) were unable to find any on the red-cell surface and concluded the labeled glycoproteins were associated with trophozoites.

Identification of the antigenic surface proteins of *P. falciparum* can be traced to studies carried out by Eaton in the 1930s (reviewed in Kyes *et al*., 2001). Eaton showed that serum from monkeys infected with *P. knowlesi* could agglutinate schizont-infected red cells. This schizont-infected cell agglutination (SICA) reaction clearly showed that antigenicity of the infected red cell was altered by the presence of the parasite. Since the SICA reaction was species and stage specific it implied (but did not prove) that the reaction was due to the presence of a parasite antigen on the red-cell surface. Eaton went on to show that there was serial expression of SICA and immunity was variant-specific. Thirty years later, Neil Brown and colleagues at the National Institute for Medical Research (NIMR, Mill Hill, London, United Kingdom) explored this phenomenon of antigenic variation, and were able to show that recrudescent parasites from a single inoculum differed in their SICA antigens (Brown and Brown, 1965). Since Eaton and Brown and colleagues worked with uncloned parasites, it was conceivable that these findings were the result of immune selection of genetically (and antigenically) distinct populations. However, Barnwell and co-workers (1983) repeated these experiments with cloned lines of *P. knowlesi* and proved that the parasite, within its haploid genome, had the genetic capacity to express successive antigenic variants. Using *in vitro* metabolic labelling and immunoprecipitation, the SICA antigens were described as high-molecular-weight, polymorphic, parasite-encoded proteins uniformly distributed on the surface of the red cell. The SICA antigens were trypsin sensitive,

accessible to surface iodination and insoluble in non-ionic detergents (i.e. Triton X-100; Howard *et al.*, 1982a, 1983). Serial passage of cloned organisms in splenectomized monkeys gave rise to parasite lines that no longer expressed SICA, however, on transfer back into spleen-intact animals the antigen was re-expressed (Barnwell *et al.*, 1982). Following the identification of the *P. knowlesi*-SICA antigens similar studies were conducted to identify whether similar surface antigens were present in *P. falciparum*.

Mary R. Galinski (1957–) began her scientific career with a long-standing interest in developing nations, global health and economic development. She was first interested in solving food shortage problems, influenced by her mother (a dietician), yet her academic interests, as well as her father's influence towards pursuing medicine and business, led instead to a career in malaria research. As an undergraduate at Binghamton University (New York, New York; bachelor of science, BS; 1979) she worked summers as a volunteer in Mercy Hospital's (Portland, Maine) Emergency Room and conducted research on vitamin D metabolism with James K. Yeh at Nassau County Medical Center (East Meadow, New York). In 1980, a college friend directed her to the big time, New York City, for a research position with Chris D. Platsoucas studying basic cancer immunology and biological response modifiers at Memorial Sloan Kettering Cancer Institute (New York, New York). There, Galinski realized her passion was for research, including all the long hours, waiting for those precious moments when experiments yielded gratification. Platsoucas instilled a drive to succeed in research and provided great training for multi-tasking and seeing the bigger picture. Monoclonal antibodies and investigations of interferon (IFN) were all new then! There was also excitement studying the first few known immunological cell determinants using OKT3, OKT4 and OKT8 monoclonal antibodies. Graduate school called in 1981, and with a full scholarship offered by the Sackler Institute of Graduate Biomedical Sciences doctoral program at New York University (NYU, New York, New York) Medical Center, Galinski began her journey away from cancer research and on to malaria. She was guided by Ruth and Victor Nussenzweig to join Enzo Enea's laboratory, where she gained respect for malaria genetics and evolution in the days before automated DNA sequencing or molecular biology kits existed. The first malaria gene had just been cloned, the circumsporozoite surface protein (CS) gene. In 1986, she defended her doctor of philosophy (PhD) with Enea as her mentor, studying the evolution of the CS gene repeated domain. While intrigued with both *Trypanosome* and *Plasmodium* antigenic variation, she was offered a post-doctoral position with John Barnwell, who pioneered the discovery of the *Plasmodium* variant

antigens along with Russell Howard. The elusive role of the spleen in the expression and switching of the *P. knowlesi*-SICA antigens *in vivo* in rhesus monkeys was most intriguing. Her post-doctoral and continued research with Barnwell as a member of NYU's faculty led her down a long path of gene and antigen discovery in *P. vivax*, *P. cynomolgi* and *P. knowlesi*, including the discovery of the *P. knowlesi* variant antigen gene family (named SICAvar). These studies also revealed relationships with *P. falciparum* homologues, whether for MSPs, reticulocyte-binding proteins (RBPs) or the SICAvar family, which she continues to pursue today. The multitude of questions, with decreasing NIH, United States Agency for International Development (USAID) and World Health Organization (WHO, Geneva, Switzerland) funds, led her, in 1992, to start the Malaria Foundation International (MFI, Stone Mountain, Georgia). There was minimal attention on this disease at the time and little information available to the public. In 1995, when the Internet was a novelty, the MFI launched the website www.malaria.org (last accessed 16 July 2008). By 1997, Galinski was recruited to Emory University School of Medicine, Division of Infectious Diseases (Atlanta, Georgia) to establish malaria research at the new Emory Vaccine Center at the Yerkes National Primate Research Center (Atlanta, Georgia). There, she broadened her research interests to include topics on malaria pathogenesis and epidemiology, and vaccine development and testing. She also embraced teaching, in essence laying the foundation for establishing and directing Emory's International Center for Malaria Research and Education. Today, her main interests remain in the molecular and cell biology of *Plasmodium*, revealing its complex survival strategies, while also stimulating the continued advancement of knowledge with students and the public through MFI projects aimed at education, the support of research and the ultimate control of malaria.

In 1984, through the use of radioiodination and immunoprecipitation with isolate-specific immune sera, a consistent surface-associated protein named *Plasmodium falciparum* erythrocyte surface protein-1 (PfEMP1) was clearly identified (Leech *et al.*, 1984). Although PfEMP1 was detected by iodination, later it was shown to be metabolically labeled with radioactive amino acids and since mature red cells are incapable of protein synthesis it was clearly a parasite-encoded protein and not a modified host protein. PfEMP1 is a high-molecular-weight antigen that can be removed from the surface of intact falciparum-infected red cells by exposure to low concentrations of trypsin and after trypsin 'stripping' the binding of infected red cells to endothelial cells was ablated. Strain-specific sera were shown to immunoprecipitate PfEMP1 from a homologous strain but not from others. In addition, these strain-specific sera also blocked the binding of homologous infected red cells to endothelial cells (summarized in Pasloske and

Howard, 1994 and Sherman *et al.*, 2003). The conclusion was inescapable: the parasite-encoded chimeric molecule PfEMP1 is responsible both for adherence and antigenic variation. For almost a decade the PfEMP1 protein itself could not be isolated (presumably because of low abundance). Indeed, because of this I boldly (and unwisely) questioned its very existence (Sherman and Winograd, 1990); however, in 1995 the gene for PfEMP1, named *var* (for variant), was cloned and sequenced in three laboratories (Baruch *et al.*, 1995; Smith *et al.*, 1995; Su *et al.*, 1995).

Russell J. Howard (1950–) is currently the chief executive officer and a co-founder of Maxygen, Inc. In 1972, Howard received his bachelors' degree in biochemistry with honors from Melbourne University (Melbourne, Vic, Australia) and his PhD on the biochemistry of marine algae in 1975. In 1976, Howard began his career in the field of molecular pathogenesis of malaria with post-doctoral studies in the Immunoparasitology Laboratory of Graham F. Mitchell at WEHI. These studies continued in 1979 with Louis Miller at the National Institutes of Health in Bethesda, Maryland. At the NIH Howard made several seminal discoveries, including the identification of PfEMP1, the molecule involved in both adherence and antigenic variation in *P. falciparum* malaria. In 1988, he created the Laboratory of Infectious Diseases at the DNAX Research Institute of Molecular and Cellular Biology (Palo Alto, California) and continued work on antigenic variation in malaria; in 1992, he joined Affymax Research Institute (Palo Alto, California) as vice president and director of cell biology, and in 1994 became its president and scientific director. At Affymax, Howard and colleagues were able to clone and sequence PfEMP1 (1995) and identify the putative binding sequences. During this period, he also led an interdisciplinary team of 200 scientists working on the Affymax combinatorial chemistry and drug discovery platform. In 1996, Howard became president and chief operating officer of Maxygen during its incubator phase and in 1998 became its president and chief executive officer. He retains his interest in infectious diseases and vaccine discovery and serves currently as chairman of the USAID Malaria Vaccine Development Program as well as a member of NIH/ National Institute of Allergy and Infectious Diseases (NIAID, Bethesda, Maryland) Malaria Vaccine Development Committee.

The PfEMP1 proteins are encoded by 50–60 *var* genes. The var genes are large (6–13 kb), localized to subtelomeric regions of all 14 chromosomes, extremely divergent in sequence and theoretically could encode proteins of around 200–500 kDa. Members of the PfEMP1 family share a similar organization with two exons. The first, a large 5′ exon, encodes the

extracellular binding region and contains two to seven copies of a motif denoted DBL (Duffy-binding-like) interrupted by one or two copies of cysteine-rich interdomains (CIDR). The second, and smaller, 3′ exon encodes an acidic region called the acidic terminal sequence (ATS) and is presumed to anchor the molecule to the red-cell surface (Flick and Chen, 2004; Horrocks *et al*., 2005a). Many of the var genes are transcribed at the early ring stage but a single dominant messenger ribonucleic acid (mRNA) coding for the surface-expressed PfEMP1 appears at a later developmental stage (Duffy and Tham, 2007; Kraemer *et al*., 2007; Kyes *et al*., 2007). For some, perhaps all, *var* genes there is a single unique *var* transcription site within the nuclear periphery (Ralph *et al*., 2005b). Once such a site is occupied active transcription at other *var* loci does not occur. A recent study has shown that in early ring stages only a single *var* gene is transcribed by ribonucleic acid (RNA) polymerase II, while the majority of other *var* genes remain silent (Kyes *et al*., 2007).

2. PfEMP1, Knobs and Adhesion

It is now more than a century since Bignami and Bastianelli, working in the Rome laboratory of Batistta Grassi (see p. 6) discovered sequestration in *P. falciparum* (i.e. the adhesion of red cells bearing the mature pigment-containing stages (trophozoites and schizonts) to the endothelial cells that line post-capillary venules of the deep tissues; Bignami and Bastianelli, 1890). Although their observation explained why these stages were absent from the peripheral blood, whereas the young, unpigmented ring-stage parasites circulate freely in the peripheral blood, it did not reveal the mechanisms for such preferential binding. Much later, with the development of transmission and scanning electron microscopes it was possible to describe differences in the red-cell surface when such cells harbored different developmental stages, and these would provide clues to an understanding of the mechanisms for the differing adhesive properties of the falciparum-infected red cell. Cells with mature-stage parasites lose their biconcave disc shape, are distorted and are covered with submicroscopic elevations of the plasma membrane (around 100 nm in diameter and 20 mm in height) underlaid by an electron-dense plaque, called ‘knobs’ (Aikawa *et al*., 1983; Gruenberg *et al*., 1983). (For a recent study using atomic force microscopy see Nagao *et al*., 2000.) The numbers of knobs per infected cell has been estimated to be as low as 500 (Gruenberg *et al*., 1983) or as high as 6000 (Nagao *et al*., 2000). By contrast, ring-stage-infected red cells have a smooth surface and are knobless. These observations were first made by transmission electron microscopy using blood samples from patients by Trager, Rudzinska and Bradbury (1966) and later Luse and Miller (1971), and Miller (1972) found the same knobby

structures on red cells from falciparum-infected *Aotus* monkeys. Miller (1969), using transmission electron microscopy, showed attachment to endothelial cells was *via* the knobs. Soon after the successful *in vitro* cultivation of *P. falciparum*, Susan Langreth (working in Trager's laboratory) was able to show that knobs were present in cultured parasites (Langreth *et al*., 1978). In addition, in 1981 when Udeinya *et al*. were able to mimic sequestration *in vitro* (called cytoadherence)by adhering infected red cellsto endothelial cells (and later to C32 amelanotic melanoma cells) and to show that adhesion was at knobs, the conclusion was inescapable: knobs mediate adhesion. Additionally, when immunocytochemical staining of falciparum-infected red cells with hyperimmune serum showed that only the knobs were labeled it became clear that the changes in the knobs were sufficient to be recognized as 'non-self' (i.e. they were antigenically distinct) (Langreth and Reese, 1979). These early studies (as well as those concerned with SICA, see pp. 186–187) led to a search for the 'non-self' molecules that were the 'glue' of the knob. In addition, there was a stated practical goal: identification of the 'glue' might provide for understanding of antigenic variation and could be the basis for a vaccine.

The biochemical changes associated with adhesion have been the subject of several recent reviews (Baruch, 1999; Baruch *et al*., 2002; Cooke *et al*., 2001, 2004b, 2005; Sherman *et al*., 2003). Included have been discussions of cytoskeletal alterations, modifications of intrinsic membrane proteins of the red cell such as band 3, exposure of phosphatidylserine (PS), parasite-encoded proteins exported into the red cell cytosol and associated with the cytoskeleton (i.e. KAHRP, *P. falciparum* erythrocyte surface protein-3 (PfEMP3), MESA, and those exposed on the surface, i.e. PfEMP1, sub-telomeric variant open reading frame (STEVOR) and repetitive interspersed family of genes (RIFINS) involved in re-modelling of the infected erythrocyte).

As noted earlier, PfEMP1s are large molecules synthesized by the parasite, are antigenically diverse and localized to the knob. Since the *var* genes appear to be transcribed at the ring stage but the full dominant product does not appear at the red-cell surface until some 16 h post-invasion (and the newly synthesized PfEMP1 at 12 h post-invasion did not appear until 8 h later) a special trafficking pathway must exist (Horrocks *et al*., 2005a; Kriek *et al*., 2003). This trafficking pathway has now been characterized in some detail (see pp. 208–210).

PfEMP1 is an adhesive and antigenic molecule (Kraemer and Smith, 2006; Kyes *et al*., 2001, 2007; Smith *et al*., 2000a) composed of three different adhesive domains: DBL, CIDR and a constant domain C2. The extracellular portion of PfEMP1 variants contains between two and seven cysteine-rich DBL domains and each can be divided into 10 semi-conserved blocks (A-J) and 10 variable blocks (1–10) (Howell *et al*.,

2006; Smith *et al*., 2000a). The semi-conserved blocks contain a large number of invariant or biochemically conserved residues that are presumed to be important for domain folding. The DBLs are grouped into five sequence types α, β, γ, δ and ε on the basis of consensus motifs plus a sixth heterogeneous group, DBL-X. Two distinct types of DBLα domains (α and α1) that differ in the number of conserved cysteines and other hydrophobic residues have been described. The CIDR region can be divided into three sequence types α, β and γ. Although there is less than 50% identity in amino acid composition between DBL domains the DBLα domain that occurs at the *N*-terminal has the highest degree of sequence conservation among all domains and for this reason it has been suggested as a possible vaccine candidate. Indeed, a recent study found that anti-DBL1α antibodies blocked red cell adhesion in four out of five monkeys and 'the presence of anti-DBL1α antibodies reduced the sequestration of infected red cells as efficiently as the removal of PfEMP1 from the infected red-cell surface' (Moll *et al*., 2007).

At least 12 receptors: CD 36, ICAM-1, PECAM-1/CD31, ELAM, thrombospondin (TSP), VCAM, E-selectin, chondroitin sulphate A (CSA), HA and alpha V/beta 3 integrin (Siano *et al*., 1998) on endothelial cells and HS, CR1 (CD35), blood group antigen A on red cells (see reviews by Sherman *et al*., 2003 and Cooke *et al*., 2004b) have been identified in the adhesion of *P. falciparum*-infected red cells to the vascular endothelium (sequestration/cytoadherence) and to erythrocytes (rosetting).

One of the endothelial cell receptors for PfEMP1, ICAM-1 (reviewed in Chakravorty and Craig, 2005), is a 90–115 kDa transmembrane glycoprotein containing five immunoglobulin (IgG) domains. It is expressed on many cell types, including the endothelial cells, and can be up-regulated in response to inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-β. It exists on the cell surface as a dimer. The ICAM-1 binding domain for PfEMP1 has been mapped to DBLβ2 and C2, where it binds to the center of the dimer sitting between two ICAM molecules (the so-called BED side of the *N*-terminal IgG-like domain). Different parasite lines bind to similar regions of ICAM-1 and there are discrete variations in the contact residues leading to changes in avidity. A model of the complex between PfEMP1 and ICAM-1 has recently been published (Bertonati and Tramontano, 2007); it provides a rational explanation for the altered adhesiveness of different strains that bind to the receptor. Based on the crystal structure of human ICAM-1, part of the ICAM-1 binding site for *P. falciparum*-infected erythrocytes was used to screen a library of compounds (Dormeyer *et al*., 2006). Thirty-six structural mimotopes were found as potential competitive inhibitors of binding. One, (+)-epigalloyl-catechin-gallate ((+)-EGCG), inhibited adhesion in a dose-dependent manner with two variant ICAM-1-binding parasite lines.

Infusion of anti-ICAM monoclonal antibodies in a rodent model (*P. yoelii*) resulted in detachment of infected red cells (Kaul *et al*., 1998).

Binding to ICAM-1 by parasitized red cells is not common amongst various isolates, and, it has been contended that when ICAM-1 is up-regulated on the brain endothelium sequestration at this site may lead to cerebral malaria (Chakravorty and Craig, 2005). A genetic polymorphism exists with ICAM-1 and some have claimed that one of these, ICAM-1^{kilifi} can be associated with disease severity in Africa (Fernandez-Reyes *et al*., 1997; Jenkins *et al*., 2005), however, studies in Thailand did not support the correlation (Ohashi *et al*., 2001).

Almost all *P. falciparum* lines bind to CD36, an 88 kDa glycoprotein found on the surface of several cell types, including platelets, monocytes, dendritic cells and microvascular endothelial cells, however, it is absent from brain endothelia. It is generally believed that CD36 provides a stable and long-lasting anchor for binding of parasitized cells (Cooke *et al*., 2001). The CD36 domain responsible for binding infected red cells has been reported to reside in amino acids 139–184 (Baruch *et al*., 2002). The binding region of PfEMP1 for CD36 has been claimed to be CIDR1 since tryptic fragments to this domain bind to CD36, and anti-CIDR antibodies block binding of parasitized red cells to CD36. The 179 amino acids containing seven cysteine residues of a recombinant CIDR protein were found to constitute the minimal adhesion motif (summarized in Baruch *et al*., 2002). Five of the seven cysteine residues all occur in the cysteine-rich core and aside from cysteine-49, all appeared to be required for correct folding and function. Since the recombinant protein blocked adhesion of all the CD36-binding strains that were tested, it was claimed that the protein with a CIDR sequence from any of the various isolates could fold into a conserved structure to mediate binding to CD36. It has been suggested that modulation of dendritic cell function contributes to the delay in acquisition of malaria immunity as well as suppression. Indeed, Urban *et al*. (1999, 2001) showed that *P. falciparum*-infected red cells inhibited the maturation of human monocytes-derived dendritic cells and interfered with the ability to activate a T-cell response. The interaction was claimed to be due to PfEMP1 and the dendritic cell scavenger receptor, CD36. However, a recent study indicated that modulation of dendritic cells required neither CD36 binding of PfEMP1 nor direct contact between dendritic cells and infected red cells (Elliott *et al*., 2007). These investigators suggested that a soluble factor (unidentified) might be responsible for the inhibition and apoptosis of dendritic cells.

TSP is a large protein consisting of three identical 140 kDa chains linked to each other by disulphide bonds and is found in the extracellular matrix, in platelet granules, on the surface of macrophages, melanoma cells and endothelial cells and binds to CD36, CD47 and falciparum-infected red cells. TSP plays roles in platelet clotting and regulation of angiogenesis. Although Baruch *et al*. (1996) claimed that CIDR1α bound TSP, Gardner

et al. (1996) suggested another molecule (possibly modified band 3 protein) was responsible for TSP binding. Their reasoning was: specific binding to ICAM-1 and CD36 appear simultaneously during the cell cycle, however, binding to TSP did not show variant-specific characteristics and appeared 2 h earlier in the cell cycle and was insensitive to immune sera. Exposure of PS on infected red cells was found to contribute to binding to CD36 and TSP (Eda and Sherman, 2002). This finding of PS binding to TSP is compatible with the suggestion that PS is involved in the adhesion of sickle red cells (Setty *et al*., 2002). In addition, as described later (see pp. 201–202) pfalhesin (HPLQKTY) binds to the RGD (arginine-glycine-aspartic acid sequence) in the type III repeat of TSP and fibronectin (Eda and Sherman, 2004). And, although Cooke *et al*. (1994) claimed that binding of falciparum-infected red cells to TSP was not stable under flow conditions, Siano *et al*. (1998) and Rock *et al*. (1988) have observed stable binding to TSP under flow using microvascular endothelial cells.

3. PfEMP1 and Rosetting

In the 1980s, it was discovered that *P. falciparum*-infected red cells were able to bind to uninfected red cells to form clumps of cells called rosettes (Wahlgren, 1986). Rosetting was also observed in *P. vivax* (Udomsangpetch *et al*., 1995), *P. ovale* (Angus *et al*., 1996) and *P. malariae* (Lowe *et al*., 1998) in the human host, *P. coatneyi* in the rhesus monkey (Udomsangpetch *et al*., 1991) and *P. fragile* in the toque monkey (Handunnetti *et al*., 1989). A rosetting model in the rodent malaria *P. chabaudi* was developed that shows some of the features found in *P. falciparum* and other primate malarias (Mackinnon *et al*., 2002). In this model, rodent malaria rosetting increased with parasite maturity and was reversed by trypsin and ethylenediaminetetraacetic acid (EDTA) suggesting that rosetting was likely due to a parasite-induced surface protein. Although rosetting in *P. chabaudi* may be phenotypically analogous to that in *P. falciparum*, the *var* genes encoding the PfEMP1 family of proteins are not detected in rodent malaria genomes (Janssen *et al*., 2004), therefore, it is highly unlikely this protein is PfEMP1 (Franke-Fayard *et al*., 2005).

Rosetting in some species may be benign, however, in others (i.e. *P. falciparum*) it may contribute to pathogenesis leading to severe malaria (Chen *et al*., 2000b; Rowe, 2005). Indeed, although rosetting was associated with severe malaria in some parts of Africa it was not in Papua New Guinea where a CR1 polymorphism prevents that particular phenotype from occurring (Cockburn *et al*., 2004; Rowe, 2005; Rasti *et al*., 2006). The DBL1α domain that contains clusters of glycosaminoglycan (GAG) binding motifs is reported to be the rosetting ligand and specific amino acid motifs were correlated with severe malaria (Normark *et al*., 2007).

The identified rosetting receptors on the red cell are: heparin sulphate, blood group antigens and complement receptor 1 (Rowe, 2005). Stable rosettes require serum whose essential components are mimicked by complement factor D, albumin and anti-band 3 IgG antibodies (Chen *et al*., 2000b; Luginbuhl *et al*., 2007). The A and B tetrasaccharides are believed to be receptors for rosetting on uninfected red cells, and although *P. falciparum* rosettes can form in group O cells, these tend to be smaller; it has been hypothesized that there is a protective effect of group O and that this operates through reduced rosetting (Rowe *et al*., 2007). Curdlan sulphate, a sulphated glycoconjugate, significantly reduced rosette formation *in vitro* in laboratory strains and clinical isolates from Africa (Kyriacou *et al*., 2007). It was proposed 'that rosette-inhibiting drugs or vaccines may have the potential to save many patients' lives' (Rowe, 2005).

In 2000, the Wahlgren laboratory at the Karolinska Institutet in Stockholm, Sweden studied the properties of the polyadhesive FCR3S1.2 clone that rosettes, autoagglutinates and binds to PECAM-1/CD31, CD36, IgM, blood group antigen A and to heparan sulphate (Chen *et al*., 2000a). The *N*-terminal head structure of the DBL1α–CIDR1α mediated adherence to all of these receptors. Using recombinant proteins, CIDR1α and DBL2δ were found to be involved in binding to PECAM-1/CD31. CIDR1α bound normal non-immune IgM, whereas DBL1α had a high affinity for HS with less involvement of CIDR1α and DBL2δ, and DBL1α had high affinity for blood group antigen A. Earlier Baruch *et al*. (1997) had shown that the CIDR1α binds to CD36 *via* a peptide–peptide interaction involving a degenerate CIDR1α sequence containing multiple conserved cysteine residues, a sequence also present in FCR3S1.2. Although binding of DBL1α, CIDR1α and DBL2δ to ICAM-1 was weak, Chen *et al*. (2000a) suggest 'CIDR1α has an affinity for a structure present in members of the IgG family, that is IgM and PECAM-1 but (there is also) a separate binding site for CD36.' Immunization with DBL1α generated functional antibodies able to disrupt rosettes, autoagglutination and to block adhesion in a rat model (Chen *et al*., 2004) and immunization with either a single DBL1α domain or with three showed a reduction in adhesion in four out of five monkeys. (Interestingly, a small degree of desequestration was obtained by infusion of depolymerized heparin; Vogt *et al*., 2006.)

Mats Wahlgren (1952–) is currently professor of parasitology at Karolinska Institutet and senior consultant at the Swedish Institute for Infectious Disease Control. Wahlgren received his medical degree from Karolinska Institutet (1979) and a Diploma in Tropical Medicine & Hygiene from Mahidol University (Bangkok, Thailand) (1979). In 1981, Wahlgren began his career in the field of molecular pathogenesis of malaria with doctoral studies in the Department of Immunology

of Peter Perlmann that led to a PhD from Karolinska Institutet (1986). Wahlgren identified the cell-phenomenon known as rosetting during his thesis work and later its association with severity from *P. falciparum*. He was appointed associate professor at Karolinska Institutet in 1990 and spent the next year as visiting guest researcher at DNAX Research Institute of Molecular and Cellular Biology in the laboratory of Russell Howard. Wahlgren was subsequently appointed professor of parasitology (1993) and has since served as chairman of the Microbiology & Tumor Biology Center of the Karolinska Institutet, director of the Multilateral Initiative on Malaria, program director for the Infection and Vaccinology Program and President of the Swedish Society of Tropical Medicine. Since his return to Stockholm, Wahlgren and colleagues have unraveled the molecular details of the interaction between the rosetting-ligand PfEMP1, its receptors on endothelial and red blood cells, and discovered the role of heparan sulphate as a receptor in rosetting-binding to red blood- and to endothelial cells. Wahlgren is one of the co-founders of Dilafor Ltd., a biotechnology company committed to the development of carbohydrate-based therapy, including that for severe malaria.

4. PfEMP1 and Placental Adhesion

Sequestration of *P. falciparum*-infected erythrocytes in the placenta is associated with pathological outcomes of pregnancy-associated malaria (Beeson and Duffy, 2005; Duffy, 2007; Duffy and Fried, 2005). Adhesion studies have shown that the parasitized erythrocytes from the placenta predominantly bind to the GAG CSA, whereas infected red cells from the peripheral blood of pregnant women bind to other receptors, primarily CD36. Such findings suggest that the placental endothelium selects for the rare CSA-binding cells. Antibodies against recombinant DBL3γ (from FCR3var1CSA) recognized a wide variety of heterologous placental isolates (Bir *et al*., 2006), however, transcription of *var1csa* or FCR3varCSA is not up-regulated in CSA-binding or placental isolates. In Malawian women where *var2csa* predominates, DBLX, DBLγ and DBLϵ domains occur (Duffy *et al*., 2006a,b). Importantly, sera from immune multi-gravid women recognize placental isolates from different geographical regions (Fried *et al*., 1998). Transcription of *var2csa* is up-regulated in parasites selected to bind to CSA (Salanti *et al*., 2003) and this gene is semi-conserved between geographically diverse isolates (Trimnell *et al*., 2006). An interesting observation is that CSA-binding lines are phagocytized at significantly lower levels than CD36 binding lines (Serghides *et al*., 2006).

The fact that knockouts of the *var2csa* gene have impaired placental adhesion (Viebig *et al*., 2007) and that a distinct pattern of surface protein expression exists on placental *P. falciparum*-infected erythrocytes (Fried *et al*., 2007) continues to fuel optimism that a CSA-binding ligand would be a prime vaccine candidate for therapeutic and preventative interventions (Avril *et al*., 2006; Costa *et al*., 2006; Rogerson *et al*., 2007). However, tempering this enthusiasm for a vaccine is the finding that in fresh placental isolates there is multiple binding to several receptors (IgM, IgG and HA) in addition to CSA (mediated by DBL2X and DBL6ε (Rasti *et al*., 2006)) and since so much diversity of DBL3X sequences was found in an isolated locale it may be difficult to produce a vaccine from a single *var2csa* DBL3X sequence (Dahlback *et al*., 2006). In addition, as shown by differential trypsin sensitivity and sequence comparison (Trimnell *et al*., 2006), polymorphic variants of var2CSA exist (Nielsen *et al*., 2007).

Mats Wahlgren has cautioned, 'it is uncertain how many different PfEMP1 variants or perhaps other unidentified ligands need to be recognized by the host's immune system before an optimal protective response against placental isolates develops . . . future success of any anti-adhesion intervention . . . necessitates an in-depth understanding of these alternative receptor ligand interactions adopted by the parasite.' In addition, although it is reasonable to suggest that conserved amino acid residues within the DBL and CIDR domains are necessary for proper folding for adhesion and receptor function, there has been no identification of the adhesive sequences. Indeed, Baruch, Rogerson and Cooke (2002) stated, 'although several adhesion domains have been defined. . . it is impossible at this time to predict adhesion phenotype of a *var* gene from its primary sequence.'

5. KNOB-ASSOCIATED PROTEINS INVOLVED WITH ADHESION

Three other parasite-encoded proteins, KAHRP, PfEMP3 and MESA, are knob associated. Although none of these are surface exposed, they do play a role in adhesion. The gene for KAHRP, located on chromosome 2, encodes an 80–108-kDa protein, insoluble in Triton X-100 that has been localized to the electron-dense plaque (Cooke *et al*., 2004b). Since the ATS of PfEMP1 interacts with the His-rich and the 5′ and 3′ highly charged repeats of KAHRP it acts as a sub-membranous attachment point and organizing region for those proteins involved in cytoadherence. The actin-binding site of KAHRP has been mapped (Kilejian *et al*., 1991). A recombinant 5′ repeat of KAHRP forms stable complexes with actin and spectrin, the two major components of the red cell cytoskeleton. Spectrin is composed of two subunits, an α (280 kDa) and a β chain (246 kDa) both made of repeating units with α having 21 and β having 16; each repeat

is composed of around 106 amino acids; the *N*-terminus of β spectrin is responsible for binding to actin and protein 4.1 and the *N*-terminus of the α spectrin chain and the *C*-terminus of the β chain are involved in tetramerization. The binding region of KAHRP to spectrin has been mapped to a 72 amino acid fragment (residues 370–441) that binds a single repeat (repeat 4) of spectrin (Pei *et al*., 2005). The interaction of KAHRP with spectrin could be critical to its membrane localization in the electron-dense plaque of the knob, located at nodular regions where spectrin, actin and band 4.1 are joined. Indeed, the distance between knobs is 30–50 nm and this corresponds to the distance between spectrin–actin–band 4.1 junctions and is also the region where band 3 protein binds to band 4.1. The knob structure has a positive charge whereas the rest of the surface of an infected red cell is negatively charged. Protrusion of the knob may be necessary to elevate this positive charge for adherence to negatively charged surfaces of endothelial cells.

Reorganization of the surface of the infected red cell may begin as early as the ring stage and is evidenced by changes in band 3 mobility, however, this immobilization has been shown to not be due to KAHRP; it may involve deposition of hemichromes, clustering of band 3 and binding of autoantibodies (Parker *et al*., 2004). Fully functional knobs require the *C*-terminal repeat region of KAHRP, and if KAHRP is removed or deleted this weakens the tethering to the membrane and cytoskeleton and adhesion is reduced under flow conditions. In knob-less lines the basic distribution and biophysical properties of PfEMP1 are similar to that of knob-bearing cells; however, in the absence of knobs the export of PfEMP1 is less efficient and less is loaded on to the surface of red cells bearing a knob-less line (Horrocks *et al*., 2005b).

Mature parasite-infected erythrocyte surface antigen (MESA) is a 250–300 kDa-fibrillar phosphoprotein encoded by a gene on chromosome 5 (Cooke *et al*., 2001). It has a 19 amino acid sequence that binds to the membrane cytoskeletal protein 4.1 (Waller *et al*., 2003). The precise function of MESA is not known but parasites that do not express MESA are viable and cytoadhere.

PfEMP3 is a large highly charged protein of 315 kDa (Cooke *et al*., 2001). The *N*-terminal contains a putative hydrophobic signal while the *C*-terminal is composed of repetitive sequence elements. PfEMP3 can be detected in knobs but is more broadly distributed under the membrane of the red cell. PfEMP3 is not essential for knob formation and transgenic parasites lacking PfEMP3 still cytoadhere although not as avidly. PfEMP3 appears to contribute to the reduced deformability of infected red cells (Glenister *et al*., 2002).

The approximately 30 STEVOR proteins are encoded by subtelomeric genes (reviewed in Sherman *et al*., 2003). In early gametocytes (stages I to IIA) when knobs and PfEMP1 are present, STEVOR proteins are localized to the parasite cytoplasm, but as gametocytes mature it traffics through the red cell

cytoplasm reaching the plasma membrane in mature gametocytes (stages IIB to V), a time when knobs are absent (McRobert *et al*., 2004). STEVOR proteins are also present in sporozoites and in Maurer's clefts in mature asexual stages but in gametocytes trafficking appears to be independent of Maurer's clefts since these structures are absent from all except the youngest gametocytes (McRobert *et al*., 2004). Although *var* and STEVOR genes are subtelomeric and close to one another on the chromosome, transcription of *var* in gametocytes is unlinked to STEVOR transcription (Sharp *et al*., 2006). STEVOR peptides bind to human red cells (Garcia *et al*., 2005) suggesting involvement in rosette formation. The expression of STEVOR in different stages in the life cycle (Florens *et al*., 2002) may indicate potential importance for parasite survival. When four STEVOR sequences were cloned and expressed in *Escherichia coli*, with His-tagged fusion proteins used to assess developing immunity, high levels of antibodies were found in clinically immune adults, however, infants with high anti-STEVOR antibody levels exhibited an elevated rise for developing infections (Schreiber *et al*., 2007). The precise role of STEVOR remains unclear, an adhesive role seems unlikely and their contribution to the immune response is transitory.

The RIFINS encoded by approximately 200 proteins of 35–45 kDa (reviewed in Sherman *et al*., 2003) were first identified by Weber (1988) as repetitive interspersed fragments and then *via* surface iodination and immuno-precipitation with immune sera (Fernandez *et al*., 1999; Helmby *et al*., 1993). Originally described as playing a role in rosetting, perhaps *via* CD31, they were named rosettins, but recent studies have shown they are not always associated with the rosetting phenotype. There are two subgroups of RIFIN variants designated A- and B-type (Petter *et al*., 2007). A-type RIFINs were associated with the parasite and transported to the surface of infected erythrocytes *via* Maurer's clefts, whereas B-type RIFINs were mostly retained inside the parasite. However, expression of both subtypes was not mutually exclusive. Both variants were also expressed in the merozoite, present either in the apical region (A-type) or in the cytosol (B-type). The presence of RIFINs in merozoites suggested to Petter *et al*. that antigenic variation in *P. falciparum* is not only restricted to parasite-derived proteins at the infected red-cell surface, but may also prevail in other life cycle stages.

6. BAND 3 AND ADHESION

Prior to 1984, our laboratory (amongst others) attempted to find parasite-encoded proteins inserted into the red cell membrane of cells infected with *P. falciparum*. Indeed, our inability to find convincing evidence for the insertion of parasite-encoded proteins in *P. lophurae*- and *P. falciparum*-infected red cells (see p. 189) led us to suggest that in addition to

plasmodial proteins the intra-erythrocytic parasite might alter the red-cell surface by modification of intrinsic proteins (Sherman, 1985). The approach we used was similar to that employed by others: surface iodination and immunoprecipitation. But, rather than use 'immune' sera from monkeys or humans, we prepared monoclonal antibodies against live *P. falciparum*-infected red cells. By immunoelectron and immunofluorescence microscopy some of these monoclonal antibodies could be localized to the external surface of infected red cells and were also able to prevent adhesion to target cells (i.e. they were cytoadherence-blockers) (Winograd and Sherman, 1989). Using Western blotting, immunoprecipitation and peptide mapping of surface iodinated red cells, it was determined that the infected red cells did not recognize a parasite-encoded protein, but instead an intrinsic membrane protein, the anion transporter band 3, albeit in a modified form (Winograd and Sherman, 1989).

Band 3 is an approximately 95-kDa membrane-spanning protein that exists as a tetramer; each monomer has two domains: a glycosylated 52-kDa external *C*-terminus with eight putative exofacial loops and a highly acidic 43-kDa cytoplasmic *N*-terminal domain that serves as a center of membrane organization interacting with proteins such as protein 4.1, protein 4.2 ankyrin and glycolytic enzymes such as glyceraldehyde-3-phosphate dehydrogenase and aldolase, and with binding sites for hemoglobin and hemichrome (Bustos and Reithmeier, 2006; Chu and Low, 2006). Band 3 is the most abundant membrane protein with a million copies per red cell. Using the primary amino acid sequence of band 3 in conjunction with Pepscan analysis, synthetic peptides corresponding to particular regions of band 3 were synthesized and antibodies to these were made in mice and rabbits. Peptides with the amino acid sequence HPLQKTY (corresponding to band 3 residues 546–553) blocked adhesion of infected red cells *in vitro*, as did antibodies to the synthetic peptides (Crandall *et al*., 1993; Crandall and Sherman, 1994). Microspheres coated with this peptide adhered to target cells as did infected red cells (Guthrie *et al*., 1995) and when peptide was infused into falciparum-infected *Aotus* or *Saimiri* monkeys there was evidence for an anti-sequestering effect (Crandall *et al*., 1993). Serum from human populations where malaria was endemic had higher reactivity to the peptide than did sera from non-endemic areas (Hogh *et al*., 1994). We named the peptide pfalhesin and went on to show that its receptor was the RGD sequence found in the type-3 repeats of TSP (Eda *et al*., 1999; Lucas and Sherman, 1998) and fibronectin (Eda and Sherman, 2004). Subsequent to this work we used the anion transport inhibitor 4,4′-diisothiocyano 2,2′-dinitrostilbene sulphonate (DIDS) to block adhesion (Winograd *et al*., 2004). In human red cells, DIDS binding occurs in a region close to that of HPLQKTY, namely YETFSKLIKIFQDH (residues 537–547 of band 3); synthetic peptides

containing this sequence (called the DIDS-binding region, DBR) and antibodies prepared in chickens and rabbits against the sequence were able to block adhesion (Winograd *et al*., 2004). The DBR peptide specifically mediated binding to CD36 and sera from individuals from an endemic region; so-called 'immune sera' were shown to contain antibodies to the DBR peptide (Winograd *et al*., 2005). We proposed that the DBR and pfalhesin were cryptic in the uninfected red cell but upon infection become exposed due to a clustering of band 3. Further, band 3 clustering and binding of autologous IgG were simulated by treatment of uninfected red cells with agents known to cluster band 3 in human erythrocytes (e.g. acridine orange, BS^3, $ZnCl_2$) (Winograd and Sherman, 2004). In another study, we were able to show that oxidative stress (by exposure to *t*-butylhydroperoxide) results in band 3 clustering and exposure of DBR. In addition, when band 3 clustering is induced in uninfected red cells by treatment with acridine orange it exposes a cryptic tryptic cleavage site (presumably close to residues 551 and/or 562); when such cells are treated with trypsin there is cleavage of band 3 (reminiscent of the 'stripping' off of the extracellular domain of PfEMP1 by trypsin) (Winograd *et al*., 2005).

Freeze-fracture studies of *P. falciparum*-infected red cells showed a clustering of intramembraneous particles (mostly composed of band 3 protein) with parasite development (Allred *et al*., 1986). The lectin concanavalin A (con A) recognizes the mannosyl residues in band 3 protein, and increased agglutination of infected red cells is effected by con A; clustering of con A on the surface of *P. falciparum*-infected red cells was demonstrated using electron microscopy (Sherman and Greenan, 1986). Taken together these studies show that membrane modifications of falciparum-infected red cells need not involve parasite-encoded proteins; however, a metabolically active parasite is essential to promote the membrane alterations (i.e. conformational change and exposure of a cryptic amino acid sequence of band 3 protein through what we believe is an oxidative mechanism; summarized in Sherman *et al*., 2003).

Abnormal hemoglobins such as HbC are associated with protection against severe malaria (Roberts and Williams, 2003). The knobs on the surface of the AC and CC cells are abnormally large and may be involved in protection. Although PfEMP1 is abnormally distributed on CC cells (Fairhurst *et al*., 2003) and may contribute to the reduced cytoadherence of such cells, a more likely explanation is that in CC cells there is increased deposition of hemichromes during parasite development and as a result there is band 3 clustering and exposure of neoantigens leading to opsonization by autologous IgG and complement. In addition, the increased knob size would decrease the binding in the microvasculature and such

cells would be removed from the circulation by passage through the spleen (Arie *et al*., 2005). Membrane alterations akin to those observed in infected red cells do occur in senescent red cells (Arese *et al*., 2005; Hornig and Lutz, 2000; Lutz *et al*., 1990; Sherman *et al*., 2004; Turrini *et al*., 1991) and can be simulated by band 3 clustering (Winograd *et al*., 2005). Since these changes also occur in sickle (Kannan *et al*., 1988), thalassaemic (Cappellini *et al*., 1999) and glucose-6-phosphate dehydrogenase (G6PD)-deficient red cells (Cappadoro *et al*., 1998) it has been suggested that with parasite development in these cells there is deposition of hemichromes and oxidative aggregation of band 3. When this occurs in ring-stage-infected red cells their removal is promoted leading to a reduced parasite burden (Ayi *et al*., 2004; Arese *et al*., 2006).

PfEMP1 and pfalhesin/DBR are not identical. The exposed band 3 sequences are neither isolate specific nor are they parasite encoded, but the DBR sequence and PfEMP1 do have a common receptor, CD36; pfalhesin binds to TSP, which is not a receptor for PfEMP1 (Gardner *et al*., 1996). Based on the available evidence, I would suggest that at a minimum the adhesion of *P. falciparum*-infected red cells is mediated by a combination of parasite-encoded proteins such as PfEMP1, exposed sequences in band 3 protein and PS (see below)—a notion expressed by Newbold and Marsh (1990) in response to my reckless challenge that antigens on the *P. falciparum*-infected erythrocyte surface were not parasite derived (Sherman and Winograd, 1990).

7. ADHESION AND CEREBRAL MALARIA

Cerebral malaria is a neuropathological syndrome that occurs in approximately 1-10% of *P. falciparum* infections and can be lethal. One of the signal characteristics of cerebral malaria is blockade of brain microvessels due to sequestration of infected erythrocytes (Haldar *et al*., 2007). In addition to erythrocytes, platelets and leucocytes may also be present in the occluded capillaries leading to brain edema, alterations in the blood–brain barrier and microhemorrhage. Other suggestions for development of cerebral malaria are decreases in the integrity of the blood–brain barrier (Tripathi *et al*., 2007) and brain inflammation involving Toll-like receptors (Coban *et al*., 2007). In Malian children, cerebral malaria has been associated with group A DBL1α (Kyriacou *et al*., 2006). (Groups A-C of DBL1α are defined by the presence of one of three conserved 5′ upstream sequences—UpA, UpB and UpC—and by the position of the gene on the chromosome). Mechanical blockage of microvessels in the brain with attendant hypoxia and lactic acidosis due to the sequestration of non-deformable infected and uninfected red cells has been hypothesized to be responsible for cerebral malaria, however, not all agree with this

hypothesis (Haldar *et al*., 2007). Another hypothesis states that cerebral malaria is an inflammatory response due to release of a malaria toxin, glycosylphosphatidylinositol (GPI) that activates monocytes with proinflammatory cytokine (IL-6, TNF) secretion and release of nitric oxide (NO) and superoxide. In this scenario, the TNF would up-regulate ICAM-1 and other endothelial cell adhesion molecules. One of the criticisms of this inflammation hypothesis is that anti-inflammatory agents have been of no benefit to patients with cerebral malaria.

Haldar *et al*. (2007) suggest that if cerebral sequestration is responsible for coma but not death in patients with cerebral malaria, then a spectrum of sequestration pathologies would be expected at autopsy and many factors may lead to death (i.e. parasitemia, parasite strain, malnutrition, hyperthermia, dehydration, anaemia, acidosis, unprotected airway, acidosis and human polymorphisms). This is consistent with the relatively low cerebral malaria mortality; however, if sequestration is responsible for the death of the comatose cerebral malaria patient then perhaps an exact anatomical lesion (i.e. sequestration in the brainstem) would lead to sudden death.

In an attempt to reconcile these differences and provide a mechanism for cerebral malaria, Grau's group (Coltel *et al*., 2006; van der Heyde *et al*., 2006; Wassmer *et al*., 2006) has proposed that initially adhesion of falciparum-infected red cells is *via* P-selectin, CD36 and ICAM-1; this then activates endothelial cells to increase their production of adhesive molecules (IFN-γ, vascular endothelial growth factor, platelet activating factor, etc.). As a consequence, there is an enhancement in the degree of sequestration and this facilitates adhesion of platelets and leucocytes to the endothelium. According to van der Heyde *et al*. (2006) platelet adhesion activates a pathogenic inflammatory cascade that causes disruption of endothelial cell junctions, apoptosis of endothelial cells, sloughing, hemorrhage, disruption of the blood–brain barrier and ultimately cerebral malaria.

Were sequestration a key element to progression to severe malaria and/or cerebral malaria then it is conceivable that immunotherapy might alter the dynamics of parasite clearance and be beneficial to the patient (Taylor *et al*., 1992). Adherence to individual receptors was blocked by monoclonal antibodies and synthetic peptides in *in vitro* adherence studies (Crandall *et al*., 1993); administration of anti-malaria immunoglobulin to children with mild malaria reversed sequestration *in vivo* and evidence of synthetic peptide (HPLQKTY) blocking of adherence *in vivo* was also reported (Crandall *et al*., 1993 and citations in Goldring, 2004). Reversal of sequestration was shown in a squirrel monkey model of malaria by administration of hyperimmune sera (David *et al*., 1983) and adherence of isolates from Malawi was reversed with a pool of local sera (Goldring *et al*., 1992). However, in a double-blind, placebo-controlled administration of antibodies as an adjunct to quinine there was no measurable effect on adherence and patient recovery was unaffected (Taylor *et al*., 1992).

Goldring (2004), in evaluating immunotherapy, concluded, 'immunotherapy aimed at either parasite ligands or host receptors to reverse sequestration in the treatment of severe malaria infections is unlikely to be successful given the complexity and number of receptors and ligands and the calculated concentrations of antibodies required.' Perhaps the use of high-avidity synthetic anti-adhesives would circumvent the problems associated with immunotherapy.

Because of the difficulties in predicting the development of human cerebral malaria and in sampling brain tissue, studies have used either autopsy material or animal models, including mice and monkeys (Aikawa *et al*., 1992; de Souza and Riley, 2002; Hearn *et al*., 2000; Kaul *et al*., 1994; Penet *et al*., 2007; Shear *et al*., 1998; Togbe *et al*., 2007). The deficiencies in the use of human autopsy and primate materials have resulted in extensive studies using murine models of cerebral malaria. However, it should be emphasized that a key difference between the murine models and human cerebral malaria is that *P. berghei* (ANKA) and *P. yoelii* (17XL) primarily induce adherence of leucocytes to the vascular endothelium, whereas in human cerebral malaria it is infected red cells that are primarily adherent to the endothelium. In addition, murine cerebral malaria is not associated with neurological impairment and coma. Despite these caveats, murine models could reveal something about human cerebral malaria as well as providing insight into the nature of murine cerebral malaria pathogenesis. A recent study compared genetically resistant and susceptible inbred strains of mice to infections with *P. berghei* (ANKA) (Lovegrove *et al*., 2007) It found that most genes were differentially expressed in susceptible mice 1–2 days before the onset of symptoms of cerebral malaria. Most of the differentially expressed genes were associated with IFN-regulated processes and neuronal apoptosis.

The past 25 years has witnessed an explosion of new research on the identification of the erythrocyte surface proteins of *Plasmodium*-infected cells, notably *P. falciparum*, as well as in defining their role in immune evasion, cytoadherence and pathogenesis. This has been achieved largely due to the availability of entire genomes, the development of microarray expression profiling, transfection systems, cloning, expression and tagging, and other sophisticated molecular tools. Yet there is much work ahead before it will be possible to prevent or reverse cytoadherence/sequestration and to circumvent pathogenesis. Understanding how signaling between host and parasite is accomplished is still beyond our ken as are the molecular/biochemical mechanisms whereby variant antigen switching occurs. Finally, it should be emphasized that until we have a clearer understanding of the manner by which immune adults manage to control their infections, there will be little prospect for developing a practical protective vaccine and as a consequence we may have to rely on chemotherapeutic interventions for many years to come.

CHAPTER 21

Trafficking

During its erythrocytic asexual cycle the malaria parasite develops in a cell that is essentially a viscous solution of hemoglobin enclosed by a plasma membrane. However, despite the mature human erythrocyte being devoid of intracellular organelles and incapable of *de novo* protein synthesis, as well as lacking in the capacity for membrane turnover, upon infection it can be re-modeled through modifications of intrinsic membrane proteins and export of parasite-encoded proteins. The dramatic morphological changes of a *Plasmodium falciparum*-infected red cell, especially the appearance of knobs, are accomplished by trafficking parasite proteins across the host cell cytoplasm to the cell surface. Understanding the export pathways and molecular mechanisms involved could contribute to the development of novel therapeutic approaches to prevent sequestration and pathological sequelae.

With the invasion of erythrocytes (as well as hepatocytes) there is invagination of the host cell plasma membrane and as a result the parasite becomes enclosed in a membrane-lined parasitophorous vacuole (PV). The parasitophorous vacuolar membrane (PVM), derived from the red cell membrane, is devoid of red cell membrane proteins, however, its lipid composition is similar to that of the host cell during invasion (Ward *et al*., 1993). Over time, the PVM enlarges to accommodate the growing parasite and is modified though the development of membranous structures, including a tubulovesicular network (TVN) and cisternae-shaped membrane vesicles termed Maurer's clefts. The identification of the molecular characteristics of these various membrane structures progressed slowly largely due to difficulties associated with isolation and assurances of purity; however, in recent years significant advances have been made by the use of reporters such as green fluorescent protein (GFP),

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fluorescent lipids and transfection techniques combined with confocal fluorescence microscopy, immunoelectron microscopy and biochemical techniques.

In 1902, using light microscopy and Giemsa-staining, Georg Maurer described stippling and dots in the red cell cytoplasm of *P. falciparum*-infected red blood cells. These structures, named Maurer's clefts, are a feature of red cells infected with mature stages of *P. falciparum* (Haeggstrom *et al*., 2007) and, when such cells were examined using the transmission electron microscope, Maurer's clefts were seen as membranous sacs with a translucent lumen and an electron-dense coat lying just below the red-cell surface (Atkinson and Aikawa, 1990; Kriek *et al*., 2003; Langreth *et al*., 1978; Trager *et al*., 1966). Initially they appear twisted and branched and near the PVM but later they relocate closer to the red cell membrane. The TVN consisting of interconnected tubules and vesicles grows during asexual development but does not undergo rapid movement. In addition, although Maurer's clefts can be distinguished from the TVN by the presence of specific parasite proteins (Hawthorne *et al*., 2004; Taraschi *et al*., 2003; Vincensini *et al*., 2005) a physical connection to other membrane systems was the subject of controversy for several years. The TVN was first reported to be involved in nutrient uptake: it contains erythrocyte raft proteins whose import was restricted when tubule development was arrested by blocking sphingomyelin synthase. Indeed, using a threo-phospholipid analogue, 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol, tubule formation was retarded, raft accumulation decreased and nutrient uptake blocked (Lauer *et al*., 1997). TEM (Langreth *et al*., 1978) and serial sections (Bannister *et al*., 2004), as well as fluorescence microscopy with lipid probes and serial sections of the *P. falciparum*-infected red cell, led to the postulate that Maurer's clefts and the TVM form a continuous meshwork (Wickert *et al*., 2003). Suggestive evidence for Maurer's clefts being a domain of the TVN came from co-localization of a putative Maurer's cleft protein, HRP2, to the tip of the TVN (Lauer *et al*., 1997). However others, using GFP chimeras of Maurer's cleft cargo, provided evidence that the two were distinct entities (Knuepfer *et al*., 2005). HRP2 is a soluble protein found in the red cell cytosol and is released upon lysis of the red cells so it cannot act as a marker for a connection. Further, it was contended that a physical barrier prevented diffusion of protein between adjacent Maurer's clefts and no evidence was found for a continuity of these structures (i.e. Maurer's clefts, TVM with the PVM) (Knuepfer *et al*., 2005), however, more recent studies suggest that nascent Maurer's clefts appear to form sub-domains of the PVM/TVN (Spycher *et al*., 2006). The rarity of finding connections between the TVN and the PVM (as well as Maurer's clefts) may be due, as Kasturi Haldar claims (Haldar *et al*., 2005), to technical difficulties associated with electron microscopy as well as the undulating nature of

the TVN tubules, or more likely that such connections are only transitory. What recent experiments do make clear is that Maurer's clefts bud from the PVM and diffuse within the cytoplasm of the red cell before 'taking up residence at the cell periphery' (Spycher *et al*., 2006).

Kasturi Haldar (1957–) grew up in India where she was first exposed to the devastating effects of parasitic diseases. She received her university education in the United States sponsored by a Government of India Merit Scholar and National Science Scholarship. From 1974 to 1978 she attended at Bryn Mawr College (Bryn Mawr, Pennsylvania) and received her bachelor of arts (BA) in 1978. Kasturi then moved to the Massachusetts Institute of Technology (Cambridge, Massachusetts) where in 1982 she completed a doctor of philosophy (PhD) in biochemistry working on *Escherichia coli* membrane vesicles ('*Proton translocation and quinone reduction catalyzed by* D-*amino acid dehydrogenase*') under the supervision of Christopher Walsh. In 1983–1984 she was a postdoctoral fellow with George Cross at the Rockefeller University (New York, New York) and after one year of leave to work in the Institute of Basic Medical Sciences at the Capitol Medical College (Beijing, China) she returned to Rockefeller as a research associate (1985–1987) working on identification of the transferrin receptor of *P. falciparum*, the transport of phospholipid (PL) analogues from the red cell to the parasite and the accumulation and metabolism of ceramides. In 1988, she moved to Stanford University (Palo Alto, California) where first as an assistant professor of microbiology and immunology, and later as an associate professor (1994–1998) she continued to carry out studies on intracellular transport: the movement of fluorescent tracers in *P. falciparum*, the inhibition of protein secretion by brefeldin A and discovery of the TVN. In 1998, she moved to the Northwestern University Feinberg School of Medicine (Chicago, Illinois) where she is presently professor of pathology and microbiology and immunology. Her current research is focused on how secretory pathways deliver proteins and lipids to organelles to support the growth of membranes especially the role of lipid rafts. Using the emerging genetic techniques in the development of functional assays she has mined databases for functional motifs of unique organelles to develop a high-throughput assay and to use microarrays to track global changes in secretory gene expression during intracellular development of *Plasmodium* and other pathogens such as *Salmonella*, *Mycobacterium*, *Chlamydia* and *Toxoplasma*. Her long-term research goals are to understand the common principles of vacuolar biogenesis in intracellular parasites in order to identify targets for immunological prophylaxis and/or chemotherapy.

Plasmodial protein transport (i.e. trafficking) has been proposed to be a multi-step process: secretion, translocation across the PVM, movement and specific sorting in the red cell cytoplasm, followed by translocation/insertion into the target site. Trafficking has recently been the subject of several reviews (Cooke *et al*., 2004a; Lanzer *et al*., 2006; Lingelbach and Przyborski, 2006; Marti *et al*., 2005; Przyborski *et al*., 2003; Przyborski and Lanzer, 2005; Tilley *et al*., 2007; Tonkin *et al*., 2006; van Ooij and Haldar, 2007).

It has been shown that entry into the parasite's default pathway (bulk flow) is *via* the endoplasmic reticulum mediated by a canonical (Burghaus and Lingelbach, 2001) or an unconventional *N*-terminal sequence (Wickham *et al*., 2001). The default pathway allows plasmodial proteins to be trafficked across the parasite plasma membrane (PPM) into the lumen of the PV. Additional sequences are required for proteins to be exported beyond the PVM (and into the red cell cytoplasm), and this is thought to occur *via* selective translocators in the PVM. Recent studies with knob-associated histidine-rich protein (KAHRP) have shown that although signal sequences with a recessed hydrophobic signal are necessary and sufficient for secretion into the PV, *N*-terminal signal sequences also mediate transport into the PV; however, a second signal is required for translocation across the PVM (Rug *et al*., 2006). Two laboratories have shown that a short plasmodial peptide motif is necessary for protein export across the PVM into the red cell cytoplasm. The motif, termed *Plasmodium* export element (PEXEL) by Marti *et al*. (2004) or host targeting (HT) by Hiller *et al*. (2004) has the consensus R/KxLxEQ with positions 1, 3 and 5 being critical. Identification of PEXEL has allowed a motif search of the *P. falciparum* genome at the website: http://www.plasmodb.org (last accessed 16 July 2008) that predicts an 'exportome' (called the 'secretome' by Hiller *et al*., 2004) of approximately 400 proteins (i.e. about 8% of the proteome) that are exported to the cell where they may be 'involved in antigenic and structural modifications of the erythrocyte membrane and cytoplasm, mediate nutrient import from the red blood cell into the parasite and provide the machinery for protein export to the erythrocyte' (Marti *et al*., 2004). Identification of signal motifs has allowed for the prediction of exported proteins, the 'exportome', for *P. yoelii*, *P. berghei*, *P. chabaudi*, *P. vivax* and *P. knowlesi* as well as *P. falciparum*; the 'core' exportome for the genus *Plasmodium* is postulated to be involved in the re-modelling of the host red cell, whereas the genes for *P. falciparum* 'probably encode proteins directly or indirectly involved in the different properties of this parasite' (Sargeant *et al*., 2006).

Lingelbach and colleagues using biochemical methods, have shown that within the parasite *P. falciparum* erythrocyte surface protein-1 (PfEMP1) exists in a soluble form and becomes increasingly insoluble as it proceeds along the pathway to its final destination (Papakrivos

et al., 2005). These observations indicate that membrane insertion of PfEMP1 occurs after the protein is transported out of the endoplasmic reticulum. Moreover, the physical properties of PfEMP1 in the erythrocyte membrane resembled proteins that are part of larger complexes rather than integral membrane proteins. With a GFP-PfEMP1 chimera evidence was found for its presence in the red cell cytoplasm. Observation of diffusion of the fluorescent chimera was interpreted to mean that PfEMP1 was present as a soluble complex in red cell cytoplasm (Knuepfer *et al*., 2005) and membrane insertion took place only after export into the host cell. Together, these data suggest a trafficking pathway of PfEMP1 that differs distinctly from the transport pathways of surface proteins in eukaryotic cells.

After PfEMP1 insertion into the Maurer's clefts membrane, either within or attached to the cytoplasmic face of the cleft, the protein moves to the membrane cytoskeleton. Here PfEMP1 becomes anchored at the knob structure *via* the acidic terminal sequence (ATS) and its extracellular binding domains become exposed on the red-cell surface. The final translocation of PfEMP1 on to the surface of the red cell has been shown to involve a Maurer's cleft protein, skeleton binding protein 1 (SBP1). SBP1 is a 48-kDa integral membrane protein that spans the cleft membrane with its *N*-terminus within the cleft and the *C*-terminus exposed to the cytoplasm (Blisnick *et al*., 2005). In clonal transgenic parasite lines in which SBP1 was not expressed (i.e. SBP1 'knockouts'), PfEMP1 was not found on the red-cell surface (and the cells did not bind to CD36), yet the number of knobs appeared to be the same in the knockout as in the wild type; cleft number and structure were also unaffected. Moreover, other proteins associated with Maurer's clefts such as *P. falciparum* erythrocyte surface protein-3 (PfEMP3), Pf332, KAHRP, REX (Hawthorne *et al*., 2004) and MAHRP (Spycher *et al*., 2006) were trafficked normally to the underside of the red cell membrane. It was hypothesized that the effect of SBP1 on PfEMP1 translocation is indirect and subtle, affecting cleft morphology and distance to the infected red-cell surface such that the final translocation step is inefficient (Cooke *et al*., 2006). Further, they reason, since other proteins were unaffected it appears these proteins have a larger margin of error for binding to the cytoskeleton. The final step in the delivery of PfEMP1 to the red cells may involve fusion with the cholesterol-rich microdomains in the red cell membrane (Frankland *et al*., 2006).

Brian M. Cooke (1964–) received an undergraduate degree from Bristol University (Bristol, United Kingdom) in 1989, and then moved to The University of Birmingham Medical School (Birmingham, United Kingdom) to begin a PhD in hematology with Gerard Nash and John Stuart. Under their guidance, he worked on malaria and sickle cell

disease receiving his PhD in 1993. During the early 1990s, Brian spent time in The Gambia, West Africa, working with Brian Greenwood to extend his studies to isolates of malaria parasites in a clinical setting. There, he was the first to study the interactions of malaria-infected red blood cells with a variety of different vascular adhesion molecules and made an assessment of the role of different adhesion molecules in the development of clinical malaria. Cooke's independent research career was launched properly when he moved to Australia in 1993 to join the malaria research group at WEHI in antigen discovery and vaccine research. During that time, under the supportive directorship of Sir Gustav Nossal, he was encouraged to set up a new laboratory with his colleague and mentor Ross Coppel at Monash University in Melbourne. Together they established a unique and internationally recognized molecular and cellular biorheology laboratory at Monash University. Over the past 15 years, Cooke's work at Monash has focused on understanding the molecular basis by which malaria parasites cause disease in humans by modifying the properties of red blood cells in which the parasites reside, including the mechanisms involved in the trafficking of virulence proteins to the red blood cell surface. He and his group are part of a worldwide consortium studying the functional properties of novel genes identified in the genome of *P. falciparum*. The Cooke and Coppel groups at Monash University are supported by national and international research grants from Australia, the United Kingdom and the United States, including major funding from The Bill & Melinda Gates Foundation, to identify novel drug targets and to develop an effective malaria vaccine. Cooke is presently a National Health and Medical Research Council senior research fellow and associate professor in the Department of Microbiology at Monash University.

In accord with the results of Cooke *et al*. (2006), Maier *et al*. (2007) found that a *P. falciparum* skeleton-binding protein 1 (PfSBP1) knockout resulted in marked reduction in surface-exposed PfEMP1 and because other cleft proteins such as KAHRP and MAHRP trafficked normally, PfSBP1 was required for trafficking of PfEMP1 from the parasite to Maurer's clefts. But, in contrast to Cooke *et al*. (2006), they contend that PfSBP1 is responsible for the transfer of PfEMP1 from the PV to the Maurer's clefts. Since PfPBSP1 lacks a PEXEL motif, it must be directly loaded into Maurer's clefts, possibly crossing the PVM by being associated with a PEXEL-containing protein.

PfEMP3, deposited on the cytoplasmic face of the erythrocyte cytoskeleton, is a component of the electron-dense plaque knob. Although truncation prevented its distribution at the red cell membrane (and knobs

appeared to be smaller), transfer of PfEMP1 to the surface was unaffected, as was cytoadherence (Waterkeyn *et al*., 2000). GFP-PfEMP3 chimeras were exported to the red cell cytoplasm as aggregates rather than in vesicles and were trafficked to Maurer's clefts. Since PfEMP3 was not required for PfEMP1 trafficking its precise role remains unclear, but what is known is that it contributes to the reduced deformability of parasitized cells (Glenister *et al*., 2002).

A variation on the trafficking of PfEMP1, KAHRP and PfEMP3 is seen with STEVOR. STEVOR proteins are expressed in gametocytes and sporozoites as well as on the surface of red cells bearing asexual stages (McRobert *et al*., 2004). The precise function of STEVOR variants is unknown. STEVOR proteins contain two transmembrane domains and a predicted signal sequence. The transmembrane domains are crucial for the targeting of STEVOR to Maurer's clefts and when this is deleted it accumulates in the red cell cytoplasm in a soluble form. Although STEVOR and PfEMP1 are both transported to Maurer's clefts, STEVOR traverses the secretory pathway as an integral membrane protein (Przyborski *et al*., 2005) and there is no co-regulation of the *var* and *stevor* gene families (Duffy and Tham, 2007; Sharp *et al*., 2006).

Trafficking of PfCG2, a non-integral high-molecular-weight protein (320–330 kDa) located on the cytosolic face of the PVM to the digestive food vacuole (FV) has been described (Cooper *et al*., 2005). The levels of PfCG2, found in electron-dense patches along the PVM, the cytoplasm and the digestive FV, increase through the trophozoite stage and decline rapidly in the schizont. Since PfCG2 does not have a PEXEL-targeting sequence (necessary for secretion from the PV into the red cell cytoplasm), the mechanism for its trafficking to the digestive FV remains an open question. Once inside the FV, PfCG2 is subjected to proteolysis by plasmepsins, and becomes associated with the membranous material between hemozoin crystals, and could serve as a nucleation site for malaria pigment formation. Although a precise function for PfCG2 is unknown, its importance is suggested by the fact that attempts to knockout the gene have been unsuccessful.

Leann Tilley (1958–) grew up in the small town of Stawell in Victoria, Australia. She moved to Melbourne to start her university studies and obtained a bachelor of science (BSc) (Hons) from the University of Melbourne (Melbourne, Vic, Australia) (1978) and a PhD in biochemistry from the University of Sydney (Sydney, NSW, Australia) (1984). After post-doctoral fellowships at the University of Utrecht (Utrecht, The Netherlands) (1985) and the Collège de France (Paris, France) (1986), she returned to Australia and the University of Melbourne (1987–1988) where she continued her studies of the proteins and lipids

of the human erythrocyte membrane using fluorescence-based biophysical methods. This led quite naturally to collaborative studies with Robin Anders and Mick Foley at Walter and Eliza Hall Institute (WEHI) and the University of Melbourne on *P. falciparum*-red cell protein interactions. In 1989, she joined the Biochemistry Department at La Trobe University (Vic, Australia) and was appointed professor in 2004. At La Trobe, Tilley established a malaria research program using a series of fluorescence-based imaging techniques to ask questions about the cell biology of *Plasmodium*. A Wellcome grant helped establish a confocal fluorescence photobleaching facility and a Linkage Infrastructure Equipment and Facilities (LIEF, Australia) grant for a confocal fluorescence correlation facility. Currently, Tilley is deputy director of a new Centre of Excellence for Coherent X-ray Science (La Trobe University, Vic, Australia). The Centre brings physicists, chemists and biologists together to develop fundamentally new approaches to probing biological structures and processes. It combines world-class expertise in imaging, structural biology, laser science and molecular theory. The project will develop novel high-resolution imaging using the Australian Synchrotron, and ultimately x-ray lasers, to determine the structures of important drug targets whose molecular architecture cannot be determined with current techniques. Her laboratory is using a range of techniques to image the ultrastructure of malaria parasite-infected erythrocytes with particular emphasis on protein trafficking and the molecular mechanisms of anti-malarials.

Although defining the molecular mechanisms involved in the regulation of trafficking of plasmodial proteins has been a study of errors slowly corrected it is now clear that this complex process will continue to provide challenges for biochemists and molecular biologists and a fuller understanding will require new methodologies and approaches.

As a teenager, after reading '*Of Microbes and Life*' by Jacques Monod, which describes his discovery of the lactose operon in *E. coli*, **Michael Lanzer (1959–)** decided he wanted to become a molecular biologist—and he did. From 1979 to 1984, Lanzer studied biology at the University of Heidelberg (Heidelberg, Germany). Hermann Bujard who, more than 20 years after the initial discovery by Jacob and Monod, had some unresolved questions regarding gene regulation of the lac operon, accepted Lanzer as a PhD student and 3 years later he published his first scientific paper on the thermodynamic parameters that determine the intricate interplay between the ribonucleic acid (RNA) polymerase, repressor, promoter and operator responsible for the tight

regulation of the operon. This study, which appeared in the *Proceedings of the National Academy of Science USA*, remains one of his most cited publications. During the period when he was pursuing his PhD (1984–1987) he spent some time at the pharmaceutical company Hoffmann LaRoche in Basel, Switzerland. At that time, Hoffmann LaRoche had a small but effective malaria research team, and many prominent malariologists of the time, including John Scaife and Ruth and Victor Nussenzweig, came to visit, and their descriptions as well as a self-realization led to Lanzer's recognition of the beauty of *P. falciparum* with its many developmental stages and its complex life cycle. The unresolved questions of its biology, the humanitarian problem and the possibility of finding a niche in a research field that was small and less competitive convinced him to try his luck at conducting malaria research. At the Memorial Sloan Kettering Cancer Center in New York in the laboratory of Jeffrey Ravetch, he found the right environment. Ravetch had just published a number of seminal papers on *P. falciparum* biology and Lanzer joined his team to study gene regulation and chromosome structure and function in this parasite. It was a very productive and inspirational time (1988–1993). For example, together they were the first to clone an entire *P. falciparum* chromosome using yeast artificial chromosomes. In 1993, Lanzer was appointed assistant member at the University of Würzburg (Würzburg, Germany) and in 1999 full professor of parasitology at the University of Heidelberg. In succeeding years he has remained true to malaria with current research focusing on mechanisms of drug resistance and on protein targeting and trafficking pathways in the parasite.

Klaus Lingelbach (1955–) studied biology at the universities of Tuebingen (Tuebingen, Germany) and Heidelberg with major subjects zoology/parasitology. Having acquired a solid basis in general parasitology, he became interested in the molecular basis of host–parasite interaction. Upon completion of his diploma thesis on tapeworms (1982, '*The immune response to* Echincoccus multilocularis') he then joined the immunoparasitology unit of WEHI where he became involved in malaria research and in the efforts towards the development of a vaccine. At that time Lingelbach realized that very little was known about the cell biology of the parasite and the infected host cell. To gain more experience in basic cell biochemistry, he joined the Biochemistry Department at La Trobe University, where he completed a PhD (1986) on mitochondrial protein import under the supervision

of Nick Hoogenraad. In retrospect, the scientific community in Melbourne provided an ideal environment for a young scientist to combine modern biochemistry with excellent parasitology. During that time, Lingelbach started to develop a scientific and experimental framework to study protein trafficking in the infected erythrocyte. In 1987, he joined Bernhard Dobberstein's group at the European Molecular Biology Laboratory in Heidelberg (Heidelberg, Germany) where he became acquainted with the molecular principles of protein secretion. In 1989, Lingelbach was offered a position as a junior group leader at the University of Hamburg (Hamburg, Germany), where he began his work on protein secretion in *P. falciparum*. In 1992, he joined the Bernhard Nocht Institute for Tropical Medicine (Hamburg, Germany) first as an independent group leader and, later, as the co-ordinator of the Molecular Parasitology Program. In 1996, he was appointed professor for parasitology at the Philipps-Universität Marburg (Marburg, Germany) Lingelbach's research interests continue to be describing the mechanisms that underlie host cell modification by secreted proteins in the *P. falciparum*-infected erythrocyte.

CHAPTER 22

Erythrocyte Membrane Lipids

The earliest investigations of changes in red blood cell lipids associated with the intracellular development of the malaria parasite date to the late 1940s; then there were attempts to understand better the mode of action of antimalarials and to find ways to improve the media being used for *in vitro* growth of *Plasmodium* spp. Then in the late 1960s and early 1970s, after a hiatus when few studies of lipids were conducted, funding by the United States Army, the Walter Reed Army Institute of Research (WRAIR) and the United Kingdom Medical Research Council (MRC), as well as the availability of new analytical tools such as gas liquid and thin layer chromatography, stimulated research on lipid metabolism of the then favored model malarias: *Plasmodium knowlesi*, *P. lophurae* and *P. berghei*. The general conclusions from the studies were: a marked increase in the phospholipid (PL) content of the malaria-infected red cell, glycerol and glucose served to form the glycerol backbone, the parasites were unable to synthesize fatty acids (FAs) *de novo* and depended on host-supplied FAs, and plasmodia had the capacity for the *de novo* fabrication of PLs (reviewed in Holz, 1977a and Sherman, 1979). The precise biochemical mechanisms the plasmodia used for PL synthesis would not be discovered for at least another decade, when it did it was largely due to the work of Henri Vial and his collaborators.

Our investigations on lipids began in 1970 at the Marine Biological Laboratory (MBL) in Woods Hole (Massachusetts) when George G. Holz Jr. (1922–1989), Clark P. Read (1921–1973) and I were instructors in the invertebrate zoology course. During the academic year, Holz was a faculty member at the Upstate Medical Center in Syracuse (New York) with a primary interest in the lipids of protozoans and Read was a faculty member at Rice University (Houston, Texas) investigating the transport

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properties of tapeworms. My research focus was on the physico-chemical properties of the membranes of malaria parasites, but since malaria parasites were not marine organisms and thus unavailable at the MBL, I apprenticed myself to Holz and his colleague David H. Beach and two of Read's post-doctoral students (George Buteau and John Simmons) as they began investigating the lipids of tapeworms from triakid sharks (Buteau *et al*., 1971). Although at first glance the differences between flatworms and malaria parasites would seem to be so great that little of value could be learned, this turned out not to be the case: both tapeworms and malaria parasites were found to be incapable of *de novo* synthesis of non-volatile saturated and unsaturated FAs and consequently they had to rely on their hosts to supply precursors for lipid biosynthesis. In subsequent summers at the MBL Holz and I shared a research laboratory and there, as well in our home institutions in Syracuse and University of California at Riverside (UCR, Riverside, California), we analyzed the lipids of *P. lophurae* and the duckling-infected red cell. One of our earliest findings was that octadecenoic FAs—principally oleic and to a lesser degree cis-vaccenic acid—were elevated in lophurae-infected red cells over uninfected cells in all classes of membrane PLs (but especially phosphatidylcholine (PC) and phosphatidylethanolamine (PE)) and in the blood plasma (Beach *et al*., 1977). Both FAs were found to be hemolytic (Holz *et al*., 1977). We suggested that the octadecenoic FAs could modify membrane fluidity and in turn the functional properties of the plasma membrane of infected red cells.

We found the principal FAs upon infection to be oleic acid (18:1) and saturated FAs. The most characteristic increases in FAs were in palmitic (16:0), which went from 19% to 28%, stearic (18:0) from 10% to 14% and oleic (18:1) from 18% to 28%. The relative paucity of polyunsaturates in PLs led us to suggest that there were defects in appropriate desaturases, chain elongation systems and acylation enzymes and we concluded that 'the malaria parasite, though it may have no capacity for *de novo* biosynthesis of lipids from acetate, can regulate its use of host cell lipids and lipid precursors in such a manner as to establish and maintain a lipid composition distinct in many respects from that of the erythrocyte' (Beach *et al*., 1977).

I would have liked to continue the lipid studies, however, attempts to obtain funding from several granting agencies were rejected for two reasons: it was suggested that we were not working with a 'real' malaria, meaning *P. falciparum*, and one grant reviewer claimed that since lipids did not show heterogeneity it was unlikely they would provide a suitable target for chemotherapeutic intervention. These criticisms have turned out to be without merit (Mitamura and Palacpac, 2003). Indeed, 'malaria parasites meet their demand for lipid species by active synthesis through *de novo* pathways using precursors that are actively transported from the host'... and the unique features in lipid biogenesis that have enabled

Plasmodium spp. to survive within the erythrocyte may also serve as rational drug targets (Vial and Calas, 2001; Vial and Ben Mamoun, 2005).

The average human red cell plasma membrane is lipid-rich with 240 million PL molecules, 190 million cholesterol molecules, 12 million glycolipid molecules and only 4 million protein molecules (Lux and Glader, 1981). The PLs of the red cell plasma membrane are asymmetrically distributed in the bilayer. The choline-containing PLs, PC and sphingomyelin represent 40% and 15%, respectively, of the total PL, and are enriched in the outer leaflet. In contrast, the amino PLs, PE and phosphatidylserine (PS) are exclusively confined to the inner leaflet and constitute 35% and 15%, respectively, of total PLs.

At the time we embarked on studies of malarial lipids a battery of biochemical methods, including phospholipase sensitivity, the amino-labeling agents TNBS (2,4,6-trinitrobenzene sulphonic acid) and fluorescamine, as well as annexin V binding, staining with merocyanine 540, and a PS-specific monoclonal antibody and prothrombinase activity, were available for monitoring alterations in PL asymmetry of red cells during parasitization by *Plasmodium* spp. In *P. yoelii*, *P. berghei* and *P. chabaudi*, no evidence was found for a redistribution of PS to the outer leaflet (Taverne *et al*., 1995) whereas with *P. knowlesi* there was (Gupta and Mishra, 1981). With *P. falciparum*, the findings were contradictory (reviewed by Vial and Ancelin, 1998). Although Taverne *et al*. (1995) found no change in PS distribution in *P. falciparum*-infected red cells, we did (Eda and Sherman, 2002; Sherman and Prudhomme, 1996). The reasons for the discrepancies, I believe, lie primarily in the sensitivity of the method used as well as the parasitemia and the developmental stage of the intracellular parasite examined, and were only secondarily due to strain/species differences. For example, we found that for annexin V binding a parasitemia of more than 20% was needed and parasites had to be at the trophozoite or schizont stage. Supporting our finding of PS externalization is the report by Facer *et al*. (1994) that anti-PL antibodies were elevated in patients with *P. falciparum* and the highest levels of IgM and IgG binding was to PS. In its externalization of PS the falciparum-infected red cell resembles an aged erythrocyte (Omodeo-Sale *et al*., 2003; Sherman *et al*., 2004). In addition to malaria-infected red cells, PS externalization has also been shown to occur with thalassaemic red cells, sickle cells and red cells from people with diabetes, all of which may be under oxidative stress. Further, in *P. falciparum*, exposure of PS in trophozoite/schizont-infected red cells has functional outcomes: enhanced adhesion to endothelial cells and CD36 (Eda and Sherman, 2004) as well as erythrophagocytosis.

The measurement of lipid molecules from one layer to the other (called flip-flop) is restricted by the activity of Mg^{2+} adenosine triphosphate (ATP)-dependent amino PL translocase; further, some investigators claim integral membranes may also play a role. Electron spin

resonance studies have demonstrated a marked increase in the flip movement of sphingomyelin, PE and PS in knowlesi-infected red cells whereas using NBD-PC, an accelerated transbilayer flip of PC, but not sphingomyelin was shown (Beaumelle *et al*., 1988). Modification in the cholesterol content of uninfected red cells promotes the externalization of PS, and cholesterol depletion of oxidatively stressed red cells significantly increases PS movement to the outer leaflet (Lopez-Revuelta *et al*., 2007). These may be contributing factors in PS externalization since as we have shown in *P. falciparum*-infected cells cholesterol levels decline (Maguire and Sherman, 1990) and infected cells are under increasing oxidative stress (Becker *et al*., 2004).

Cholesterol represents around 45% (by weight) of the membrane lipids of human red cells and is enriched in the outer leaflet of the bilayer. Malaria parasites lack the capacity for the *de novo* synthesis of cholesterol (Trigg, 1968b). Cholesterol is required in the medium for *in vitro* growth of *P. knowlesi* (Trigg, 1969) and both *in vivo* and *in vitro* is in dynamic equilibrium with the cholesterol in plasma lipoproteins (Holz, 1977b; Nawabi *et al*., 2003). The absence of cholesterol in the membranes of the parasite results in a cholesterol to PL molar ratio of 0.1 as compared to that of the erythrocyte membrane where the ratio is 0.8. The levels of cholesterol in the membrane of malaria-infected red cells were previously reported to be unaltered with parasitization in *P. falciparum* or reduced in *P. lophurae* and *P. falciparum* or increased in *P. knowlesi* (references in Maguire and Sherman, 1990 and Vial and Ancelin, 1998). As noted above, depletion of cholesterol could affect PS distribution, and in normal cells scrambling of the PLs by raising the cytoplasmic Ca^{2+} levels with ionomycin or the ionphore A23187 or low ionic strength buffer resulted in increased surface activity of cholesterol through enhanced 'bobbing' frequency (Lange *et al*., 2007). The enhanced 'bobbing' may allow cholesterol to leave the surface of the falciparum-infected red cell at an increased rate and could account for the findings of Maguire and Sherman (1990).

The two major PLs of *Plasmodium* are PC and PE with minor amounts of PS, phosphatidylinositol (PI) and cardiolipin. As with other organisms biosynthesis of PC begins with two acylation steps of glycerol-3-phosphate (formed from phosphorylation of glycerol by glycerokinase or reduction of the glycolytic intermediate dihydroxyactone-3-phosphate by dihydroxyactone-3-phosphate dehydrogenase). The pathway is illustrated in Vial *et al*. (2003) and Vial and Mamoun (2005) as well as in the website http://sites.huji.ac.il/malaria/FramLipids.html (last accessed 16 July 2008).

The first acylation, catalyzed by acyl-coenzyme A-glycerol-3-phosphate acyltransferase (GPAT), results in the formation of 1-acyl-glycerol-3-phosphate (lysophosphatide, LPA). The LPA serves as the substrate for the second acylation reaction, catalyzed by acyl-CoA-1-acylglycerol-3-phosphate transferase (AGPAT), to form phosphatidic acid (PA).

The AGPAT enzyme has been characterized biochemically in *P. knowlesi* (Beaumelle and Vial, 1988). Two GPAT genes, *P. falciparum* glycerol-3-phosphate acyltransferase (PfGAT) and PfPlsB, have been identified in the *P. falciparum* genome. The 583 amino acid protein encoded by PfGAT shares greatest homology with the yeast enzyme and is localized to the endoplasmic reticulum; attempts to knockout this gene have been unsuccessful suggesting it is essential (Santiago *et al*., 2004). PfPlsB encodes a 400 amino acid sequence and is probably in the apicoplast (see Chapter 13, p. 105), where it is involved in the type II FA synthesis pathway (see p. 109). Only one AGPAT gene, *PfPlsC*, has been identified in the *P. falciparum* genome and predicted to be localized to the apicoplast.

PA is converted to cytidine diphosphate-diacylglycerol (CDP-DAG) *via* CDP-DAG synthase (CDS). The CDS activity in *P. falciparum* has been characterized and the gene, present as a single copy on chromosome 14, has been cloned and sequenced (Martin *et al*., 2000). It encodes a protein of 667 amino acids with a predicted relative molecular mass (M_r) of 78 kDa with a unique *N*-terminal domain. The full-length protein undergoes rapid proteolytic processing during intraerythrocytic development of the parasite. Diacylglycerol (DAG) is produced through dephosphorylation of PA by 3-phosphatidate phosphohydrolase, and the *P. falciparum* genome reveals the presence of a putative gene on chromosome 6 that encodes a 461 amino acid protein with a phosphohydrolase signature domain; it lacks an apicoplast targeting sequence.

PC can be synthesized by three pathways: (1) from choline *via* the *de novo* synthesis pathway (also called the Kennedy pathway), (2) from ethanolamine (Etn) *via de novo* synthesis of PE and transmethylation and (3) from incorporation of serine followed by PS decarboxylation. The first two pathways involve a DAG-dependent reaction. The cytidine diphosphate-choline (CDP-choline) *de novo* pathway begins when choline is transported into the red cell (see p. 162) and converted into phosphocholine by a cytosolic, plasmodial choline kinase (CK). A gene, *P. falciparum* choline kinase (*PfCK*), encodes a polypeptide sharing amino acid sequence similarity with yeast CK. The next step, catalyzed by CTP phosphocholine cytidyl transferase (CCT) produces CDP-choline. The falciparum CCT gene has been cloned and sequenced; the protein, containing 370 amino acids, exists in an inactive soluble form and a membrane-bound active form that can be activated by anionic lipids (Yeo *et al*., 1997). Similar structures with two CCT domains are found in homologues of PfCCT in *P. knowlesi*, *P. yoelii* and *P. berghei* (Vial *et al*., 2004). The reaction of CDP-choline with DAG is catalyzed by CDP-choline: 1,2-DAG choline phosphotransferase and results in PC. Of some interest is that only a single gene, *P. falciparum* choline/ethanolamine-phosphate transferase (*PfCEPT*), localized to chromosome 6 and encoding a protein with 390 amino acids

exists in the *P. falciparum* genome and possibly catalyzes the last steps in the synthesis of both PC and PE (Vial and Ben Mamoun, 2005).

Catherine Braun-Breton (1953–) As a high school student studied the work of Monod, Jacob and Lwoff and was captivated by the power and elegance of bacterial genetics in deciphering the essential processes of cell biology. She was a successful candidate at the prestigious *Ecole Normale Supérieure* in 1972 and received a masters degree in genetics and microbiology (1974). Following attendance in the famous microbiology course of the Institute Pasteur (Paris, France), she joined the laboratory headed by Maxime Schwartz and Maurice Hofnung and started seven years of rewarding studies of the phage lambda receptor in *Escherichia coli*, supervised by the former students of the researchers whose very clever work had piqued her interest. In 1981, after a one-year post-doctoral position working on the red cell Na^+/K^+ ATPase in Guido Guidotti's laboratory at the Harvard Biological Laboratories (Cambridge, Massachusetts) she decided to capitalize on her expertise of membrane proteins and microbiology by joining the Pasteur Institute MalariaLaboratory in Paris, France, headed by Luis H. Pereira da Silva, as a research assistant. Her work led to the identification of GPI-anchored *P. falciparum* membrane proteins and the novel role this type of membrane anchor has in the regulation of serine-protease activity, its involvement in parasite entry into the red blood cell and its presence in the rodent malaria parasite *P. chabaudi*. Further studies using the enzyme activity purified from *P. chabaudi* and *P. falciparum* merozoites showed how the serine protease was involved in the formation of the PV. For the next 10 years, the molecular mechanisms of red cell invasion by malaria parasites became the main focus of her research, particularly the characterization of enzymes, phospholipases and proteases, central to this process. She eventually became research associate and later head of laboratory at the Institute Pasteur and chief of the Experimental Parasitology Laboratory when Luis H. Pereira da Silva retired in 1997. Her supervision of a PhD student at the Institute Pasteur of French Guyana gave a new and unexpected turn to her research interests with the characterization of a membrane protein of the Maurer's clefts, a membrane compartment transposed by the parasite into the cytoplasm of its host cell. The result was the discovery of interactions between Maurer's clefts and the red cell membrane that might play a role in the trafficking of parasite proteins to the red blood cell cytoplasm and plasma membrane and in preventing premature rupture of the red blood cell during the intraerythrocytic parasite development. Following 30 years of research and 10 years as the head of the microbiology course

at the Institute Pasteur, Braun Breton had, thanks to Henri Vial, the opportunity to apply for a full professorship position at the University of Montpellier (Montpellier, France) where she is currently heading the laboratory of dynamics of normal and pathological membrane interactions, teaching microbiology and parasitology and investigating further the biological roles of Maurer's clefts as an interface between the parasite and its host cell. This is in close collaboration with Michael Lanzer, Klaus Lingelbach and Marta Ponzi, all principal investigators (PIs) of the Biology and Pathology of Malaria Parasites (BioMalPar) European Network of Excellence.

Using biochemical studies, Vial's team discovered that *L*-serine can also provide substantial amounts of PE *via* a newly detected pathway that involved direct serine decarboxylation in Etn and subsequently phosphoryl-Etn (P-Etn). Serine decarboxylase activity determination includes the incorporation of radiolabeled serine, extraction of labeled metabolites and subsequent identification by reverse phase high-pressure liquid chromatography (HPLC) and/or three thin-layer chromatography methods) (Elabbadi *et al*., 1997). They show that formation of P-Etn cannot be supplied *via* the sphingolipid pathway, because of the lack of appreciable labeled sphingomyelin (or any metabolic intermediates) after incorporation of ^{3}H-serine or ^{3}H-palmitate, and a non-spontaneous, pyridoxal phosphate (PLP)-dependent serine decarboxylation reaction emerges as the only mechanism, most probably enzymatic, producing Etn and P-Etn. This activity was then described in plants. Later on, the available *P. falciparum* genome revealed the presence of one putative plant-like *S*-adenosyl-*L*-methionine-dependent three-step methylation phosphoethanolamine methyltransferase (PfAMT) (Gardner *et al*., 2002). This enzyme was then shown to convert P-Etn to phosphocholine but not choline and was localized to the Golgi apparatus (Witola *et al*., 2006). The gene encoding this activity, *PfPMT*, encodes a polypeptide of 266 amino acids with an *S*-adenosyl methionine-binding site having homology to the enzyme from plants, and its M_r is 31 kDa. Miltefosine, or hexadecylphosphocholine, a phosphocholine analogue, inhibits the enzyme with an IC_{50} of around 80 μM. Thus, this pathway consists of five enzymes of which three (ethanolamine kinase, CTP-phosphocholine phosphoethanolamine cytidyl transferase and choline transferase) are components of the CDP-choline pathway and two (serine decarboxylase and phosphoethanolamine methyltransferase) catalyse two plant-like reactions to produce phosphocholine, a PC precursor. Although this pathway could explain the experiments of Divo *et al*. (1985) and Mitamura *et al*. (2000) who showed that *P. falciparum* could be grown in the absence of choline in the medium, a connection between the different pathways leading to PC still needs to be proved.

Since a major route for the synthesis of PC requires host-supplied choline it was hypothesized that compounds that mimic choline structure would be effective anti-malarials (Vial and Calas, 2001). Of the hundreds of chemically synthesized choline analogues, the compound G25 (1,16-hexadecamethylenebis (*N*-methylpyrrolidinium) dibromide) efficiently inhibited pharmacoresistant malaria *in vitro*, with an IC_{50} of less than 1 nM (Ancelin *et al*., 2003) and eliminated *Plasmodium* infection without recrudescence in rodent (Ancelin *et al*., 2003), and primate models (Wengelnik *et al*., 2002) at very low doses.

To solve the high toxicity and low oral absorption issues of bis-thiazolium salts, and their non-ionic precursors, which *in vivo*, after enzymatic transformation can lead to thiazolium drugs, were synthesized. The bis-thiazolium compounds, T3 (3,3′-dodecane-1,12-diylbis[5-(2-hydroxyethyl)-4-methyl-1,3-thiazol-3-ium]dibromide) and their neutral bio-precursors, exert a very rapid cytotoxic effect against malarial parasites in the very low nanomolar range. They are able to cure highly infected mice with an ED_{50} lower than 0.2 mg/kg as well as retain full activity against *P. cynomolgi* with no recrudescence at lower doses (Vial *et al*., 2004). The bisthazolium drug, T16, inhibited PC metabolism of red blood cells infected with *P. falciparum* and *Babesia* (Richier *et al*., 2006). These compounds show high levels of protection against the two major human malaria parasites, *P. falciparum* and *P. vivax*, and have shown high efficacy against multi-resistant *P. falciparum* malaria as well as activity against sexual stages of *P. falciparum*. They mimic the choline structure, and were shown to be specifically accumulated inside infected erythrocytes, thus ensuring both potency and selectivity. They blocked the plasmodial phosphatidylcholine biosynthesis as well as interacting with heme/hemozoin (plasmodial hemoglobin metabolites) (Biagini *et al*., 2003). Their dual mechanism of action should limit the risk of emergence of resistance. Although the data indicate that the lethal effect is closely related to the blockage of phosphatidylcholine biosynthesis, the exact steps that mediate selective inhibition of this metabolic pathway have yet to be clarified (Vial, personal communication). Clinical trials (phase I) on T3 were initiated in May 2007.

Although PS accounts for less than 5% of *Plasmodium* lipids, it is a key intermediate in the synthesis of PE, which serves as a precursor to PC (see above). PS is synthesized by a condensation of serine with CDP-DAG catalyzed by PS synthase (PSS). Initially, PSS was characterized biochemically in *P. knowlesi* and later *P. falciparum* (Elabbadi and Vial, personal communication). Two putative genes that possibly encode PSS have been identified in the *P. falciparum* genome base and a recombinant protein has been made. In *P. falciparum*, the putative PSS gene is transcribed during the ring and trophozoite stages. PS decarboxylation *via* PS decarboxylase (PSD) was first demonstrated in *P. knowlesi*-infected red cells where it was found that radiolabeled serine was incorporated into Etn (Elabbadi *et al*., 1997).

The gene, *P. falciparum* phosphatidylserine decarboxylase (*PfPSD*), was identified in 2004 and its product localized to the endoplasmic reticulum (Baunaure *et al*., 2004). Since the structural and catalytic properties of the PSD from human and *P. falciparum* are so similar it is unlikely to be a suitable drug target. Thus, *L*-serine is likely to play a major role in the plasmodial PL biosynthesis. PE from the *P. falciparum* parasites can be biosynthesized from Etn *via* a direct serine decarboxylation and the *de novo* CDP-Etn pathway. In the light of the PS conversion into PE and PC, PS is a PL that is very abundantly biosynthesized by *Plasmodium*-infected erythrocytes, and serine metabolism through its direct decarboxylation or through enzymes involved in PS synthesis and PS decarboxylation are likely to be of importance in PL biosynthesis in the malaria parasite (Vial and Ben Mamoun, 2005).

In eukaryotes, sphingomyelin biosynthesis involves adding a phosphocholine from PC to ceramide (a precursor for complex sphingolipids, glycolipids or sulphatides) through the action of sphingomyelin synthase. The sphingomyelin can then be broken down into ceramide *via* sphingomyelinase. When radioactive serine and palmitic acid were used as precursors, there was no evidence for the synthesis of ceramide by *P. falciparum*, however, using the fluorescent ceramide analogue or a radiolabeled short-chain ceramide there was incorporation into sphingomyelin suggesting the presence of sphingomyelin synthase. Indeed, ceramide analogues have been proposed as potential antimalarials (Labaied *et al*., 2004). Using a bioinformatics approach a new class of sphingomyelin synthase (SMSs) has been identified in the falciparum genome (Huitema *et al*., 2004). Two genes, PfSMS1 and PfSMS2, located on chromosome 6, encode a protein with 461 amino acids and are expressed throughout the life cycle of asexual erythrocytic stages as well as in gametocytes and sporozoites (Hanada *et al*., 2002).

A perplexing question has been: what supplies the ceramide? Recently with the demonstration of sphingomyelinase in *P. falciparum* it was proposed that the plasmodium degrades host sphingomyelin to provide ceramide (Hanada *et al*., 2000). In support of this is the observation that the sphingomyelin content of *P. falciparum*-infected red cells declines with parasite development (Maguire and Sherman, 1990) and this coincides with the transcription of the sphingomyelinase gene (Hanada *et al*., 2002). Another possibility is that a plasmodial sphingomyelinase is involved in the turnover of already assembled sphingomyelin in the falciparum-infected red cell. This unique sphingomyelinase (a lysocholine PL phospholipase C)—an isozyme—may be a possible new antimalarial target. Indeed, scyphostatin, an inhibitor of mammalian sphingomyelinase impaired the maturation of *P. falciparum* trophozoites at around 3 μM (Hanada *et al*., 2000).

PI is involved in membrane anchoring of proteins *via* GPI and the inositol moiety is a substrate for lipid kinases yielding phosphoinositides involved in cytoskeletal organization, vesicle transport and signal transduction.

In *P. falciparum*, PI is the PL showing the highest relative increase (3% to 11%) after infection. PI is synthesized from CDP-DAG and myo-inositol, a reaction catalyzed by PI synthase. This enzyme has been characterized biochemically in *P. knowlesi* and *P. falciparum* and recently the gene has been cloned and sequenced (Wengelnik and Vial, 2007). Both genes are highly spliced, with nine exons of up to 40–135 bp length of which five exons are shorter than 60 bp, and encoding a protein of 209 and 207 amino acids, respectively. Two protein kinase sites are present. The high sequence conservation allowed prediction of the gene in *P. chabaudi*, *P. berghei* and *P. vivax*.

The PE content of plasmodial membranes is high relative to most eukaryotes and 60% of the plasmodial PE comes from the CDP-Etn pathway and 40% from decarboxylation of PS. PS decarboxylase activity is high in falciparum- and knowlesi-infected red cells (Elabbadi *et al*., 1997) and a parasite-specific PLP-dependent serine decarboxylase was found in the erythrocytic stages of *P. knowlesi* and *P. falciparum* (see above). PE can also be formed *de novo via* the CDP-Etn pathway. The first reaction is *via* a cytosolic ethanolamine kinase. A putative gene, named *PfEK*, has been identified in the *P. falciparum* genome that encodes a 428 amino acid protein (Vial and Ben Mamoun, 2005). The next step, catalyzed by CTP-phosphoethanolamine cytidyl transferase, converts the phosphoethanolamine into CDP-Etn, and a putative transferase gene, identified on chromosome 12, encodes a polypeptide of 573 amino acids that is expressed throughout the life cycle. The final step in the formation of PE might be catalyzed by the same enzyme as in the CDP-choline pathway, namely choline-Etn phosphotransferase. However, biochemical studies for this enzyme are lacking.

Blood serum is the source of cholesterol as well as FAs for all species of *Plasmodium*. The components of serum that support the intraerythrocytic growth of *P. falciparum* have been reported to be high- and low-density lipoproteins, long-chain saturated and unsaturated FAs associated with bovine serum albumin (BSA) and a mixture of lysophosphatidylcholines associated with BSA as well as palmitic and oleic acids associated with BSA. Recently using radiolabeled FAs and gas chromatographic analyses it was possible to show that the parasite can desaturate and elongate serum-derived FAs with little modification, that *de novo* FA synthesis makes a very limited contribution to the acyl group, and that the parasite's overall FA composition reflects that of the medium. In this regard, *Plasmodium* differs from most eukaryotes that maintain their FA composition by coordinating *de novo* biosynthesis, scavenging and saturation/desaturation. Further, because palmitic, oleic and stearic acids can be generated by *de novo* biosynthesis in humans and these are the same FA combinations that favor the *in vitro* growth of falciparum it has been speculated that the parasite has adapted to human hosts in which other FAs vary according to diet and health (Mi-Ichi *et al*., 2006).

The discovery of the apicoplast was a major breakthrough in the understanding in the evolution and biology of *Plasmodium* (see p. 105). The *P. falciparum* genome contains the group of highly conserved proteins known as type II fatty acid synthase (FAS II) with distinct enzymes for the different reactions. FAS II has as initial substrate, acetyl coenzyme A (acetyl-CoA), which is presumably either transported into the organelle or synthesized there from a source of free acetate. Incorporation of the precursor acetate into FA chains could be demonstrated, attesting that the *de novo* biosynthetic pathway was functional.

This indicates that some *de novo* FA biosynthesis occurs in the apicoplast. It may occur in a discrete compartment or be related to a specific but crucial type of lipid (predominantly C-10 to C-14, as shown by Surolia and Surolia, 2001). This pathway is probably quantitatively minor but appears to be critical to parasite survival. It could be involved in the synthesis of lipids required by the apicoplast or a specific lipid that may be provided to the parasite. A major interest resides in the fact that FAS II is structurally and functionally distinct from the equivalent pathway in the vertebrate host, and would explain the susceptibility of the malarial parasite to herbicides targeting the enzyme. It is considered today as a validated target that still needs to be developed into a practical antimalarial.

Whereas neutral lipids (NLs) are found only in traces in normal erythrocytes, upon infection there is a high increase (by more than five-fold) in NLs in content (including FA, DAG and triacylglycerol (TAG)) irrespective of the species (see Holz, 1977a; Sherman, 1979). The increase, expressed as a percentage, is exceptionally high, but the final amounts remain very low (see TAG biosynthesis). During the last few years, there has been re-newed interest in the functions of these NLs that are probably parasite-associated. Electron and fluorescence microscopic studies have documented the presence of lipid bodies of 1–2 μM in diameter in the intraerythrocytic stages of *P. falciparum*. TAG is a major lipid species stored in lipid bodies in the late trophozoite and schizont stages of *P. falciparum*, localized in the PV and/or the digestive vacuole (Vielemeyer *et al.*, 2004). TAG may be utilized to provide acyl groups for lipid synthesis and may be used when local resources fail or when specific kinds of FAs or lipid precursors are required. TAG degradation might become active particularly from early schizont to late schizont stages with subsequent FA released into the medium during schizont rupture and/or merozoite release (Palacpac *et al.*, 2004).The most interesting point is that the NLBs appear closely associated with the food vacuole (FV), as confirmed by electron microscopy and also by isolation of the FV (Jackson *et al.*, 2004). The association of lipid bodies and substantial amounts of DAG and TAG with the FV suggest that these NLs may function in the detoxification of heme through the formation of crystals of hemozoin (see p. 56). These NLs and their precursors might play a role in heme detoxification during the early trophozoite stage of infection in the FV before

their incorporation into NLB (Jackson *et al.*, 2004; Pisciotta *et al.*, 2007). Lipid bodies are most prominent in more mature-stage parasites and DAG levels have been reported to be maximal in the trophozoite stages but are apparently converted to TAG by the schizont stage (Nawabi *et al.*, 2003). Enzyme(s) mediating their degradation remain to be elucidated.

Henri Vial (1948–) obtained a doctor of philosophy (PhD, 1978) in molecular and cellular biology and pharmacology from Claude Bernard University (Lyon, France) and the National Institute of Applied Sciences (INSA, Lyon, France) on the mechanisms of action of drugs acting on the central nervous system. During his time as a post-doctoral researcher in the laboratory of Jean Philippot (Montpellier, France) he was contacted by the World Health Organization (WHO, Geneva, Switzerland) to elucidate the possibility of cholesterol biosynthesis in *Plasmodium* spp.; this triggered his interest in health problems due to malarial parasites. In 1980, after Holz, Beach and Sherman published major observations about the lipid content in several *Plasmodium* spp., Vial re-oriented his research on these other lipids found in substantial amounts in malarial parasites. The idea was to clarify how the parasite acquires these new molecules and then, provided they were crucial, to design compounds that could disturb the acquisition machinery to prevent the parasite from developing within the host.

In 1979, Henri obtained a permanent position at INSERM (French National Institute of Health) and very quickly his biochemical studies revealed that malarial parasites possess a bewildering and unexpected variety of metabolic pathways to synthesize lipid components of their membranes and, in addition, their relative contribution to the final bulk of lipids was quantified. Lipid trafficking inside infected erythrocytes was unraveled in collaboration with the Dutch group of J. Op den Kamp (Utrecht, The Netherlands). Vial and his group also investigated the limiting steps in biosynthetic pathways and through the use of commercial compounds revealed that some pathways were crucial. Providing proof of concept, he initiated collaboration with different chemistry teams to design and synthesize anti-PL effectors. Michele Calas, a professor of organic chemistry, devoted part of her life to coordinating this directed pharmacology strategy.

In 2003, Vial and co-workers established a collaboration with the pharmaceutical company Sanofi Aventis and its highly motivated teams led by J. P. Maffrand and L. Fraise to ensure the clinical development of their prize compound in humans, a task beyond the scope of their academic expertise.

During 1994–2006, Vial was director of the Molecular Dynamics of Membrane Interactions laboratory, a joint laboratory between the French

Centre National de la Recherche Scientifique (CNRS, Paris, France) and the University of Montpellier II (Montpellier, France). Presently he is research director (DR1) at INSERM, director of CNRS University Montpellier 2 Laboratory (UMR 5539), Molecular Dynamics of Membrane Interactions, Health Department, biochemistry/pharmacology section.

Vial's research has pinpointed a series of compounds that possess powerful anti-malarial properties. Whatever the future of these compounds holds, there is no doubt that lipid metabolism is of interest for the development of new antimalarial therapies and that designing a novel chemical scaffold for enzyme-shaped inhibitors represents a promising new avenue for plasmodial chemotherapy.

Our understanding of *Plasmodium* membrane lipids and their synthesis has increased remarkably in the past two decades thanks to advances in gene sequencing and expression; however, sub-cellular fractionation still remains difficult and although much has been achieved recently through the use of fluorescently labeled lipids and cells' transfected cells' with green fluorescent protein (GFP), better techniques for subcellular localization still need to be developed to allow mechanisms of trafficking to be elucidated. Mass spectrometry such as that employed by Enjalbal *et al*. (2004) may find a useful role in this regard. Critical to the design of new and effective antimalarials will be a better understanding of the structural characteristics and the mechanisms of action of the enzymes involved in membrane lipid biosynthesis.

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CHAPTER 23

Invasion of Erythrocytes

Being an obligate intracellular parasite, host cell entry is a necessary and specific requisite for *Plasmodium* in the vertebrate host. As such, the molecular mechanisms of invasion have been considered the Achilles' heel that might be exploited for the development of new therapies for malaria. Despite half a century of 'invasion research' a practical and effective means for interrupting the entry process into red blood cells has not been achieved. However, hope for novel interventions remains.

Electron microscopic studies demonstrated a complex of organelles at the apical end of the invasive stages of malaria parasites. In sporozoites these included 'conoid bodies and paired apical bodies' (Garnham *et al*., 1961; 1963). Similar structures were seen with erythrocytic merozoites (reviewed by Rudzinska, 1969). In 1969, the invasion of erythrocytes by merozoites was described in electron microscopic studies carried out by Ladda, Aikawa and Sprinz (1969) working at the Walter Reed Army Institute of Research (WRAIR). Their morphological study found 'no evidence of a special type of interaction which might lead to parasite entry into the red cell' and they went on to state 'no specific receptor sites exist on the red cell which might initiate attachment . . . for penetration.' Ten years later, however, with improvements in electron microscopy techniques and membrane sieving methods that allowed for isolation of large numbers of *Plasmodium knowlesi* merozoites (Dennis *et al*., 1975), a detailed description of the morphological characteristics of invasion was achieved (Aikawa *et al*., 1978; Bannister *et al*., 1975). As other electron microscopic studies have shown similar structural characteristics for erythrocytic merozoites it has been assumed that the steps in invasion are similar for all species of malaria parasites, including *P. falciparum* and *P. vivax*.

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At the apical end of the *Plasmodium* merozoite and within the cytoplasm is a pair of membrane-bound pear-shaped and ducted structures called rhoptries; more numerous (up to 40) fusiform bodies that converge around the tips of the rhoptry ducts, called micronemes, and spheroidal membranous vesicles, the dense granules. The surface of the merozoite is covered with an electron-dense fibrillar coat. In an elegant series of studies using both videomicroscopy (Dvorak *et al*., 1975) and electron microscopy the dynamics of the entry process have been described in considerable detail (summarized in Bannister *et al*., 2005; Preiser *et al*., 2000; Topolska *et al*., 2004b). Primary attachment of the merozoite occurs at any point on the merozoite surface, and then there is re-orientation so that the apical end is juxtaposed to the erythrocyte surface. At the point of contact of the apical end of the merozoite with the erythrocyte, an electron-dense area is seen beneath the erythrocyte membrane bilayer (Aikawa *et al*., 1978). The electron-dense area, termed a 'tight junction', forms a circumferential ring around the merozoite. (The components and constituents of the junction are not known.) During invasion the rhoptries discharge their contents, the tips of the ducts fuse with each other and the merozoite plasma membrane, and then they collapse. The micronemes also release their contents by membrane fusion. Concomitant with rhoptry-microneme discharge, the tight junction moves from the apical end of the merozoite to the posterior end powered by its actin–myosin motor (Keeley and Soldati, 2004) and in the process the merozoite's fuzzy coat is shed due to proteolysis via a micronemal serine protease, SUB2 (Harris *et al*., 2005). As the tight junction moves posteriorly it is thought that the ligands mediating the invasion process (see below) are removed by one or more serine proteases of the rhomboid family (Cowman and Crabb, 2006; O'Donnell and Blackman, 2005; O'Donnell *et al*., 2006). With the discharge of the contents of the rhoptries, the red cell membrane begins to expand allowing the merozoite to induce an invagination; when this is sealed it forms an enclosing parasitophorous vacuolar membrane (PVM) separating the parasite from the red cell cytoplasm. Although initially the PVM phospholipids (PLs) are derived from the host (Ward *et al*., 1993), after the dense granules release their contents into the parasitophorous vacuole (PV) and presumably these materials are inserted into the PVM, the vacuole enlarges. It should be noted that the protein and lipid contents of the PVM remain largely uncharacterized, however, host-derived proteins appear to be absent, but present are detergent-resistant raft proteins (DRM) containing 10 raft proteins, including flotillins, B2 adrenergic receptors, aquaporin, G proteins and tyrosine kinases (Haldar and Mohandas, 2007; Murphy *et al*., 2007; Nagao *et al*., 2002). The entire process of invasion is accomplished in less than 30 seconds (Dvorak *et al*., 1975).

Chetan Chitnis (1961–) did undergraduate work in physics at the Indian Institute of Technology (IIT), Bombay, India and was attracted to the life sciences through independent reading and some elective courses at IIT in biochemistry and molecular biology. After obtaining a masters degree in physics at Rice University (Houston, Texas) (1985), he found his way into an interdisciplinary biophysics program at University of California, Berkeley (Berkeley, California) where he obtained a PhD (1990) working on the biosynthetic pathway of the polysaccharide alginate, an important virulence factor in *Pseudomona aeruginosa*. Chetan then decided to continue to work on the molecular basis of microbial pathogenesis for post-doctoral research but looked for laboratories working on diseases relevant to India where he wanted to return eventually. He joined Miller's laboratory at the NIH for post-doctoral work in 1991. When he arrived in the laboratory, John Adams had just identified the genes encoding PkDBP and PvDBP and Kim Lee Sim working at WRAIR had cloned the gene encoding EBA175. Moreover, they had just demonstrated by sequence analysis that all these genes contain conserved cysteine-rich regions and belong to a family of erythrocyte-binding proteins (EBPs). It was an exciting time and Miller gave Chetan the task of identifying the receptor-binding domains of these EBPs. Because of the presence of cysteines, there were problems expressing fragments of EBPs in their functional form in *Escherichia coli*. He developed an assay in which domains of EBPs could be expressed on the surface of mammalian COS cells as fusions to *Herpes simplex* virus glycoprotein D (HSV gD) and showed that COS cells expressing region II of PvDBP and PkDBP on the surface could bind Duffy-positive human red blood cells with specificity. Just a few weeks before these results were obtained, Kim Lee Sim who was working on EBA175 came to spend 1 year in Miller's laboratory. Kim Lee tried the HSVγD fusion and CV-1 (immortalized cells from green monkey kidney) carrying SV40 (simian virus) (COS) cell expression system for EBA-175 and showed that the receptor-binding domain of EBA175 also lies in region II. This assay has now been used widely in malaria to study receptor–ligand interactions. These studies showed that malaria parasites use conserved cysteine-rich domains, that are now referred to as Duffy-binding-like (DBL) domains after the first binding domains identified from PkDBP and PvDBP. Chetan returned to India in 1995 to start his own laboratory at the International Center for Genetic Engineering and Biotechnology (New Delhi, India) where he continues to work on these parasite ligands, to understand their functional roles and how they interact with their receptors as well as to explore the possibility of developing vaccines based on these functional domains involved in receptor recognition for red blood cell invasion.

The earliest light microscopic investigations, as well as the visualization of the steps in invasion (i.e. merozoite attachment, apical re-orientation, junction formation and merozoite enclosure, as revealed by the electron microscope) has suggested the presence of parasite ligands and red cell receptors. However, unraveling the molecular mechanisms that allow invasion to proceed in a stepwise fashion required functional studies. R. Barclay McGhee was the first to discover the basis for host cell specificity by malaria parasites and in doing so he provided clues to the nature of the red blood cell receptors. In the 1950s, McGhee, who was Trager's associate at the Rockefeller Institute, attempted to determine the biochemical basis for host susceptibility to a particular species of malaria. In order to avoid the complications of the host immune response, McGhee (1953b) used chicken embryos. When washed uninfected duckling erythrocytes were introduced into the circulation of *P. lophurae*-infected chick embryos the duckling red cells were preferentially invaded. McGhee concluded that if there were random contact between the lophurae merozoites then the number of infected red cells in chick cells would be three to five times greater than in duck cells. He wrote: there was '. . . selective invasion. . .of susceptible cells . . .[because] only certain areas. . .are receptive to penetration.' In a seminar given at the Rockefeller Institute he used the term 'receptor' for these areas, much to the distress of the virologists in the audience (McGhee, 1983). McGhee then went a step further. To identify the molecular nature of the receptor he attempted to remove it by treating duckling red cells with a battery of enzymes (McGhee, 1953a). None, however, affected invasion. A decade later, when I arrived in Trager's laboratory, (and after McGhee had left the Rockefeller Institute and was a professor at the University of Georgia, Athens, Georgia) I was told of the discriminating taste of *P. lophurae* merozoites for duckling red cells that had been discovered by McGhee; however, since the identity of the duckling red cell receptor was still unknown, I was encouraged to identify it. Rather than chick embryos I used an *in vitro* culture system that had been developed by Trager (and which was a modified Harvard rocker system). Following McGhee's lead, attempts were made to determine whether enzyme treatment of duckling red cells could remove specific surface receptors and thus prevent *P. lophurae* invasion (Sherman, 1966b). None of the enzyme (i.e. trypsin, chymotrypsin, neuraminidase) treatments I tried had any effect. This lack of success led to my abandoning further research on merozoite invasion. However, I learned an important lesson from this futile experience: success can depend on the choice of a particular experimental system. Fortunately, there were other investigators who were not so faint of heart and they chose more wisely.

In 1973, Butcher, Mitchell and Cohen (with support from the United Kingdom Medical Research Council (MRC) and World Health Organization (WHO, Geneva, Switzerland)) used an *in vitro* short-term culture system to study invasion and found that red cells from Old World monkeys

(kra and rhesus) were susceptible to *P. knowlesi* merozoites, whereas New World monkey (*Aotus*) and human red cells were less susceptible and non-primates were completely resistant (Butcher *et al*., 1973). Although these workers were convinced these results were due to differences in erythrocyte susceptibility and were the basis of host-cell specificity, they did not identify the red cell receptor. However, in a series of what are now considered to be classic studies, Miller and co-workers (1975, 1976) used enzymatic treatment of red cells (as prepared by McGhee and Sherman) to define the chemical nature of an erythrocyte membrane receptor. When human red cells were treated with chymotrypsin or pronase, invasion of *P. knowlesi* merozoites was blocked, whereas neuraminidase and trypsin were ineffective. However, rhesus red cells treated with the chymotrypsin remained susceptible. Miller *et al*. (1977) suggested the differences in invasion between the two kinds of red cells were due to the 'high affinity between *P. knowlesi* merozoites and (rhesus) monkey erythrocytes [which] may require greater alteration of the receptor to inhibit invasion.' This interpretation for rhesus cells would subsequently turn out to be incorrect (see below). However, the study did allow for the formulation of a hypothesis for invasion (Miller, 1977) and subsequently led to the identification of a human red cell receptor (Miller *et al*., 1976).

Because human red cells are one-quarter as susceptible to *P. knowlesi* invasion as are rhesus red cells, enzymatic 'stripping' was effective in removing a receptor; by contrast, our failure to find a receptor for lophurae merozoites was probably a result of the much greater susceptibility of duckling red cells to *P. lophurae* merozoites so that enzymatic 'stripping' failed to remove the receptor. Perhaps, if McGhee and I had used a less susceptible cell for 'stripping' an erythrocyte receptor might have been identified a decade earlier.

Miller and co-workers took advantage of the availability of human red cells that differed genetically in their surface molecules (something lacking for duckling and chicken erythrocytes) and was able to show that *P. knowlesi* merozoites could invade human red cells that have the Duffy factor antigen (called Duffy positive) on their surface, but if human red cells lacked the Duffy antigen they were refractory (Miller *et al*., 1975). Then using enzyme 'stripping' (Mason *et al*., 1977; Miller *et al*., 1977) it was found that when Duffy antigen was removed from human red cells by chymotrypsin (but not trypsin or neuraminidase) treatment the erythrocytes became Duffy negative and these cells resisted invasion by *P. knowlesi* merozoites. These laboratory studies were extrapolated by Miller to explain the absence of *P. vivax* in West Africa (where most of the population is Duffy negative). Indirect evidence in support of Miller's proposal came from the following: (1) West Africans and black Africans who are 95% and 70% Duffy negative, respectively, are resistant to *P. vivax*, (2) Duffy-negative volunteers failed to become infected when

bitten by mosquitoes carrying *P. vivax*, (3) Of 13 black Americans who had vivax malaria in Vietnam, all were Duffy positive (Miller *et al*., 1976). Final and direct proof of Miller's postulate came when a short-term *in vitro* culture system for *P. vivax* showed that these parasites could not invade Duffy-negative red cells (Barnwell *et al*., 1989).

Human Duffy antigen is a 36-kDa acidic glycoprotein predicted to have 7 α-helical transmembrane domains with an extracellular *N*-terminal domain of 60 amino acids having two *N*-glycosylation sites at residues 17 and 28. Duffy negativity has been determined to be due to a point mutation (cytosine to thymine) on the Duffy gene promoter 33 bases from the initiation codon that does away with the binding site for the GATA-1 erythroid transcription factor and as a result transcription of Duffy messenger ribonucleic acid (mRNA) in red blood cells, but not other cell types, is abolished (Tournamille *et al*., 1995). Although the Duffy determinant has been shown to be crucial for the entry of vivax merozoites (Tournamille *et al*., 2005), recent observations indicate that this pathway is not absolute (i.e. *P. vivax* infections were found in two Duffy-negative individuals from South America (Cavasini *et al*., 2007) and in four Duffy-negative individuals from East Africa (Ryan *et al*., 2006)). This suggests that *P. vivax* merozoites are able (albeit infrequently) to infect Duffy-negative red cells and in this species, as in *P. falciparum*, alternate pathways for erythrocyte invasion exist.

Recent studies using immunoelectron microscopy and recombinant deoxyribonucleic acid (DNA) technology have shown that there are two Duffy-binding proteins (DBP), PvDBP and PkDBP, found in *P. vivax* and *P. knowlesi*, respectively, and these are present in the micronemes (Fang *et al*., 1991; Miller *et al*., 1988; Wertheimer and Barnwell, 1989; and summarized in Chitnis, 2001). The PvDBP, a 140-kDa protein, specifically binds to the human Duffy blood group antigen *via* a conserved *N*-terminal cysteine-rich region called region II (Chitnis and Miller, 1994). Region II contains 330 amino acids and binds to a 35 amino acid region of the *N*-terminus of the Duffy antigen (Chitnis *et al*., 1996) with recognition by PvDBL/PkDBLα achieved by a single contiguous and fully exposed site on sub-domain II with residues Tyr 94, Asn 95, Lys 96, Arg 103, Leu 168 and Ile 175 being required for recognition (Singh *et al*., 2006). Site-directed mutagenesis of each of these residues abrogates or disrupts the Pv/PkDBLα binding (Hans *et al*., 2005; VanBuskirk *et al*., 2004). The sulphated tyrosine 41 of the Duffy antigen has been shown to be essential for binding of PvDBP and PkDBP (Choe *et al*., 2005).

Louis H. Miller (1935–) received a bachelor of science (BS) degree from Haverford College (Haverford, Pennsylvania) in 1956 and a doctor of medicine (MD) from Washington University (St. Louis,

Missouri) in 1960. Miller's interest in tropical medicine was piqued by reading the autobiography, '*Out of My Life and Thoughts*' by Nobelist Albert Schweitzer. After an internship and residency in internal medicine at the Mount Sinai Medical Center in New York (1960–1964) he spent a year at Cedars-Sinai Medical Center (Los Angeles, California) as a renal metabolism fellow, training that would stand him in good stead for later studies on renal pathology in human and experimental malarias. Returning to New York City he worked with Harold Brown (head of parasitology and tropical medicine at Columbia University School of Public Health (New York, New York) who had spent most of his life working in tropical medicine) receiving a masters degree in parasitology in 1965. Drafted into the United States Army as a captain in the Medical Corps he was sent to WRAIR's SEATO Laboratory in Bangkok, Thailand where he intended to study tropical sprue, however, after encountering only a single case in 2 years he felt it wise to change career. At that time chloroquine-resistant malaria was a significant problem for the troops fighting in Vietnam and so Miller, the clinician, turned his attention from tropical sprue to malaria. Knowing little about malaria he spent one month in the library in Bangkok learning all he could. Comparative studies on the pathology and renal physiology using monkeys and rodents, as well as deep vascular schizogony and sequestration, were carried out with Robert Desowitz (who had been a student of the great malariologist Colonel Henry E. Shortt at the London School). After completion of military service Miller joined the faculty at the Columbia University School of Public Health as an assistant professor and he began to move further away from the clinical aspects of the disease and to focus on more basic questions that, he felt, would have an impact on malaria control. To follow up his work on sequestration, Miller contacted Craig Canfield, a friend from Bangkok, and then director of experimental therapeutics at WRAIR, to obtain two *Aotus* monkeys to be infected with *P. falciparum*. The result was the discovery of the presence of knobs on the surface of red cells in organs where sequestration occurs. In 1971, Miller, now an associate professor, left Columbia University to establish a malaria laboratory at the National Institutes of Health (NIH, Bethesda, Maryland) where he eventually became chief of the NIH Laboratory of Parasitic Diseases (LPD, Bethesda, Maryland).

Shortly after joining the NIH, Miller attended a conference organized by Elvio Sadun at WRAIR and there he met Sydney Cohen (from Guy's Hospital, London, United Kingdom) who was carrying out short-term cultures of *P. knowlesi* to study invasion. This led to Miller's seminal studies of host-cell specificity and the discovery that *P. knowlesi* was unable to invade Duffy-negative red cells. A visit to the library that night made him realize he had found the missing factor for

the resistance of West Africans to *P. vivax*. Later, he was able to show that *P. vivax* did not invade Duffy negative red cells. For the next 20 years, the biochemistry and molecular mechanisms of invasion became the major focus of his research. In this work, his collaborators were John Adams, Chetan Chitnis and Kim Lee Sim.

At the NIH, Miller was joined by Robert Gwadz, Richard Carter and Mary Nijhout who provided a description of the factors involved in gametocytogenesis. In collaboration with Peter David, Russell Howard, Stephen Aley, John Barnwell, James Leach, Dror Baruch and Tom Wellems, antigenic variation and cytoadherence were investigated, *P. falciparum* erythrocyte surface protein-1 (PfEMP1) was discovered and its gene cloned and sequenced. In the late 1970s, when monoclonal antibodies were discovered, Gwadz, Carter and Miller developed monoclonal antibodies that blocked infection in the mosquitoes. Strategies to block mosquito transmission and development of blood-stage vaccine are the current focus of his research. Presently, Miller is chief of the Malaria Vaccine Development Branch at NIH.

The PkDBP, a 135-kDa protein, binds to both the human and rhesus Duffy blood group antigens. Antibodies to the 135-kDa PkDBP were used to screen a deoxyribonucleic acid (cDNA) expression library from mRNA and the gene encoding the PkDBP cloned and sequenced (Adams *et al*., 1990). The PkDBP is encoded by an α-gene. The region II of the β and γ proteins, encoded by two other homologous genes, bind to rhesus red cells but not to Duffy-positive or Duffy-negative red cells. Initially, objections were raised as to Miller's identification of the Duffy antigen as a determinant for *P. knowlesi* invasion since paradoxically Duffy-negative red cells treated with trypsin were susceptible to invasion yet they remained Duffy negative. In addition, chymotrypsin-treated rhesus red cells that had lost the Duffy antigen could still be invaded by *P. knowlesi* merozoites. This paradox has now been explained by *P. knowlesi* having alternate pathways for invasion of rhesus red cells that do not depend on the Duffy antigen: the β and γ proteins mediate this (Chitnis, 2001). The DBP-β binds to sialic acid residues in a stretch of 53 amino acids between the fourth and fifth cysteines; the receptor for DBP-γ is still unknown. Since it is possible to produce large amounts of the recombinant receptor binding domain of PvDBP (PvRII) (Yazdani *et al*., 2006) and rabbit antibodies raised against a recombinant PkDBP-α inhibited the invasion of human and rhesus red cells by *P. knowlesi* merozoites it has been suggested that PvRII may serve as a possible vaccine candidate (Singh *et al*., 2002).

Knockouts of PkDBPα invade rhesus erythrocytes as well as wild-type parasites, however, when the PkDBP α-gene was deleted, *P. knowlesi*

merozoites were unable to invade Duffy-positive human red cells. Since both wild-type and knockout lines invade rhesus erythrocytes treated with neuraminidase, trypsin, proteinase K and neuraminidase plus trypsin it appears that deletion of the α-gene does not affect invasion of rhesus red cells by pathways other than the Duffy receptor pathway (Singh *et al*., 2005).

Earlier, electron microscopic studies showed that when *P. knowlesi* merozoites were treated with cytochalasin B (which blocks polymerization of actin) there is an arrest of invasion at the step of junction formation (Miller *et al*., 1979). When cytochalasin B treatment was carried out with merozoites from the PkDBPα knockouts, no junction was visible with Duffy-positive human red cells (but a junction is formed with rhesus red cells) suggesting that the interaction of PkDBP with the human Duffy antigen is necessary for junction formation and when this critical step is blocked invasion does not occur (Singh *et al*., 2005).

P. vivax preferentially invades reticulocytes. Two vivax reticulocyte-binding proteins, PvRBP-1 and PvRBP-2, have been identified and the genes for these cloned and sequenced (Galinski *et al*., 1992; summarized in Galinski *et al*., 2005). Both are large proteins—325 kDa and 330 kDa, respectively—and both have a two-exon structure: the first exon encodes the signal peptide followed by a short intron and a large 6–9-kb exon. The reticulocyte-binding proteins (RBPs) have been localized to the apical region of the merozoite but whether they are located in the micronemes or rhoptries has not been precisely determined, though the pattern of fluorescence with antibody is consistent with a microneme location. PvRBP-1 and PvRBP-2 have been postulated to form a complex that mediates adhesion and recognition of the reticulocyte independent of the Duffy phenotype (reviewed in Iyer *et al*., 2007). Since there is no evidence for the Duffy-like ligands being exposed at the surface prior to initiation and formation of a tight junction, it has been suggested that binding may be necessary to trigger (signal) the timely release of the sequestered microneme proteins so that a tight junction forms and entry can proceed (Galinski *et al*., 2005). In this way, by acting prior to RBP binding and junction formation (i.e. during apical end orientation) the *P. vivax* merozoite is able to target reticulocytes, which usually represent less than 1% of the total red cell population. The receptor molecule on the reticulocyte to which the PvRBP binds remains unknown (Galinski *et al*., 2005).

John Wesley Barnwell (1948–) resumed his undergraduate studies in 1972 after a three-year hiatus in the United States Army and a tour of Vietnam. Graduating from Oregon State University (Corvallis,

Oregon) with a degree in medical microbiology (1974) he traveled in the summer of 1974 again to tropical climes to study public health in Hawaii. After 18 months of epidemiology and biostatistics courses and collecting mongoose scat, local sea snails and giant African land snails to study *Capillaria*, bird schistosomes and *Angiostrongylus*, John emerged with a master in public health (MPH) and a notion of doing graduate research in tropical medicine under the tutelage of Robert S. Desowitz, continuing a lineage going back to Sir Ronald Ross, through H. E. Shortt and R. Christophers. Four years later, having now dabbled with dog filariasis, *Toxocara* and malaria in rats, he was awarded his PhD in tropical medicine from the University of Hawaii (Honolulu, Hawaii) and joined the malaria section of the Laboratory of Parasitic Diseases at NIH as a staff fellow working under Lou Miller and directly with Russell Howard. The initial project was to immunochemically identify the variant antigen(s) exposed on the surface of malaria-infected erythrocytes that had been first demonstrated by Neil Brown at National Institute for Medical Research (NIMR, London, United Kingdom) some 15 years earlier and to be responsible for antigenic variation in simian malaria parasites (and later in human malaria). However, the agglutination test necessary to demonstrate variant antigen specificity of antisera was not working with the *P. knowlesi* parasites being used at the NIH. Tracing the lineage of that parasite line John determined the one variable that stood out was passage in splenectomized monkeys. In the next few months it was conclusively demonstrated that in the environmental absence of a spleen, the parasites failed to express variant antigens. It was also shortly demonstrated, with a working schizont-infected cell agglutination (SICA) assay, that the SICA-variant antigens were large proteins synthesized by the parasites and inserted into the membranes of infected erythrocytes. This quickly led, using similar methods, to the identification of the corresponding antigens on the surface of *P. falciparum*-infected erythrocytes and evidence that these antigens were connected to the adhesion of the infected erythrocytes to endothelium. In 1983, John went to New York to take up a position as an assistant professor at New York University (NYU, New York, New York) Medical Center with a group led by Ruth Nussenzweig, which shortly afterwards became the Department of Medical and Molecular Parasitology. Continuing an interest in *P. falciparum* antigenic variation and endothelial cell adhesion, John and Chris Ockenhouse, a newly minted PhD in the department, demonstrated that the host cell protein (later designated as CD36) recognized by the OKM5 monoclonal antibody was a major receptor for the adhesion and sequestration of *P. falciparum*-infected cells. After a period of working with sporozoites and blood stages of simian malaria parasite species,

John set up a program that has continued for over 20 years to study the cellular and molecular biology of *P. vivax*, a human parasite unable to be propagated by *in vitro* culture and which requires New World monkeys for laboratory studies. In John's laboratory the parasite ligand necessary for *P. vivax* merozoite invasion of erythrocytes, the DBP, was first identified by a graduate student, Samuel Wertheimer, and a merozoite ligand complex that also functions in *P. vivax* invasion was identified and characterized at a molecular level by Mary Galinski, who remains his scientific partner and spouse. This work continued after John left NYU to join the Centers for Disease Control and Prevention (CDC) and Mary Galinski for a faculty position at Emory University (Atlanta, Georgia) in 1998, has led to the discovery of a new merozoite ligand family of considerable importance that is present in *P. falciparum*, and other primate malarias and rodent species of *Plasmodium*. John's interest in malariology has continued at CDC where he is currently in the Senior Biomedical Research Service as chief of the Malaria Laboratory Research and Development Unit and is responsible for programs on drug resistance, drug development, immunology and genetics, non-human primate malaria models, vaccine development and evaluation and biology of parasite interactions in primate hosts and mosquito vectors.

Genes sharing homology with the PvRBP have been found in the genomes of *P. falciparum* and *P. knowlesi* although these species do not invade reticulocytes; to distinguish these from the RBPs they are called normocyte-binding proteins (NBPs). One orthologue of the PvRBP, PfNBP1 (*P. falciparum* normocyte-binding protein-1) has been shown to bind to a sialic acid-dependent trypsin-resistant receptor; antibodies to PfNBP1 blocked invasion of trypsinized red cells and two truncated versions were unable to invade trypsinized red cells (Rayner *et al*., 2001). Although the receptor recognized by PfNBP1 is a sialoglycoprotein, its identity remains unknown, as does its role in invasion.

In the late 1970s and early 1980s—the time when *P. falciparum* could be cultured continuously in the laboratory—there was mounting evidence that the receptor for *P. falciparum* differed from that of *P. vivax* and *P. knowlesi*. Several laboratories reported that a receptor for *P. falciparum* merozoites was sialic acid (*N*-acetylneuraminic acid) since human erythrocytes treated with neuraminidase or trypsin resisted invasion by *P. falciparum* merozoites and this was unlike the situation with *P. vivax* and *P. knowlesi* where 'stripping' by chymotrypsin but not trypsin blocked invasion. Further, Tn erythrocytes, which lack a glycosyltransferase that results in cells deficient in sialic acid, were also refractory to invasion, as were En (a-) cells; and when and S-s-U (-) cells, which were

relatively resistant to invasion, were treated with trypsin they became totally resistant (Pasvol, 1984; Pasvol *et al*., 1982). These studies suggested that glycophorin, a sialic acid-bearing glycoprotein, was the red cell receptor for *P. falciparum* (Pasvol *et al*., 1989). Glycophorins occur in three main types: A, B and C, with glycophorins B and C representing approximately 10% and glycophorin A representing around 90% of the total. Indeed, it is estimated there are two million molecules of glycophorin A on the surface of a human red cell. Because En (a-) cells are deficient in glycophorin A, and S-s-U (-) cells lack glycophorin B, the former are almost totally resistant and the latter partially resistant to invasion. S-s-U (-) cells are found at a frequency above 1% in West Africa suggesting a degree of protection for populations having this trait (Pasvol and Wilson, 1982; Pasvol *et al*., 1982).

Geoffrey Pasvol (1948–) is currently professor of infection and tropical medicine in the Faculty of Medicine, Imperial College London (London, United Kingdom). After receiving his bachelor of medicine (MB) degree from the University of Cape Town (Cape Town, South Africa) (1972) he was awarded a Rhodes Scholarship to the Oxford University (Oxford, United Kingdom), and was poised to 'find the cure for acute leukemia' by identifying tumor-specific markers on the surface of malignant cells. However, a chance telephone conversation between professor Sir David Weatherall, Nuffield Professor of Medicine at Oxford, and professor Herbert Gilles at the Liverpool School of Tropical Medicine (LSTM, Liverpool, United Kingdom) sent him on a 'short trip' to the MRC laboratories in The Gambia, West Africa (1974–1979) where he met Iain Wilson with whom he collaborated for a number of years. From that point on he never looked back.

He completed his doctor of philosophy (DPhil) at Oxford (1978) on '*Fetal hemoglobin and Malaria*' and in the process, established a rapid method of concentrating falciparum schizonts using a gelatin solution (Plasmagel®), determined that even falciparum parasites have a predilection for younger-aged red cells, grow more luxuriantly under low-oxygen tensions in normal, but not sickle trait, red cells and that growth is retarded in cells containing fetal hemoglobin. At Oxford and during an MRC Fellowship that took him to the Harvard School of Public Health in Boston (Massachusetts) (1983–1984), he worked on the role of glycophorin and sialic acid in the invasion of red cells using multiple but well-defined genetic red cell variants as well as the invasion and development of parasites in thalassaemic red cells.

He then turned to the clinical problem of malaria in young children and was scientific team leader at the University of Oxford and Kenyan

Medical Research Institute (KEMRI, Nairobi, Kenya) research unit funded by the Wellcome Trust at Kilifi Kenya (1989–1990) where he worked on the optimal dosing of quinine for the treatment of severe malaria. In 1991, he was appointed as professor of infection and tropical medicine at Imperial College, London, United Kingdom, where he investigated the role of rosetting and tumor necrosis factor (TNF) generation in the pathogenesis of severe malaria as well as clinical aspects of a large cohort of almost 700 cases of malaria admitted to his unit.

Currently he is a consultant physician at Northwick Park and St Mary's Hospitals (London, United Kingdom) and director of the Imperial College Wellcome Centre for Clinical Tropical Medicine and dean of the Faculty at Imperial College London. He is now involved mainly in clinical research on malaria and tuberculosis in London and in Mumbai, India and Cape Town, South Africa.

Nevertheless, there were discrepancies in results from different laboratories working with invasion into enzyme 'stripped' or naturally occurring and poorly sialylated red cells. The discrepancies in invasion behavior were partly resolved when Graham Mitchell was invited to visit Miller's laboratory and given the task of examining invasion into both enzyme-stripped and Thomsen-Friedenreich antigen (Tn) cells. Starting with *P. falciparum* parasite populations that were uncloned, and so retained some natural diversity, cultures were maintained with only Tn cells added. Selection occurred for parasites able to use a non-sialic acid-dependent invasion pathway, as could be shown by subsequent comparison with sibling cultures which had been maintained with 'normal' red cells, when both populations were isolated and allowed to invade into stripped and other experimental cell preparations. Clearly Mitchell found there was receptor diversity and heterogeneity in at least one step in the invasion pathway (Mitchell *et al.*, 1986).

Graham Mitchell (1948–) had come from Sydney Cohen's laboratory at Guy's Hospital. He had trained as a zoologist (bachelor of science, BSc; 1969, Exeter University, Exeter, United Kingdom), and worked initially for his PhD on the immune response to merozoites, and their use as a vaccine in *P. knowlesi* infections, in close collaboration with Geoffrey Butcher and under Cohen's supervision. His thesis was entitled '*Experimental studies on acquired malarial immunity*'. Dave Dennis and Lawrence Bannister were in the team, and together they isolated viable merozoites and studied their invasion at the ultrastructural level; this work continues well into the present century. The *P. knowlesi*

vaccine work eventually matured into the discovery of Pk66/AMA-1, once Alan Thomas and Judith Deans had joined the laboratory. Mitchell currently runs the laboratory at Guy's, having had postings to New Delhi (1975), The Gambia (1976–1977), and the University of New Mexico (Albuquerque, New Mexico) (1977–1978) as well as the period at the NIH (1984) working on invasion.

Contemporaneously with the findings of receptor heterogeneity, attempts were made to identify the merozoite ligand for glycophorin using classical biochemical methods (reviewed in Hadley and Miller, 1988; Hadley *et al*., 1986). When radiolabeled merozoites were extracted and allowed to react with immobilized glycophorin A, several proteins, including those of 155, 140, 70 and 35 kDa were found to bind (e.g. see Perkins, 1984). Since the latter two proteins could be eluted by *N*-acetylgalactosamine, GlcNAc (previously reported by Jungery *et al*., 1983 to block invasion) it provided suggestive evidence that they were merozoite ligands. However, when it was found that inhibition by GlcNAc was due to toxicity rather than receptor blockade and that was probably GlcNAc did not block invasion by free merozoites (Howard *et al*., 1982b and reviewed in Hadley *et al*., 1986), it was concluded that GlcNAc binding was probably at least in part *via* the α2-3 linked sialic acid on the *O*-linked tetrasaccharides of glycophorin. The role of the 140, 70 and 35 kDa antigens has never been determined. Similarly, soluble proteins of 175, 120, 65 and 46 kDa that bound to normal human red cells were identified in culture supernatants and these required the presence of sialic acid. However, save for the 175-kDa protein, the role of these other proteins as putative glycophorin-binding ligands remains suspect (Hadley *et al*., 1986).

The 175-kDa protein (named EBA-175, for erythrocyte-binding antigen), the first merozoite ligand for glycophorin, was described by Camus and Hadley (1985), working at WRAIR, in 1985. Schizont-infected red cells were radiolabeled, culture supernatants collected and incubated with intact erythrocytes (to preserve the natural environment of the receptor and to avoid the use of detergents) and then the red cells were washed, lysed and precipitated with a serum from a Nigerian donor. Of the four antigens recovered, only EBA-175 bound to intact normal human red cells, not to neuraminidase-treated cells or Ena (a-) or Tn cells; it specifically bound to glycophorin A. Later, the assay was modified to avoid washing and immunoprecipitation: erythrocytes with bound proteins were centrifuged through a silicone gel cushion to remove unbound proteins and then the bound proteins were eluted with 2-M NaCl (Haynes, 1988). To add further support for EBA-175 being the falciparum ligand, when antibodies were made against EBA-175, they were able to block the invasion of *P. falciparum* merozoites by 90% (Pandey *et al*., 2002).

Although glycophorin B contains the same *O*-linked oligosaccharides as glycophorin A, EBA-175 did not bind to it. Later, the reason for this would be shown to be that the binding of EBA-175 to glycophorin A is not solely determined by sialic acid residues but also requires specific amino acid sequences (Sim *et al*., 1994).

EBA-175 is a micronemal protein encoded by a single copy gene located on chromosome 7. As is the case with other members of the erythrocyte-binding family of proteins (i.e. PvEBP, PkEBP, Pk β and γ proteins), EBA-175 has an extracellular *N*- and *C*-terminal cysteine-rich regions in addition to transmembrane and cytoplasmic domains. The two cysteine-rich regions (referred to as II and VI) bear numerous conserved cysteine residues with region II having the erythrocyte-binding function. Targeted disruption of EBA-175 in the clone W2mef, whose invasion is sialic acid dependent, was associated with a switch to a sialic acid-independent pathway of invasion (Duraisingh *et al*., 2003; Gilberger *et al*., 2003; Reed *et al*., 2000). However, when EBA-175 was disrupted in another cloned line (Dd2/Nm) selected on neuraminidase-treated red cells, the knockouts invaded the treated cells, as did the wild type. Such findings suggest that *P. falciparum* can invade red cells without EBA-175 except in one parasite clone (Gaur *et al*., 2003, 2004). Indeed, the *P. falciparum* reticulocyte homology 4 (PfRH4) protein, is uniquely up-regulated in Dd2/NM compared with Dd2, and both native PfRH4 and a recombinant 30-kDa protein to a conserved region of PfRH4 (rRH4(30)) bound to neuraminidase-treated erythrocytes. rRH4(30) blocked both the erythrocyte binding of the native PfRH4 and invasion of neuraminidase-treated erythrocytes by Dd2/NM (Gaur *et al*., 2007). This finding indicates that PfRH4 is a parasite receptor involved in sialic acid-independent invasion of erythrocytes. Since antibodies to rRH4(30) blocked binding of the native protein to erythrocytes, but failed to block invasion, it suggests that, although PfRH4 is required for invasion of neuraminidase-treated erythrocytes by Dd2/NM, it is inaccessible for antibody-mediated inhibition of the invasion process.

In field isolates from The Gambia and Brazil, the sialic acid-dependent pathway seems to predominate whereas in India and Kenya neuraminidase- and trypsin-sensitive, chymotrypsin-resistant receptors (characteristic of EBA-175 and EBA-140 mediated invasion) were rarely used (Deans *et al*., 2007).

The *P. falciparum* genome project has identified four paralogues of EBA-175 (named EBA-140 (BAEBL), EBA-181 (JESEBL), EBA-165 (PEBL) and EBL-1 (BAEBL), identified on the basis of sequence similarity to EBA-175 encoded by a single copy gene on chromosome 13 which has been localized to the micronemes (Treeck *et al*., 2006). Using enzyme-treated red cells as well as mutant erythrocytes deficient in surface molecules (as prepared by Miller and Pasvol) the red cell receptors for BAEBL

and several of the other paralogues were identified. A glycophorin C variant known as Gerbich negative (Ge–) results from a deletion of exon 3 in the glycophorin C gene and occurs at a frequency of 47% in Papua New Guinea; certain falciparum lines invade Ge– cells at around 60% of the rate of normal red cells (Pasvol, 1984). In general, BAEBL binds to glycophorin C and binding is sialic acid dependent as BAEBL failed to interact with neuraminidase-treated red cells. The ligands for BAEBL are an *N*-linked oligosaccharide or a cluster of *N*- and *O*-linked oligosaccharides, and soluble glycophorin C blocked invasion (Mayer *et al*., 2006). It is the region II domain of BAEBL that binds red cells and mutations in only four amino acids in 20 clones sequenced define the five BAEBL variants: VSTK, VSKK, ISKK, INKK and INRE. Three of the BAEBL variants (INRE, ISKK and INKK) lack specificity for glycophorin C demonstrating that glycophorin C is the ligand for only the VSTK variant (Mayer *et al*., 2006). This finding contradicts that of Lobo *et al*. (2003) who claimed INKK was the ligand for glycophorin C. The BAEBL receptor from clone Dd2/Nm binds to glycophorin C between residues 14 and 22. However, in another strain (E12) the binding of BAEBL to red cells was sialic acid dependent but resistant to trypsin suggesting its receptor is not glycophorin A, B, or C and knockouts of BAEBL in the 3D7 and W2mef clones were as invasive as wild-type parasites (Maier *et al*., 2003). Clearly, there are strain differences, and the binding properties between the BAEBL variants is due to amino acid changes in the region II of BAEBL and 'suggests that a single . . . ligand can function in multiple invasion pathways' (Gaur *et al*., 2004).

The gene for JESEBL was identified by a genome search and exists as a single copy on chromosome 1 (Adams *et al*., 2001). JESEBL is a 190-kDa micronemal protein whose binding was found to be dependent on a trypsin resistant, chymotrypsin and neuraminidase-sensitive, molecule that differs from glycophorin B. In the W2mef line, targeted disruption of the *JESEBL* gene did not affect invasion suggesting that it is redundant and not critical to invasion. However, in the 3D7 line JESEBL appears to be critical for invasion since disruption was lethal. Based on work with EBA-175 and other host cell binding proteins, there is a strong likelihood that all these ligands need to be shed during invasion by a rhomboid-like protease (Baker *et al*., 2006; O'Donnell *et al*., 2006), perhaps to disengage the tight-binding interactions required for host cell binding.

The merozoite surface coat consists largely of glycosylphosphatidylinositol (GPI)-anchored proteins. Currently there are 10 GPI-anchored proteins known or predicted to be located on the surface: MSP-1, MSP-2, MSP-4, MSP-5, MSP-8, MSP-10, Pf 12, Pf 38, Pf 92 and Pf 113. Besides the GPI anchor these proteins have other similarities: they contain cysteine-rich domains that are of potential significance in adherence, including epidermal growth factor (EGF)-like modules. MSP-1 is the most abundant merozoite surface protein (Holder, 1994; Holder and Blackman, 1994; Holder

et al., 1999). MSP-1 is encoded by a single copy gene on chromosome 9 in *P. falciparum*. Sequence analysis shows that MSP-1 contains conserved regions interspersed with polymorphic regions. MSP-1 undergoes extensive proteolytic processing during schizogony and soon after merozoite release. The resulting four fragments, 83, 30, 38 and 42 kDa, remain as a complex on the merozoite surface with the 42-kDa fragment being attached *via* the GPI anchor. A final processing step mediated during invasion by the subtilisin-like protease SUB2, cleaves the 42 kDa fragment into 33 kDa and 19 kDa fragments (= MSP-1_{19}) that remain on the merozoite surface during invasion. Full-length MSP-1 has been suggested to bind to red cells in a sialic acid-dependent manner and antibodies directed against MSP-1_{19} block invasion and also prevent proteolytic processing of MSP-1 (Blackman *et al.*, 1994). It has not been possible to 'knockout' MSP-1 suggesting that it is essential for invasion and parasite survival. MSP-1 is located on the merozoite surface of *P. vivax* and its solution structure has been determined using nuclear magnetic resonance (NMR; Babon *et al.*, 2007). Since immunization with recombinant MSP-1_{19} protects mice and monkeys against challenge it is a blood-stage vaccine candidate.

Anthony Holder (1951–) completed undergraduate studies in biological sciences, graduating in biochemistry at the University of East Anglia in Norwich, United Kingdom (1972). Subsequently, for his PhD studies (1972–1975) he worked with John Fincham, a well-known geneticist at the University of Leeds (Leeds, United Kingdom), using protein-sequencing methodologies to obtain amino acid sequences of nicotinamide-adenine dinucleotide phosphate (NADP)-dependent glutamate dehydrogenase from the wild type and various *am* mutants and revertants of *Neurospora crassa*. At that time, DNA sequencing was not routinely available and the dansyl-Edman procedure for peptide sequencing was very slow and extremely laborious; several person-years were devoted to obtaining the 452-amino acid sequence of this protein. Wellcome Trust and Royal Society Fellowships allowed him a very enjoyable post-doctoral post (1975–1978) at the newly rebuilt Carlsberg Laboratory in Copenhagen (Denmark) with studies on the evening primrose, barley and potato–tomato hybrids, as well as generous contributions from the brewery in the form of their products! However, after three years, a new job was needed and a serendipitous flick through the back pages of *Nature* revealed a job to work on African trypanosomes with George Cross who had recently moved to the Wellcome Research Laboratories, a pharmaceutical company whose research facilities in South London were often referred to as the 'University of Beckenham'. In this newly founded molecular parasitology group the variant surface glycoprotein

(VSG) was the molecule of choice and molecular biology was starting to have its pervasive influence. However, VSG glycosylation and the nature of the so-called 'cross-reacting determinant' was the area of choice leading to one of the first studies of what subsequently turned out to be a ubiquitous membrane anchor of proteins, the glycosyl phosphatidylinositol moiety. At the same time, in 1978, Robbie Freeman joined the group with the specific remit to establish malaria research, particularly using rodent models and developing monoclonal antibodies to define parasite proteins. This led to a productive five-year collaboration and a conversion to malaria research that has continued ever since. In 1981, Holder and co-workers were able to purify two parasite proteins and, for the first time, show that they could be used individually to immunize mice against subsequent challenge infection with *P. yoelii*, establishing the principle of a subunit vaccine against malaria. One of these proteins is now known as merozoite surface protein 1 (MSP-1) and has been worked on extensively by many researchers worldwide. The second, Py235, is an example of a family of proteins that includes the reticulocyte-binding proteins of *P. vivax* and the reticulocyte-binding homolog (RH) proteins of *P. falciparum* and is the focus of increasing interest for its role in cell invasion.

In 1988, Holder moved to head the Division of Parasitology at the National Institute for Medical Research at Mill Hill in North London, United Kingdom, a center of helminth and protozoal parasite research over many years. The initial studies on MSP-1 were rapidly expanded to other *Plasmodium* spp., including sequencing of the gene in *P. falciparum*. How could they be sure it was the right gene? Interestingly it was protein sequence from a fragment of MSP-1 released into culture supernatants that provided the definitive proof. Studies on the proteolytic processing of MSP-1 followed, particularly with M. J. (Mike) Blackman. The discovery that a monoclonal antibody isolated by Jana McBride in Edinburgh directed against the *C*-terminal region of the molecule inhibited merozoite invasion of red blood cells focused attention on this part of the molecule. Human studies, with Eleanor Riley in The Gambia and Roseangela Nwuba in Nigeria, supported the view that this part of the molecule was important as the target of protective immunity and a vaccine candidate. With Irene Ling and Sola Ogun, in parallel to work being carried out by Carole Long, a recombinant around 100 amino acid *C*-terminal fragment (MSP-1_{19}) of *P. yoelii* MSP-1 was shown to be effective in protecting mice against a parasite challenge infection. Structural studies using NMR carried out by Bill Morgan at Mill Hill provided the first three-dimensional structure of *P. falciparum* MSP-1_{19} and then, using similar techniques, he mapped the binding sites of inhibitory antibodies.

The early studies on Py235 suggested that it was a target of antibodies that inhibited invasion (reviewed in Gruner *et al*., 2004). Later studies showed that it was coded by a multi-gene family, was related to the reticulocyte binding proteins of *P. vivax* and important in red cell binding. Further molecular characterization showed that different members of the family were expressed at different stages of the life cycle and identified those that bound to red blood cells. Py235 was the first of the apical proteins looked at but it was not the last. Other microneme and rhoptry proteins have been characterized and many more are in the pipeline following the genomics revolution. Insights into the role of merozoite surface and apical organellar proteins and the functioning of the acto-myosin motor promise to provide a fascinating future for the study of invasion! Holder continues as head of the Division of Parasitology at the NIMR with a particular research focus on how merozoites invade red blood cells and applying the knowledge gained to promote potential therapeutic interventions.

Although the EGF domain of MSP-1_{19} has been suggested to mediate the initial contact of the merozoite to the red cell, it can be altered without affecting invasion (Drew *et al*., 2004; O'Donnell *et al*., 2000) which suggests that perhaps the *N*-terminal regions may be involved in early recognition. It has been proposed that MSP-1 mediates initial interaction with the red cell *via* specific regions of band 3 protein (reviewed in Oh and Chishti, 2005). Indeed, peptides containing amino acids 720–761 and 807–826 blocked *P. falciparum* invasion. Further, these peptides also interacted with two regions of MSP-1 and when recombinant proteins of the two regions were added to cultures, invasion was blocked. Based on these findings the authors have hypothesized that in the sialic acid-dependent pathway band 3 complements glycophorin whereas in the sialic acid independent pathway band 3 acts alone.

Peripheral proteins belonging to the MSP-3 group, the MSP-7 family, the serine-rich antigen (SERA) protease family as well as ABRA and Pf41 have also been suggested to serve as merozoite ligands. Although there is an abundance of putative candidates for merozoite ligands that mediate binding, the MSP-7 family members as well as Pf41 are favored in effecting primary contact with the red cell largely because they are strongly associated with the merozoite surface. However, the possibility remains that MSP-3 and MSP-6 also play roles in merozoite invasion but what these may be remains undetermined (Cowman and Crabb, 2006).

The apical merozoite antigen (AMA-1) is a merozoite protein presently under consideration as a candidate for inclusion in a blood-stage vaccine (Cowman *et al*., 2000). First identified at Walter and Eliza Hall Institute (WEHI) using human antibodies purified on a recombinant protein

(Peterson *et al*., 1989), its complete amino acid sequence was deduced from the gene sequence; initially incorrectly localized to the rhoptries (Crewther *et al*., 1990), more recently shown to be in the micronemes (Bannister *et al*., 2003). AMA-1 is encoded by a gene located on chromosome 11 (Topolska *et al*., 2003). Early studies with the *P. knowlesi* homologue, Pk66, showed invasion could be inhibited by a monoclonal antibody to AMA-1 (Deans *et al*., 1982; Thomas *et al*., 1984). The gene sequence of AMA-1 has been determined for a number of different isolates of *P. falciparum* and several other *Plasmodium* spp. AMA-1 is a type 1 integral membrane protein with an *N*-terminal signal sequence, and a presumed transmembrane domain towards the *C*-terminus. AMA-1 contains a highly conserved cytoplasmic domain with an *N*-terminal ectodomain having 16 cysteine residues forming eight disulphide bonds; the entire ectodomain is now known to form three closely packed structural domains which include prekallikrein/coagulation factor (PAN) domains, known to be involved in protein–protein or protein–carbohydrate interactions (Bai *et al*., 2005; Pizarro *et al*., 2005).

Michael Blackman (1959–) was born in Stockport near Manchester, United Kingdom, and obtained a BSc (Honors) in microbiology from the University of Leeds (1981). He went on to work in Alan Morris' group at the University of Warwick (Warwick, United Kingdom), where he obtained a master of science (MSc) in 1985. His work at Warwick, on the role of interferon (IFN)-γ on viral antigen expression, was absorbing, but Mike felt a need to work in an area more directly relevant to infectious diseases of the developing world. Therefore, later that same year, he leapt at the opportunity to take up a post as a research officer in the Medical Research Council's unit in The Gambia, West Africa, where he worked in Hilton Whittle's group on production of human monoclonal antibodies and the role of antibodies in protection against malaria. These were formative years, and it was here, through the experience of living and working in a malaria-endemic area, that he witnessed at first hand the devastating impact of malaria on the lives of those exposed to the disease. This experience further developed his interest in malaria and in particular a deep fascination with the mechanistic basis of host cell invasion by the malaria parasite.

Mike returned to the United Kingdom in 1988 to study for a PhD in Holder's laboratory at the NIMR. His work focused on the make-up and fate during invasion of the large *P. falciparum* merozoite surface protein complex, merozoite surface protein-1 (MSP-1). Particularly memorable during these enjoyable years was a short spell in Hans Heidrich's laboratory at the Max Planck Institute in Martinsried, Munich, Germany, where he was taught the 'secrets' of merozoite isolation and the necessity of long hours in the laboratory when

working with cultures of *P. falciparum*, as well as the delights of Bavarian beer and sausages! Over the years of his PhD and subsequent work, Mike and his colleagues gradually assembled a picture of the proteolytic processing of the MSP-1 complex during invasion, and came to a realization that this probably corresponded to the 'shedding' of the merozoite surface coat during invasion, a phenomenon that had been observed many years earlier in pioneering electron microscopic studies by Masamichi Aikawa and colleagues in the United States, as well as Lawrence Bannister and colleagues in the United Kingdom. Using a simple cell-based assay, it soon became clear that the shedding of the MSP-1 complex was mediated by a membrane-associated parasite serine protease, and that the process was probably a critical step in red blood cell invasion by the parasite. This finding triggered a program of work aimed at identifying this elusive enzyme, as well as other proteases involved in the complex process of host cell invasion. This resulted in the identification of a small family of three *P. falciparum* subtilisin-like enzymes, one of which, called PfSUB2, was recently identified as the MSP-1 sheddase.

Mike took up a career track appointment at NIMR, and was awarded tenure in 2000. He also holds a position as honorary reader in the Department of Medical Microbiology of University College London (London, United Kingdom). Mike's present research interests still focus on host cell invasion by the malaria parasite and the role in this of the subtilisin-like proteases and a class of intramembrane serine proteases called rhomboids. The work is very much driven by a desire to understand the workings of this remarkable organism, but also by a quote from a now retired ex-colleague, Don Williamson, who once remarked 'The only good reason to study a pathogen is to learn how to kill it!' Research in Mike's laboratory makes extensive use of biochemical techniques and cell biology, with much use of heterologous expression systems and genetic modification by transfection of the human malaria parasite *P. falciparum*. His group collaborates widely with other groups within the Division of Parasitology at NIMR, as well as the Divisions of Protein Structure and Physical Biochemistry, and it is this highly collegial, yet scientifically rigorous, atmosphere that has made his time at NIMR so stimulating and exciting. He also has extensive extramural collaborations that include research groups in the United States and a network of laboratories across the European Union and Africa which make up a large consortium called BioMalPar.

AMA-1 is expressed in the late schizont stage as well as in sporozoites; it is reported to be essential for invasion of hepatocytes (Silvie *et al.*, 2004). PfAMA-1 is synthesized as an 83-kDa precursor from which the

N-terminal 'prosequence' is cleaved, by an as yet unidentified protease, to produce a 66-kDa micronemal mature form that is translocated to the merozoite surface where it is uniformly distributed. The AMA-1 ectodomain is then proteolytically shed during invasion in a 44–48 kDa form, by the same SUB2 enzyme responsible for shedding MSP-1 and its associated proteins (Harris *et al*., 2005; Howell *et al*., 2003). AMA-1 may be responsible for apical re-orientation (Mitchell *et al*., 2004) and likely functions in partnership with one or more rhoptry proteins during formation of the moving junction at invasion. It has not been possible to knockout the gene for PfAMA-1, suggesting it is essential for invasion and studies in the apicomplexan parasite *Toxoplasma gondii* using a conditional expression system have confirmed its critical role in this process (Mital *et al*., 2005).

A large number of rhoptry proteins/peptides have been identified and some partially purified, however, the precise functions of the majority remains unknown. One of the major molecules within the rhoptries of merozoites is a complex of high-molecular-weight proteins, the RhopH complex (Kaneko, 2007). In *P. falciparum*, the RhopH complex associates with the red-cell surface on contact with the merozoite and then distributes to the PVM. Antibodies to the PfRhopH complex were found to inhibit falciparum growth *in vitro* and *in vivo*. RhopH1 proteins may serve to confer some degree of specificity to the roles of the individual complexes (Kaneko *et al*., 2005). RhopH2 and RhopH3 are associated with Maurer's clefts. Attempts to disrupt *pfrhop3* were unsuccessful suggesting the complex is essential for parasite growth. The rhoptry-associated membrane antigen (RAMA) is synthesized as a 170-kDa protein in early trophozoites, several hours before rhoptry formation and is transiently localized within the endoplasmic reticulum and Golgi within lipid-rich microdomains. Regions of the Golgi membrane containing RAMA bud to form vesicles that later mature into rhoptries in a process that is inhibitable by brefeldin A. RhopH3 and RAP1 are found in close apposition with RAMA. It has been suggested that RAMA is involved in trafficking of these proteins into rhoptries. In rhoptries, RAMA is proteolytically processed to give a 60-kDa form that is anchored in the inner face of the rhoptry membrane by means of the GPI anchor. The p60 RAMA form is discharged from the rhoptries of free merozoites and binds to the red blood cell membrane by its *C*-terminal region. In early ring stages, RAMA is found in association with the PV (Topolska *et al*., 2003, 2004a,b,c).

Proteases may be promising targets for drug intervention since proteolytic events have been shown to be critical for successful invasion of the red cell by the merozoite (Blackman, 2000; O'Donnell and Blackman, 2005). For example, *in vitro* invasion by falciparum merozoites can be blocked by the serine and cysteine protease inhibitors, chymostatin and leupeptin; moreover, these inhibitors act at different steps in the invasion process. Chymostatin inhibited both invasion and rupture of

the schizont-infected red cell whereas leupeptin inhibited only rupture (Hadley *et al*., 1983). Proteases may affect merozoite release by intensive degradation of the cytoskeleton leading to abrupt vesiculation of the red cell membrane to release merozoites (Glushakova *et al*., 2005; Roggwiller *et al*., 1998; Salmon *et al*., 2001). Protein phosphatase-1 has also been implicated in the release of merozoites by modulating the phosphorylation levels of *P. falciparum* skeleton-binding protein 1 (PfSBP1), a transmembrane protein of Maurer's clefts (Blisnick *et al*., 2006). Proteolytic modification of the red cell has also been shown to be necessary for invasion (Braun-Breton and Pereira da Silva, 1993; Braun-Breton *et al*., 1994). Chymotrypsin treatment of red cells resulted in degradation of band 3 protein and the resultant localized disruption of the membrane may allow for the insertion of parasite- derived proteins/lipids into the PVM (Roggwiller *et al*., 1996). Significantly, peptides corresponding to the predicted cleavage site inhibited merozoite invasion (Roggwiller *et al*., 1996).

Clearly over the past 30 years a staggering array of molecules involved in invasion have been identified (Cowman and Crabb, 2006; Galinski *et al*., 2005; Topolska *et al*., 2003). In addition, some species such as *P. falciparum* and *P. knowlesi* (unlike *P. vivax*) use multiple invasion pathways. The use of multiple pathways may provide those species with a 'survival advantage when faced with host immune responses or receptor heterogeneity in host populations' (Chitnis, 2001). Because of the heterogeneity of merozoite ligands, exploiting specific invasion pathways as a vaccine target may be an unrealistic goal, however, other mechanisms involved in invasion (i.e. proteolytic processing) could be a suitable target for drug and other therapeutic interventions. In addition, although it has been seductive to consider using sophisticated molecular approaches to block invasion, one should be mindful of the value of simpler strategies. For example, a novel intervention used polyethylene glycol-coated red cells; these resist invasion, possibly by masking glycophorin A (Blackall *et al*., 2001). Additionally, cyclodextrins inhibited *P. falciparum* merozoite entry *in vitro* and reduced *P. berghei* infections in a mouse model (Crandall *et al*., 2007).

Brendan Crabb (1966–) began his research career as a virologist and performed his PhD and first post-doctoral investigations at the Veterinary School at the University of Melbourne (Melbourne, Vic, Australia) in Michael Studdert's laboratory. There he focused on animal viruses, initially herpes viruses and later a different virus family, the picornaviruses. The most significant aspect of his studies was the finding, together with PhD student Feng Li, that a particular virus thought to represent a 'common cold'-type infection of horses was in fact the only known relative of the foot-and-mouth disease virus,

probably the world's most important animal pathogen. The significance of this discovery was enhanced by further work showing that a non-cultivatable form of this same virus was responsible for significant outbreaks of disease in horses, suggesting that the virus was a much more important pathogen than had been previously recognized. He has continued an active interest in virology up to very recent years where he and a number of PhD students have characterized key pathogenic determinants of this and other viruses. In 1995, Brendan began a short but career-changing post-doctoral period in Alan Cowman's laboratory at WEHI. This move was accompanied by a major shift in research focus to the study of *P. falciparum*. He was centrally involved in development of stable transfection technology in this parasite and together with Cowman and colleagues described the first gene knockout in *P. falciparum*, that of the knob-associated histidine-rich protein. In mid-1996, he moved to the position of lecturer then senior lecturer in medical virology in the Department of Microbiology and Immunology, The University of Melbourne where he established his own laboratory focussing on both virology and parasitology.

In 2001, Brendan and his laboratory moved back to WEHI. He is currently a principal research fellow in the National Health and Medical Research Council of Australia (Canberra ACT, Australia) and an international fellow of the Howard Hughes Medical Institute (Chevy Chase, Maryland). The central theme of his studies is to address the molecular basis of host–parasite interactions, especially to aid vaccine discovery. He is especially interested in determining the function of merozoite antigens and their prospects as vaccines. He retains a focus on the development of genetic technologies to characterize the function of *P. falciparum* proteins, especially of those that are essential to blood-stage growth.

CHAPTER 24

Vitamins and Anti-Oxidant Defenses

Contents

The role of vitamins in malaria infections has been studied for more than a half century. In many of the reports on the effects of vitamin deficiency it is not clear whether the deficiency affected the growth of the parasite directly or whether host nutrition was compromised leading to an indirect effect. At a USAID/World Health Organization (WHO, Geneva, Switzerland) Workshop on the Biology and *In vitro* Cultivation of Malaria Parasites held at the Rockefeller University (New York), Trager (1977) tersely summarized what was known about vitamins, 'Relatively few co-factors have so far been demonstrated to be essential for the erythrocytic stages of malaria.' In the succeeding 30 years, although much has been learned about vitamins (reviewed in Müller and Kappes, 2007) whether they are biosynthesized

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or salvaged, and in some instances what roles they play in the life of *Plasmodium*, the complete picture still remains far from clear.

1. VITAMIN B_1 (THIAMIN)

Vitamin B_1 is an essential co-factor for several enzymes of carbohydrate metabolism such as transketolase, pyruvate dehydrogenase (PDH), pyruvate decarboxylase and α-ketoglutarate dehydrogenase. To become the active co-factor: thiamin pyrophosphate (TPP), thiamin has to be salvaged by thiamin pyrophosphokinase or synthesized *de novo*. In *Escherichia coli* and *Saccharomyces cerevisiae* thiamin biosynthesis proceeds *via* two branches that have to be combined. In the pyrimidine branch, 4-amino-5-hydroxymethy-2-methylpyrimidine (HMP) is phosphorylated to 4-amino-2-methyl-5-hydroxymethyl pyrimidine diphosphate (HMP-PP) by the enzyme HMP/HMP-P kinase (ThiD); however, the step can also be catalyzed by pyridoxine kinase (PdxK), an enzyme also responsible for the activation of vitamin B_6 (see below). The second precursor of thiamin biosynthesis, 5-(2-hydroxyethyl)-4-methylthiazole (THZ), is activated by THZ kinase (ThiM) to 4-methyl-5-(2-phosphoethyl)-thiazole (THZ-P), and then the thiazole and pyrimidine moieties, HMP-PP and THZ-P, are combined to form thiamin phosphate (ThiP) by thiamin phosphate synthase (ThiE). The final step, pyrophosphorylation, yields TPP and is carried out by thiamin pyrophosphorylase (TPK).

Mammalian and avian hosts salvage thiamin from their diet, however, in spite of the fact that *Plasmodium falciparum* is able to synthesize this vitamin (by a pathway similar to that found in bacteria and yeast), the cultivation of the parasite in minimal medium is dependent on the provision of HMP or thiamin but not THZ suggesting that HMP and thiamin are transported into the parasite (Wrenger *et al*., 2006). However, such transporters have not been identified and a Basic Local Alignment Search Tool (BLAST, a program for database searching) search of known thiamin transporters of other organisms found no homologies in the *P. falciparum* genome database (Walter, personal communication). The fact that *in vitro* growth is supported by the addition of HMP suggests an absence of a link of the B_1 and B_6 pathways. In *P. falciparum*, the three thiamin biosynthetic genes ThiM, ThiD and ThiE (which were predicted from the genome) have been cloned, sequenced and functionally characterized (Wrenger *et al*., 2006). The ThiM protein, encoded by a gene on chromosome 12 and with a molecular mass of 34 kDa, is a monomer whereas the PfThiD (located on chromosome 5 and with a molecular mass of 35 kDa) and PfPdxK are dimers consistent with the structure of their bacterial and mammalian counterparts. PfThiE located on chromosome 6 and with a molecular mass of 68 kDa is a monomer. To become the active co-factor, HMP-PP

and THZ-P have to be converted by ThiE to Thi-P, and the latter needs to be dephosphorylated and then activated by TPK to form Thi-PP. Although a TPK orthologue has been found the specific phosphatase has not.

Using Northern blotting transcripts for ThiM, ThiD and ThiE, genes were found to be expressed during the entire erythrocytic cycle in good agreement with the microarray data (Bozdech *et al*., 2003; Le Roch *et al*., 2003). ThiM did not phosphorylate HMP and had strict substrate specificity for THZ. PfPdxK was able to accept all B_6 vitamers (pyridoxine, pyridoxal and pyridoxamine) as substrates and also phosphorylates HMP though not HMP-P. When the medium used for the *in vitro* culture of *P. falciparum* was depleted of thiamin, parasite growth ceased, however supplementation with HMP and thiamin (but not THZ) restored parasite proliferation 'suggesting uptake of and astonishingly dependence on HMP or thiamin' (Wrenger *et al*., 2006). In support of this the enzymes responsible for the formation of HMP were not found in the *P. falciparum* genome. However, these experiments also suggest that thiamin depletion merely has an inhibitory and not a toxic effect on the parasites, not an ideal situation for a future drug target.

PfTPK was expressed throughout the erythrocytic cycle and, by virtue of an *N*-terminal extension, is larger than TPKs from other organisms (Eschbach *et al*., 2006). PfTPK is found in the cytosol which implies that TPP is formed in the cytosol before it is transported into the mitochondrion and apicoplast where it acts for instance as a co-factor for the α-keto acid dehydrogenase complex. It was, therefore, suggested that there must be specific transporters for the relocation of TPP into both organelles (Eschenbach *et al*. 2006). Such transporters are still to be found.

2. VITAMIN B_6

Vitamin B_6 is an essential co-factor for many enzymatic reactions, including amino acid decarboxylation, elimination and amino transfer. This vitamin is a requirement for an essential enzyme, serine hydroxymethyl transferase (SHMT) in the folate metabolism of *Plasmodium* spp. (see p. 124–125). Mammalian cells are unable to synthesize the vitamin *de novo* hence they must rely on salvage. To become the active co-factor pyridoxal-5'-phosphate, vitamin B_6, pyridoxine or pyridoxal or pyridoxamine has to be phosphorylated by pyridoxine/pyridoxal kinase (PdxK). In 1977, Trager suggested that PdxK might be lacking in malaria parasites because there is a much lower activity of this enzyme in the red cells of African people than those of Caucasians (whites). When Platzer and Kassis (1978) found PdxK activity in *P. lophurae*, Trager's proposal appeared to be without support; however, since they also found the lophurae enzyme to have a lower affinity for pyridoxine than the host enzyme it was suggested that if

the host were limited in the availability of pyridoxine the parasite could be deprived of this co-factor.

Evidence for an active vitamin B_6 biosynthetic pathway in *P. falciparum* was shown by metabolic labeling experiments (Cassera *et al.*, 2004) and a sequence analysis of the parasite genome revealed the presence of three genes: *pdx1*, *pdx2* and *pdxK*. The genes encoding PfPdx1, PfPdx2 and PfPdxK have been cloned, sequenced and expressed (Müller and Kappes, 2007). PfPdxK is a protein with a molecular mass of 58 kDa, the *Pfpdx1* gene, localized to chromosome 6, encodes a 33-kDa protein, and Pfpdx2, found on chromosome 11, encodes a 24-kDa protein. The deduced amino acid sequence of all three proteins had a high degree of similarity to the enzymes of yeast. A BLAST search of the *P. falciparum* genome database (http://www.plasmodb.org; last accessed 16 July 2008) with homologues of other organisms found no other homologous genes suggesting these are single copy genes in *P. falciparum*. Northern blot and Western blotting found evidence for the expression of these genes and PfPdxK in the trophozoite stage (Gengenbacher *et al.*, 2006; Wrenger *et al.*, 2005). This is in agreement with the microarray data (Bozdech *et al.*, 2003; Le Roch *et al.*, 2003). All three plasmodial proteins lack a targeting sequence to either the apicoplast or the mitochondrion suggesting the proteins for *de novo* biosynthesis of pyridoxal by PfPdx1 and PfPdx2 and salvage of pyridoxine/pyridoxal or pyridoxamine and their subsequent phosphorylation by PfPdxK are cytosolic. This prediction was corroborated experimentally by immunofluorescence studies (Gengenbacher *et al.*, 2006). Since a BLAST search of the *P. falciparum* genome has not revealed polypeptides similar to the vitamin B_6 transporter in yeast or plants, it appears *P. falciparum* relies on *de novo* synthesis of vitamin B_6. Although PfPdxK accepts all B_6 vitamers it has preference for pyridoxine, whereas the human enzyme prefers pyridoxal. This difference might be exploitable for future drug design should the salvage of the vitamer be essential for parasite survival.

The functionality of PfPdx1 and PfPdx2 has been analyzed using recombinant proteins (Gengenbacher *et al.*, 2006). Recombinant PfPdx1 and PfPdx2 form a complex that functions as a class I glutamine amidotransferase with two domains: Pdx2, the glutaminase domain, produces ammonia from glutamine and the Pdx1 acts as the pyridoxal-5′-phosphate synthase domain that tunnels the reactive intermediate to the second active site, where pyridoxal phosphate (PLP) is formed. Pdx1 alone had no detectable PLP synthase activity with any of its substrates when glutamine was used as the ammonia donor; ribose-5-phosphate and ribulose-5-phosphate as well as glyceraldehydes-3-phosphate and dihyroxyacetone phosphate were substrates for the PLP synthase.

An elevated transcription of Pfpdx1 and Pfpdx2 was found after incubation of parasites with methylene blue suggesting that the proteins

encoded by these genes play a role in defense against singlet oxygen (Wrenger *et al*., 2005).

3. PANTOTHENATE

The need for pantothenate for good *in vitro* growth of *P. falciparum* (Divo *et al*., 1985) and the 1946 observation by Brackett that a dietary deficiency of pantothenate in chickens infected with *P. gallinaceum* resulted in a marked reduction in parasitemia (quoted in Trager, 1977) suggested that the malaria parasite was unable to synthesize pantothenate. Pantothenate is taken up by the infected red cell through the new permeation pathway (see p. 162) and is transported into the parasite by an H^+ pantothenate symporter (Saliba and Kirk, 2001). Once inside the parasite, the pantothenate is phosphorylated by pantothenate kinase; the first step in the formation of coenzyme A. Humans have four distinct forms of pantothenate kinase, however, *P. falciparum* has only one form. *P. falciparum* has two genes (annotated as hypothetical) that encode the second enzyme in the pathway: phosphopantothenylcysteine synthase. Genes that encode orthologues of pantothenoyl carboxylase have been identified, as have the next two enzymes, pantetheine phosphate adenyl transferase and dephospho CoA-kinase (http://www.plasmodb.org; last accessed 16 July 2008) which, unlike the host's, are separate enzymes. In the synthesis of coenzyme A (CoA) from pantothenate, *P. falciparum* is unlike *P. lophurae*, where it was shown that the host cell synthesizes all five of the pathway enzymes and the parasite lacks all; as a consequence, *P. lophurae* is reliant on the uptake of pre-formed coenzyme A from the host red cell (Brohn and Trager, 1975).

In short-term *in vitro* experiments with *P. falciparum* and *P. vivax*, antimetabolites of pantothenate inhibited parasite development (Trager, 1977). Pantothenol, a pro-vitamin of pantothenic acid, significantly reduced the parasitemia of mice infected with *P. vinckei* and the pantothenate analogue CJ-15, 801 inhibited the *in vitro* growth of *P. falciparum* (Saliba *et al*., 2005). Eight of 10 pantothenate analogues inhibited the proliferation of *P. falciparum in vitro* with an IC_{50} of 15–200 μM, however, when tested for *in vivo* activity in *P. vinckei*-infected mice more than half of these compounds were toxic (Spry *et al*., 2005).

4. BIOTIN (VITAMIN B_7)

Biotin has been shown to be a coenzyme for four carboxylases:

- acetyl coenzyme A carboxylase catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, is a precursor of 14 of the 16 carbons of palmitic acid, an immediate precursor of all fatty acids (FAs) up to palmitic acid, and is the rate limiting step in the biosynthesis of FAs,
- pyruvate carboxylase catalyses the carboxylation of pyruvate to form oxaloacetate (OAA),
- methylcrotonyl-CoA carboxylase involved in the metabolism of *L*-leucine and
- propionyl-CoA carboxylase involved in the metabolism of *L*-isoleucine, *L*-valine, *L*-threonine and *L*-methionine.

All four carboxylases use bicarbonate as their one-carbon substrate and, in all, the biotin is covalently linked by an amide bond between the carboxyl of biotin and an epsilon amino group of a lysyl residue in the holocarboxylase synthase (= biotin ligase) that catalyzes the formation of the covalent bond. Biotinylation of histones is involved in regulation of gene transcription and may also play a role in packaging of deoxyribonucleic acid (DNA). Biotin has also been found to inhibit the generation of reactive oxygen species (ROS) by neutrophils *in vitro*.

Studies of a biotin requirement for *Plasmodium* spp. have been inconsistent. *P. cathemerium* infections in biotin-deficient ducklings were reported to progress slowly suggesting that biotin was required for growth of the parasites (Trager, 1977) and Siddiqui *et al*. (1969) found biotin to favor the *in vitro* growth of *P. knowlesi*. By contrast, there was no effect with *P. lophurae* in biotin-deficient ducklings and blood from biotin-deficient monkeys did not adversely affect *P. knowlesi* growth (quoted in Trager, 1977). *P. falciparum* has been shown to lack the ability to synthesize biotin *de novo* but does have a biotin-dependent acetyl-CoA carboxylase (ACCase) localized to the apicoplast (Müller and Kappes, 2007). The ACCase is formed by biotin being covalently attached to acetyl-CoA *via* a biotin ligase and the *P. falciparum* genome has two orthologues that could potentially encode this ligase.

5. VITAMIN B_3 (NIACIN)

Nicotinamide adenine dinucleotide (NAD; and its reduced form dihydronicotinamide adenine dinucleotide (NADH)) as well as nicotinamide adenine dinucleotide phosphate (NADP; and its reduced form nicotinamide adenine dinucleotide phosphate hydrogen (NADPH)) are essential co-factors for many dehydrogenases. The presence of NAD was first demonstrated in *P. gallinaceum* (Speck and Evans, 1945) and later studies showed increased levels of these pyridine nucleotides in *P. lophurae*, *P. berghei* and *P. falciparum*. Trager (1977) reported that high levels of

nicotinamide favored the extracellular growth of *P. lophurae*. Taken together, these studies provided circumstantial evidence for the *de novo* synthesis of NAD and NADP. NAD is synthesized by the following process: nicotinic acid is converted to nicotinic acid mononucleotide (NAMN) *via* nicotinic acid phosphoribosyl transferase (NAPRT) and the NAMN is then adenylated to give nicotinic acid adenine dinucleotide (NAAD) *via* NAAD pyrophosphorylase; this is then amidated to NAD *via* NAD synthase. Alternatively NAD synthesis can occur from nicotinamide either by deamidation to nicotinic acid through nicotinamide deaminase or by being converted to nicotinamide mononucleotide (NMN) *via* nicotinamide phosphoribosyl transferase and then to NAD *via* NAD phosphorylase.

Activity of NAPRT was increased in *P. falciparum*-infected red blood cells as was nicotinamide synthase and nicotinamide deamidase: NAAD and NAD phosphorylase remained at similar levels upon infection. It was concluded that the majority of NAD synthesis in *P. falciparum* occurs from both nicotinic acid and nicotinamide *via* nicotinamide deamidase, NAPRT, NAAD phosphorylase and NAD synthase (Zerez *et al*., 1990). None of the genes for these enzymes has been cloned neither have the enzymes been isolated. However, evidence for them can be found in the *P. falciparum* genome database (http://www.plasmodb.org; last accessed 16 July 2008).

6. P-AMINOBENZOIC ACID

In 1945, Ball *et al*. found *p*-aminobenzoic acid **(**pABA) to favor the *in vitro* growth of *P. knowlesi* and Hawking (1954) found a similar effect on *P. berghei in vivo* (quoted in Trager, 1977). Hosts fed on a milk diet deficient in pABA suppressed *P. knowlesi*, *P. berghei* and *P. falciparum* infections and when the milk diets were supplemented with pABA the infections developed (quoted in Ferone, 1977). Jacobs (1964) carried out a detailed study of pABA in *P. berghei* infections and concluded '... the parasite is unable to utilize pre-formed folic or folinic acids and the parasite utilizes pABA to form a substance similar to but not identical with folic acid.' This notion of pABA dependence persisted until the 1990s. Recent biochemical and genetic studies have shown that *Plasmodium* spp. can synthesize pABA *de novo* utilizing phosphoenolpyruvate **(**PEP) (from glycolysis) and erythrose-4-phosphate (from the pentose phosphate pathway, PPP) *via* the shikimate pathway. The pABA is then incorporated into dihydropteroate by dihydropteroate synthetase (DHPS). All sulpha drugs (dapsone, sulphadoxine, sulphalene) work by competing with pABA for the binding site on DHPS. In addition, resistance to these drugs is generally ascribed to mutations in DHPS. It has

been found that the malaria parasite is capable of overcoming this competitive inhibition by sulpha drugs by switching over to other metabolic pathways (i.e. direct salvage from the host blood stream). Platteeuw (2006) suggests that were this the case then increased folic acid administration *via* diet or supplementation would reduce the effectiveness of sulpha drugs more than mutations.

Since malaria parasites are capable of synthesizing pABA (McConkey *et al*., 1994), why the requirement for an exogenous supply? Kicska *et al*. (2003) postulate the parasites are unable to synthesize sufficient quantities of pABA to survive *in vivo*. In addition to *de novo* synthesis of folates, malaria parasites are able to salvage folate (McConkey *et al*., 1994); indeed, both have comparable activity provided the salvage pathway is provided with reduced folate (i.e. dihydrofolate (DHF) or folinic acid (the 5-formyl derivative (N^5 formyl-5,6,7,8-tetrahydrofolic acid)) both of which can be converted to THF; Wang *et al*., 2004a). Wang *et al*. (2004b) showed that *P. falciparum* dihydrofolate reductase (DHFR)-TS converts folic acid in an enzyme- and co-factor-dependent fashion to a compound that behaves like THF. In *P. falciparum*, folinic acid is taken up and processed efficiently by the parasite providing a much better source of folates in culture than the usual supplementation with folic acid in RPMI 1640 (Nzila *et al*., 2005; Wang *et al*., 2004b) promoting a more robust growth. These findings may explain the favorable effects of added folic and folinic acid for *P. lophurae* growing intracellularly and extracellularly (Siddiqui and Trager, 1966). In addition, it may be that in these cases the folic and folinic acid samples contained 4-aminobenzoyl glutamate, or the host or red cell extract contained enzymes that broke down the added folates and this was converted into pABA to be used in the synthesis of THF (Ferone, 1977). However, how the folinic acid is used so efficiently by the parasite remains to be explained.

7. RIBOFLAVIN

Riboflavin, the precursor for the coenzymes flavin mononucleotide (FMN) and flavin adenine denucleotide (FAD) was first reported to be present in *P. knowlesi* (Ball *et al*., 1948). Riboflavin uptake and the rate of synthesis of FMN and FAD were enhanced in *in vitro* grown *P. falciparum* (Dutta, 1991). A correlation between the number of schizonts and riboflavin uptake by *P. falciparum*-infected red cells was taken to be suggestive of the greater sensitivity of these stages to oxidative stress. Although riboflavin deficiency had been shown to be associated with lower parasitemias in experimentally infected animals and humans, a recent re-investigation did not support the relationship between flavin concentration and parasitemias in *P. falciparum*-infected subjects in Gabon (West Central Africa) and claimed

the older work was suspect because of its methodology (Traunmuller *et al*., 2003). The presence of riboflavin (25–100 μM) in *in vitro* cultures of *P. falciparum* inhibited the production of hemozoin and affected parasite maturation to schizogony as well as being gametocytocidal (Akompong *et al*., 2000a,b), and when combined with mefloquine, quinine or pyrimethamine the antimalarial effects were potentiated. Cowden *et al*. (1987) found that the riboflavin analogue 10-(4'chlorphenyl)-3-methylflavin had *in vivo* activity against *P. vinckei* and inhibited the *in vitro* growth of *P. falciparum*. Possibly, the mode of action could be by reducing the levels of glutathione (GSH) in red cells due to decreased glutathione reductase (GR) activity, which is also reduced with riboflavin deficiency (Becker *et al*., 1990). It was suggested by Dutta (1991) that, although a marginal deficiency of riboflavin may be of little consequence to the well being of the host, it could be detrimental to the parasite since the deficiency could result in enhanced vulnerability to oxidative stress.

8. VITAMINS A, C, D AND E

The role of the vitamins A, C, D and E in *Plasmodium* spp. have been summarized recently (Müller and Kappes, 2007). Malaria infections are accompanied by decreases in vitamin A, and in rodent malarias there is an inverse correlation between parasitemia and vitamin A concentration in the host. Vitamin A supplements partially protect against malaria and this may be due to an indirect effect: the up-regulation of CD36 and the down-regulation of cytokines. It is not clear how vitamin A deficiency of the host is caused or whether the parasites can take up the vitamin.

An increase of vitamin C and E concentrations in *P. vinckei*-infected red cells led to the hypothesis that the parasites were able to synthesize these vitamins (quoted in Müller and Kappes, 2007), however, this is not supported by examination of the *P. falciparum* genome since genes for the synthesis of ascorbate or tocopherol were not found. A deficiency of vitamin C or E has a protective effect in malaria patients possibly because a lack of this anti-oxidant renders the parasites more vulnerable to oxidant stress.

There is no clear evidence that vitamin D_3, critical to calcium homeostasis in mammals and birds, is beneficial or adverse to malaria infections. Vitamin D_3 analogues were shown to have a pronounced inhibitory effect on mature stages of intraerythrocytic *P. falciparum* growing *in vitro* possibly by affecting phospholipid (PL) biosynthesis (Vial *et al*., 1982). None of the genes involved in the synthesis of vitamin D_3 have been characterized in *P. falciparum*, neither has a gene encoding a vitamin D receptor been found in the *P. falciparum* genome database (http://www.plasmodb.org; last accessed 16 July 2008).

9. ANTI-OXIDANT DEFENSES

Malaria parasites are subject to oxidative stress largely as a result of their living in the hemoglobin-containing erythrocyte, an environment rich in oxygen and iron, key prerequisites for the formation of highly ROS such as superoxide anions, hydrogen peroxide and hydroxyl radicals *via* the Fenton reaction (reviewed in Becker *et al*., 2004; Müller, 2004). In the acidic food vacuole (FV) ingested hemoglobin is spontaneously oxidized (ferrous to ferric iron) to form superoxide anions. This leads to the generation of hydrogen peroxide and hydroxyl radicals. In addition to the oxidative stress placed upon the parasite the erythrocyte is also exposed to such stressors. This leads to changes in membrane fluidity, probably the result of alterations in lipids and protein cross-linking as well as the accumulation of hemichrome on the inner surface of the plasma membrane with band 3 protein aggregation; these changes are similar to those found in senescent red cells as well as in sickle cell anemia, α and β-thalassaemias and glucose-6-phosphate dehydrogenase (G6PD) deficiency (reviewed in Becker *et al*., 2004). These mutant red blood cells have been shown to possess a degree of resistance to infection thereby limiting the severity of the disease either through a direct impairment of parasite growth or to the more efficient phagocytic removal of red cells infected with ring-stage parasites due to precocious aggregation of band 3 protein and the early appearance of anti-band 3 antibodies. In consequence, the parasite burden is reduced (Arese *et al*., 2006).

The redox state of a cell is mediated by the ratios of reduced and oxidized pyridine nucleotides (NADH/NAD/NADPH/NADP) and thiols such as reduced and oxidized GSH (GSH/GSSG) and thioredoxin/thioredoxin disulphide. In addition, anti-oxidants such as vitamin E (tocopherol) and ascorbate (vitamin C) can reduce ROS non-enzymatically.

Sylke Müller (1961–) graduated from the University of Hamburg (Hamburg, Germany) with a diploma in biology (1987) and subsequently studied for a PhD in parasitology at the Bernhard Nocht Institute for Tropical Medicine (Hamburg, German, awarded 1991). During this time, she worked on the polyamine metabolism of *Onchocerca volvulus*, a filarial parasite. In 1992, Sylke left the Bernhard Nocht Institute and went to London (United Kingdom)where she worked in Alan Fairlamb's group at the London School of Tropical Medicine and Hygiene (London, United Kingdom), funded by a Wellcome Trust Travelling Research Fellowship. Here she became interested in redox enzymes, particularly in GR, and studied the gene and protein in filarial worms. In 1994, after returning to the Bernhard Nocht Institute for Tropical Medicine, her research focused primarily on redox and anti-

oxidant systems of the malaria parasite *Plasmodium falciparum* although work continued on polyamines and redox enzymes in nematode parasites into 2000. In 1999, she was awarded *Habilitation* (in recognition of research) and the *venia legendi* (in recognition of teaching contributions at the University of Hamburg). In 2001, she moved to the University of Dundee (Dundee, United Kingdom) and took up a Wellcome Trust senior fellowship in basic biomedical science to investigate the role of redox and anti-oxidant systems for the survival of *P. falciparum*. In 2004, she moved to the University of Glasgow (Glasgow, United Kingdom) as a member of the Wellcome Centre of Molecular Parasitology and the Division of Infection and Immunity. In 2005, the senior fellowship was re-newed for an additional 5 years to study different aspects of the metabolism of the malaria parasite and in June 2006, she was awarded a personal professorship of molecular and biochemical parasitology at the University of Glasgow. Her current interests are in redox metabolism and redox signaling processes in *Plasmodium* as well as understanding the role of key metabolic processes in the mitochondrion and apicoplast in addition to defining the mechanisms of co-factor acquisition and elucidating their importance for malaria parasites.

No gene coding for a plasmodial catalase has been found in the *P. falciparum* genome and it is doubtful that one exists. Although *Plasmodium* spp. lack catalase (and GSH peroxidases) their armamentarium of anti-oxidant defense mechanisms includes:

- a GSH system comprising NADPH, GR and glutaredoxins,
- a GSH-dependent glyoxalase system and a glutathione-*S*-transferase with peroxidase activity,
- a thioredoxin system consisting of NADPH, thioredoxin reductase (TrxR), thioredoxins,
- superoxide dismutase (SOD) and
- lipoamide dehydrogenases (Becker *et al*., 2005; Rahlfs and Becker, 2005).

9.1. Superoxide dismutase

The first line of intracellular defense against ROS are SODs that breakdown superoxide anions into hydrogen peroxide, oxygen and peroxidases that reduce the hydrogen peroxide to water and oxygen. *P. falciparum* has two genes that encode SODs: PfSOD1 is Fe dependent, cytosolic, and transcribed and expressed throughout the erythrocytic life cycle and PfSOD2 is a mitochondrial protein. Since PfSOD1 is cytosolic, it is unlikely to act on superoxide anions in the FV, however, it is possible that host Cu/Zn-SOD ingested by the parasite (Fairfield *et al*., 1983) can contribute to detoxification. Further, superoxide anions generated in the FV may spontaneously dismutate

because of its acidic pH. PfSOD2 is assumed to be critical to the survival of the parasite because the respiratory chain (see Chapter 11, p. 98) leaks superoxide anions; however, convincing experimental proof for this is required. The gene for SOD1 has been cloned and sequenced in *P. vivax*, *P. ovale* and *P. malariae* (Baert *et al.*, 1999).

The transcript for the cytosolic PfSOD1 peaks at the trophozoite stage (approximately 30 h post-infection), whereas the mitochondrial PfSOD2 is expressed in lower amounts and peaks later in schizogony suggesting that leakage of ROS from the respiratory chain may be highest at this stage of parasite development (Bozdech and Ginsburg, 2004). *In vitro* screening of a chemical library of synthetic compounds led to the discovery of 15 compounds that have the ability to inhibit recombinant *P. falciparum* iron SOD (Soulere *et al.*, 2003).

9.2. Glutathione and glutathione reductase

The cysteine-containing tripeptide, glutathione (γ-glutamyl-cysteinyl-glycine, GSH), is the most abundant low-molecular-weight antioxidant in malaria parasites. When I studied the levels of GSH in *P. lophurae*-infected duckling erythrocytes (Sherman, 1965) it had already been shown to have a beneficial effect on the intracellular development of the parasite (McGhee and Trager, 1950) and contributed to the cysteine requirement of *P. knowlesi* (Fulton and Grant, 1956). However, when I found no decline in GSH levels with parasitemia, the conclusion I drew was that the *Plasmodium* did not disturb the capacity of the host cell to maintain its redox state. At that time, working in a laboratory adjoining that of Trager (1950), who had shown a beneficial effect of adding reduced GSH to cultures of extracellular *P. lophurae*, I asked whether he would like me to measure the amounts before and after 24 h of culture. He agreed. When I reported to him that after one day in culture there was no longer any reduced GSH, and therefore it was unlikely to favor growth, he shrugged his shoulders and said, 'All I can say is the parasites grow better when I add it to the red cell extract.' I did not doubt Trager's findings of a beneficial effect—he was a meticulous worker—however, the experience led me to believe that one should be cautious in defining a nutritional requirement when using a complex medium such as red cell extract.

In most cells, the ratio between GSH and its oxidized form, GSSG, is usually between 10:1 and 100:1 and is mainly achieved by the action of GR (Becker *et al.*, 2003b). In the host cell compartment containing trophozoites of *P. falciparum*, the GSH/GSSG ratio was decreased to approximately 28 whereas in the normal red cell and the *Plasmodium* the value is around 300, indicating a redox burden on the host cell compartment. In addition, this oxidative burden is reflected by a

considerable loss of GSSG probably mediated through the new permeability pathways (NPPs) (see Chapter 18, p. 151). GSH can be taken up from the extracellular space and into the red cell cytosol *via* the NPP. Luersen *et al*. (2000) suggest that GSH uptake into the *Plasmodium* occurs by some unknown route, possibly *via* the FV.

In the cytosol of a *P. falciparum* trophozoite, the GSH concentration is around 2 mM and this level is achieved by *de novo* synthesis. The rate of synthesis was determined at 184 nmol/10^{10} cell/h (Atamna and Ginsburg, 1997; Becker *et al*., 2005). A functional GSH synthesis pathway is present in *Plasmodium*—the genes for γ-glutamylcysteine synthetase (γ-GCS) and GSH synthetase that catalyse the ligation of glutamate and cysteine followed by the addition of glycine, as well as GR, have been cloned and sequenced (reviewed in Bozdech and Ginsburg, 2004). GCS has been characterized for *P. falciparum* and *P. berghei* (Luersen *et al*., 2000).The transcription of the *PfGCS* gene correlates well with the intensity of hemoglobin metabolism, it begins at the young trophozoite (12–18 h post-infection), is highest in the trophozoite stage and diminishes in the schizont stage (Bozdech and Ginsburg, 2004).

GR is a 550 amino acid flavo disulphide oxidoreductase. The primary structure of the falciparum GR contains parasite-specific insertions in the FAD, central and interface domains (Farber *et al*., 1998). When the first two insertions were deleted, the stability and FAD co-factor binding were reduced. Overall, the structures of the human and plasmodial GR are very similar, however, subtle differences do occur in the dimer interface region and this may allow for the design of specific inhibitors such as isolloxazine derivatives and methylene blue, which have been shown to inhibit enzymatic activity in the micromolar range (Sarma *et al*., 2003). Interestingly, a strongly synergistic action of methylene blue, which is active on *P. falciparum* in the low nanomolar range, and artemisinins has been reported on malarial parasites in cell culture (Akoachere *et al*., 2005).

When the *de novo* synthesis of GSH was inhibited, half of the GSH in the *P. falciparum*-infected red cells was lost within 2 h suggesting that the parasitized cell is highly dependent on its synthesis (Müller, 2004). *P. falciparum*-infected red cells have been reported to contain about half the GSH concentration of uninfected erythrocytes possibly due to rapid efflux of GSSG from infected red cells (Bozdech and Ginsburg, 2004). The rapid efflux of GSSG suggests that the increased oxidation of GSH cannot be met by the GR of the parasite and host cell, and maintenance of an adequate GSH/GSSG thiol redox state is achieved by increased GSSG efflux.

As well as its role as a general thiol redox buffer, GSH acts as a co-factor for a variety of enzymes, including GSH-dependent peroxidases, glutathione-*S*-transferases (GST), glutaredoxins and glyoxalase.

GSTs catalyse the conjugation of GSH to toxic by-products of oxidative stress. *P. falciparum* has a single gene encoding GST and two roles have been suggested: (1) to sequester ferriprotoporphyrin IX and (2) to act as an alkyl hydroperoxide peroxidase in addition to its role as detoxification enzyme (Becker *et al.*, 2003b).

Since G6PD-deficient red cells can have decreased levels of GSH (due to the decreased availability of NADPH), it may be that it is the inadequate concentrations of intracellular GSH that underlies the increase in oxidative damage seen in such cells when infected by *P. falciparum* and hence their selective removal from the circulation (Arese *et al.*, 2006).

9.3. Glutaredoxins and plasmoredoxins

Glutaredoxins are thio-disulphide oxidoreductases that protect the cell from oxidative damage and are involved in redox regulation (Müller, 2004; Rahlfs *et al.*, 2001). *P. falciparum* has a classic GSH/glutaredoxin redox system and a second glutaredoxin-like protein with a PICOT homology domain as well as a number of glutaredoxin-like proteins (Deponte *et al.*, 2005). Glutaredoxin is able to reduce ribonucleotide reductase and can act as an efficient reductant for plasmoredoxin which is a thioredoxin-like protein unique to *Plasmodium*. Plasmoredoxin itself also reduces ribonucleotide reductase but its precise function remains unknown (Becker *et al.*, 2003a).

9.4. Thioredoxins

P. falciparum has been shown to contain a thioredoxin redox system—thioredoxin reductase (TrxR), thioredoxin and thioredoxin peroxidases (Nickel *et al.*, 2006). *P. falciparum* thioredoxin reductase is similar to GR in that it is homodimeric, NADPH dependent and binds one FAD per subunit. TrxR appears to be essential to parasite survival as knockout of the gene is lethal (Krnajski *et al.*, 2002). In a screen of 350,000 compounds, several unsaturated Mannich bases were identified as potential inhibitors of TrxR. The screen, as well as structure–activity relationship studies, resulted in the development of the first specific PfTrxR inhibitors (Andricopulo *et al.*, 2006; Davioud-Charvet *et al.*, 2003).

9.5. Peroxiredoxins

P. falciparum possesses four peroxiredoxins (Prx) (reviewed in Becker *et al.*, 2005; Deponte *et al.*, 2007; Kawazu *et al.*, 2007). Three of them, thioredoxin peroxidase 1 (Tpx1), 1-Cys-Prx and 2-Cys-Prx, are cytosolic whereas the fourth protein, thioredoxin peroxidase 2 (TPx2), has a mitochondrial targeting sequence that was experimentally shown to be functional and

expressed during the erythrocytic stages of *Plasmodium* development (Boucher *et al*., 2006). According to the microarray data, the protein is primarily transcribed throughout the erythrocytic cycle with higher levels in gametocytes and sporozoites (Le Roch *et al*., 2003). The 2-Cys cytosolic Prx reacts with plasmoredoxin and peroxynitrate (Nickel *et al*., 2005). Further, it has been reported to accept thioredoxin as a reducing substrate and to efficiently reduce hydrogen peroxide *in vitro* with kinetic parameters that suggest a crucial function as a hydroperoxide peroxidase *in vivo*. However, in spite of its potential importance, *P. falciparum* Prx null mutants were only slightly more susceptible to oxidative and nitrosative stress than wild types (Komaki-Yasuda *et al*., 2003). In *P. berghei*, it was also shown that their sexual development was affected (Yano *et al*., 2005). These results suggest that there might be a high degree of redundancy of hydroperoxide removing enzymes during the erythrocytic development of *Plasmodium* but that this situation changes in the gametocytes. The crystal structure of the 1-Cys-Prx with a putative apicoplast import sequence has been reported (Sarma *et al*., 2005).

9.6. Lipoic acid and isocitric dehydrogenase

The apicoplast (see Chapter 13, p. 105) contains the *de novo* biosynthesis pathway for lipoic acid which, apart from its role as essential co-factor of the PDH, is highly redox active and might, therefore, act as a non-enzymatic anti-oxidant in the organelle (Müller, 2004). In fact, thioredoxin is reduced *in vitro* by a dihydrolipoamide dehydrogenase/lipoamide redox system and it has been postulated that either free lipoic acid or protein-bound lipoate can act as reductant for thioredoxin or glutaredoxins present in the apicoplast. Lipoic acid, also present in the mitochondrion, is a co-factor of α-ketoglutarate dehydrogenase, branched chain α-keto acid dehydrogenase and the H-protein of the glycine-cleavage system. Again, it is feasible that the protein-bound co-factor can interact with redox active disulphide transferases to provide protection against oxidative damage of the organelle (Müller, 2004). Another interesting finding is that *Plasmodium* only possesses an NADP-dependent isocitrate dehydrogenase, which is up-regulated when *P. falciparum*-infected red cells are exposed to oxidative stress (Wrenger and Müller, 2003). This may serve to maintain the mitochondrial anti-oxidant capacity and redox environment.

It has been speculated that malaria parasites have adopted a largely fermentative lifestyle because of their inability to cope with the oxidative stresses imposed by a fully aerobic metabolism, a reliance on hemoglobin degradation, and a lack of catalase and GSH peroxidases. A driving force that may have caused *Plasmodium* to adopt this way of life may be

that the parasitization of erythrocytes leads to a reduction in levels of reduced GSH.

Lacking GSH-dependent peroxidases, *Plasmodium* spp. rely on a Prx-linked detoxification for hydroperoxides and reduced GSH acts primarily as the principal redox buffer. It is also important in detoxification reactions as a co-factor for GST and glyoxalase and has been shown to be involved in the breakdown of free ferriprotoporphyrin IX. The lack of two major anti-oxidants present in other cells (catalase and GSH peroxidase) suggests that malaria parasites would be vulnerable to disturbances in their anti-oxidant systems. As a consequence, pro-oxidant drugs such as the artemisinins, which increase the oxidative stress, are efficient antimalarials. It has been proposed that a novel approach to malaria chemotherapy would be to develop drugs that disrupt the anti-oxidant and redox system of *Plasmodium* (Müller, 2004; Nickel *et al*., 2006; Rahlfs and Becker, 2005).

Katja Becker (1965–) received her medical education at Heidelberg University (Heidelberg, Germany). In 1988–1989, she spent 8 months in Australia working as a DFG (Deutschen Forschungsgemeinschaft—German Research Foundation) scholar with Nicholas Hunt (Department of Pathology, Sydney University, Sydney, NSW, Australia), on cerebral malaria. During this time, she also did several weeks of clinical work with the Royal Flying Doctor Service (Katherine, NT, Australia) and in Internal Medicine at the Royal Prince Alfred Hospital (Sydney, NSW, Australia) as well as on the Friendly Islands of Tonga. She did her doctor of medicine (MD)/doctor of philosophy (PhD) (1991) in the group of Heiner Schirmer at the Institute of Biochemistry, Heidelberg University working on, '*Glutathione reductase and its apoenzyme*: *contributions to chemotherapy of malaria and FAD-deficiency syndromes*'. Following this, Katja did internships at the Kantonsspital Basel (Basel, Switzerland; in internal medicine), Heidelberg University (in cardiac surgery), and at the John Radcliffe Hospital (Oxford, United Kingdom; in pediatrics). In 1992–1993, she was medical assistant at the University Children's Hospital (Heidelberg, Germany). Clinically, she focused on tropical medicine, infectious diseases, oncology and neonatology. Her research work on redox-associated processes in severe malnutrition took her to Nigeria for 3 months. From 1993 to 1999, she was assistant professor at the Centre of Biochemistry, Heidelberg University. During this time she spent 6 months at the Institute of Pathology, Sydney University (Sydney, NSW, Australia). In 1996, she did her habilitation in biochemistry focussing on structural and functional characterization of *P. falciparum* proteins at Heidelberg University. In 1998, she did her medical specialization in biochemistry and became an associate professor at the Center of

Biochemistry, Heidelberg University. In 1999, she took over an independent junior group at the Research Center for Infectious Diseases, Würzburg University (Würzburg, Germany). After only 11 months in Würzburg, she was offered the chair of biochemistry at the Interdisciplinary Research Center, Giessen University (Gießen, Germany); a position she has held since 2000. In 2005–2006, she spent a sabbatical at the Department of Cell Biology, The Scripps Research Institute (La Jolla, California) with John Yates III.

For almost 20 years, Katja Becker has been teaching biochemistry, pathobiochemistry and molecular biology to medical and natural science students. She has produced 150 scientific publications and a number of patents, mainly in the field of malaria, redox metabolism and anticancer drug development.

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CHAPTER 25

Shocks and Clocks

One of the clinical hallmarks of malaria infections is periodic fever (Gravenor and Kwiatkowski, 1998; Hawking, 1975). Fever, which coincides with the time merozoites exit from the red cell, occurs periodically because there is the tendency for parasites to develop synchronously so that merozoite release takes place at around the same time (Kwiatkowski and Greenwood, 1989). Periodicity of fever in malaria, recognized 2,000 years ago by the Greek physician Hippocrates, has perplexed investigators as to how parasite development is synchronized. One hypothesis, formulated by Leslie A. Stauber during his doctor of philosophy (PhD) studies with William H. Taliaferro (University of Chicago, Chicago, Illinois), was that synchrony was related to a host daily rhythm (Stauber, 1938). In the canary, *Plasmodium cathemerium* showed synchronous periodicity of asexual reproduction that was affected by alternate 12 h periods of high and low temperature and by light (received through the eyes and not the skin) that caused the host to be active. According to Stauber's work, synchronicity of asexual reproduction was dependent on the host's body temperature and the young trophozoite seemed to be the most susceptible to experimental influences and acted to orient the synchronous rhythm.

Nye (1961), working with *P. gallinaceum*, found that the asexual period was 36 h and remained so even when birds were infected with sporozoites at three consecutive 12-h intervals. When birds were cooled to around 30 °C the growth of the parasite was delayed causing the period of development to exceed 36 h. No correlation was found between the temperature of the birds and any phase of parasite growth. This corroborated the earlier work of Hewitt (1942) with *P. lophurae* in ducklings. Synchrony in *P. gallinaceum* was unaffected by continual though varying illumination as opposed to 12 h light and 12 h dark. Nye suggested, 'some factor other than temperature may be responsible for regulating development.'

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When I began my graduate studies at Northwestern University (1957), the chair of the Biology Department was Frank A. Brown Jr. (1908–1983), who was a well-respected invertebrate endocrinologist who had studied the daily rhythm of pigment changes in the fiddler crab *Uca* with a superimposed tidal rhythm. This led Brown to investigate the control of the timing of rhythms using a variety of plants and animals kept under constant conditions of darkness and temperature. Brown held to the view that 'pervasive geophysical factors', not temperature, acted as the exogenous timer. An opposing view was that of Colin Pittendrigh (1919–1996) who, during World War II, had been posted by the British government to Trinidad to explore ways to protect the troops from malaria. There he studied the daily activity of malaria-transmitting *Anopheles* and noted the daily peaks of activity for different species at different levels in the forest canopy. He surmised that such activity was entrained by something other than variations in humidity (Pittendrigh, 1993). Pittendrigh's experience with malaria-transmitting mosquitoes did not seduce him into becoming a malariologist but instead led to a lifelong interest in biological clocks. After World War II, Pittendrigh emigrated to the United States. He studied with Theodosius Dobzhansky working on the differential daily activity cycles of *Drosophila pseudoobscura* and *D. persimilis* and received a PhD from Columbia University (New York, New York) (1951). From that time onwards, he developed a variety of clock theories using principally *Drosophila* and concluded the eclosion rhythm was 'inherent, synchronized by light, to persist in constant darkness and to not be greatly different at different but constant temperatures.' Later studies showed this clock could be slowed by slowing protein synthesis by application of cycloheximide. By the early 1960s, behavioral rhythms for a variety of organisms had been shown to be due to an endogenous oscillator that runs at a frequency slightly deviating from one per 24 h (i.e. circadian) with the deviation corrected by a phase shift. Brown, however, undeterred argued for an exogenous timer even after clock genes were discovered (see below).

Neither Stauber (whom I knew when I was a post-doctoral student with Trager at the Rockefeller University, New York), nor Pittendrigh nor Brown led to my studying synchrony and periodicity in malaria. Instead, it was 'connectivity' and summers spent at the Marine Biological Laboratory (MBL) in Woods Hole (Massachusetts). In 1961, I became an instructor in the summer invertebrate zoology course at the MBL. A fellow instructor was George G. Holz Jr. (1922–1989), a protozoologist who worked on various aspects of *Tetrahymena* biochemistry. Holz had spent a sabbatical year (1960–1961) with Erik Zeuthen at the Carlsberg Laboratory in Copenhagen, Denmark. Zeuthen who had previously shown that division in the free-living ciliate *Tetrahymena* could be synchronized by heat shocks (Scherbaum and Zeuthen, 1954, 1955) expected that fever in malaria could be a natural synchronizer of cell division and he recruited Milan

Kovic, a veterinarian from Yugoslavia, to study the effects of febrile temperatures (i.e. heat shocks) on malaria synchrony. In 1964, when Holz learned that Kovic had died suddenly, he suggested to Zeuthen that I might be a suitable candidate to follow up Kovic's work. Knowing that Kovic had had trouble in setting up the infections in chicken embryos I spent a few weeks with Barclay McGhee (Trager's former Rockefeller Institute associate) at the University of Georgia (Athens, Georgia) learning how to handle malaria parasites in chicken embryos before I joined the Carlsberg Laboratory (Copenhagen, Denmark). In January 1967, under a fellowship from the Guggenheim Foundation, I arrived at the Zeuthen laboratory where I proceeded to inject chicken embryos with *P. lophurae* and at 12-h intervals shifted the incubator temperature from 38 °C to 43 °C. Kovic and Zeuthen (1967) found a single heat shock to be subject to individual modification (i.e. there was variability in the host response) and although one shock slightly reduced or did not change the multiplication rate, a piling up of the young ring-stage parasites was observed. Two shocks produced somewhat better results with the percentage of rings increasing from 20% to 70%; they speculated that, had it been possible to apply more than two shocks, a more pronounced periodicity deserving of the designation 'synchrony', might have been achieved. Their interpretation was that elevated temperature inhibited growth of the parasites from rings to schizonts, yet, when I employed similarly timed heat shocks no such effect was found. However, I did find that heat shocks lengthened the period of asexual development by 8–10 h with the schizont stage being the most susceptible. Although the length of the asexual cycle was the same in the chick embryos as in the duckling, the infection was highly synchronous in the duckling (sent from New York by Trager) but when adapted to the chicken embryos it had become asynchronous. Zeuthen was disappointed in my findings, which led to a falling out between us, but I did assist him in the preparation of a manuscript describing Kovic's results. My negative findings were not mentioned in their paper (Kovic and Zeuthen, 1967) and neither Zeuthen nor I ever again investigated heat shocks and synchrony in malaria.

Several years after my experiments in Copenhagen were completed, and one year after the publication by Kovic and Zeuthen (1967), Frank Hawking (1905–1986) proposed that periodicity had become established in the course of evolution of the malarial parasite in order to coincide with the biting habits of the mosquitoes that transmit the disease, the so-called Hawking phenomenon (Garnham and Powers, 1974). He wrote, 'the synchronously accurately timed asexual cycle is to make the gametocytes match the mosquitoes' (Hawking, 1975). Hawking, Worms and Gammage (1968) carried out experiments with two primate malarias, *P. knowlesi* and *P. cynomolgi*, and found these plasmodia take their cue from the temperature cycle of the host. *P. cathemerium* in chick embryos that were subjected

to alternating 35 °C and 40 °C showed entrainment of the asexual cycle by temperature (Hawking and Gammage, 1970), although the cycle tended to be 27–31 h instead of 24 h as it was in the chick. However, when they investigated *P. lophurae* in ducklings and in embryos, it did not conform to the Hawking phenomenon and was found to be 'unsuitable for this kind of study since its natural cycle ... is 36–40 h which does not correlate well with any 24 h rhythm ... the synchronism is observed when it is transmitted by syringe every seven days [and] is an artifact produced by the investigator who subjects the parasites to a 'cold shock' at seven day intervals.' Moreover, *P. lophurae*, isolated 30 years earlier from a Borneo fireback pheasant in the New York Zoo (Coggeshall, 1938b), had lost its capacity to produce gametocytes in chicks and ducklings possibly from continuous syringe passage. Hawking, Gammage and Worms (1972) went on to study the circadian rhythm of *P. gallinaceum* which had a 36-h asexual cycle in chickens when infections were produced by intravenous inoculation (Lumsden and Bertram, 1940) with peak schizogony occurring alternately at midday and midnight. They concluded its schizogonic cycle was, as with *P. lophurae*, orientated more to the hour of inoculation than to the circadian rhythm of the host and was an artifact produced of syringe passage.

For over a decade, Hawking and co-workers studied the rhythmicity of gametocytes in a variety of malarias by measuring infectivity for mosquitoes, exflagellation rates and morphology of gametocytes (summarized by Gautret and Motard, 1999; Hawking, 1975). Measuring exflagellation rates in *P. cathemerium*, *P. coatneyi*, *P. knowlesi* and *P. cynomolgi*, Hawking (1975) concluded that with each of these species the timing of schizogony was adapted to the maturation of the gametocytes so that eventually the ripeness of the gametocyte corresponded to the usual time of biting by the mosquito vector. Hawking *et al.* (1972) showed a peak in exflagellation in *P. chabaudi* at the time of schizogony (0–4 a.m.) and later a 24-h periodicity for gametocyte production was shown for *P. vinckei vinckei* and *P. vinckei peteri* by Gautret *et al.* (1995). When *P. yoelii* was synchronized by Percoll concentration of young stages, it had a 24-h rhythm of gametocyte production (Gautret *et al.*, 1995, 1996). However, as Hawking noted, there were 'special cases' (i.e. *P. gallinaceum*, *P. berghei* and *P. falciparum*). If *P. berghei* was synchronized by repetitive passage in *Thamnomy*s, exflagellation had a peak dependent on the time of inoculation (Hawking *et al.*, 1972). Infections of *Anopheles gambiae* by *P. falciparum* were similar by night or day (Bray *et al.*, 1976; Githeko *et al.*, 1993), although no periodicity was seen with the asexual stages of *P. falciparum*, gametocytes did show a diurnal periodicity with a peak between 3:30 and 7:30 p.m. However, the peak did not coincide with the biting activity of *An. gambiae* (Magesa *et al.*, 2000). Perhaps this was due, as Hawking suggested, to asynchronicity of the infections and the long-lived

P. falciparum gametocyte. Gautret and Motard (1999) provided another possibility; the existence of a circadian preferential distribution (sequestration) of the infective gametocytes in the capillaries that prevented them from being available to the mosquito. *P. chabaudi* did not show circadian variation in infectivity for *Anopheles* because 'schizogony and production of gametocytes coincided' and timing of schizogony was set by the circadian rhythm of the host (Gautret *et al*., 1995). With *P. berghei*, because of its asynchronicity of development, mature gametocytes were produced independent of time (Gautret and Motard, 1999). The 'special nature' of *P. gallinaceum* was explained by Hawking and co-workers as it not being 'particularly adapted to night-biting mosquitoes and perhaps the relevant mosquitoes bite equally at dusk and dawn when an asexual cycle of 36 h would be adequate' (Hawking *et al*., 1972).

After 1976, and with the continuous *in vitro* culture of *P. falciparum*, it became possible to eliminate the use of chick embryos for heat shock experiments since temperature changes could be carried out *in vitro*. In 1989, Kwiatkowski found that initially asynchronous cultures became synchronized when exposed to 40 °C on alternate days of the 48-h growth cycle and suggested that this was due to 'periodic disruption of the latter part of the erythrocytic cycle' by elevated temperature. Grovenor and Kwiatkowski (1998) showed that a stepwise increase in temperature (6-h periods of 37.7, 38.9, 39.4 and 38.8 °C) gave partial synchrony and were able to develop a mathematical model to explain the observed periodicities of fever and parasitemia *in vivo*. They calculated that the free-cycle times at 37 °C for various isolates of *P. falciparum* ranged from 38 to 45 h. Because late developmental stages were inhibited by heat (40 °C) a selective depletion of the more mature stages occurred. This resulted in a preponderance of young stages—the progeny of the ruptured schizont—that were more temperature resistant. This observation was put to practical use: synchronization of asexual development has been automated by the use of a temperature cycling incubator (Haynes and Moch, 2003).

Between 1960 and 1970 there was accumulating evidence that the biological clock was endogenous. The physical nature of the oscillator, however, remained unknown. In 1971, through the application of mutagens, a gene that affected the biological rhythm of *Drosophila* was found (Konopka and Benzer, 1971). A second clock was detected in *Drosophila* 13 years later (Reddy *et al*., 1984) and in 1997 the first clock gene in a mammal (mouse *Mus musculus*, was identified (Antoch *et al*., 1997). Over the years, clock genes have been found in other multi-cellular organisms—plants and animals—as well as in microbes. Indeed, it has been shown that rhythms occur within a single body cell and that the genes work together to result in what has been referred to as a 'living clock'.

Biological rhythms persist with a species–specific period even under constant conditions and the phase can be reset (entrained) by an

environmental pulse. Basically, clock genes encode proteins that form active complexes that interact with their own genes in a feedback loop and this provides the basis for perpetual and cycling regulation of clock-controlled genes. In a simplified form, and as understood primarily from *Drosophila*, the genes *clk* (clock) and *cry* (cryptochrome) are transcribed in the nucleus and the proteins CLK and CRY are synthesized in the cytoplasm where they combine. The CLK–CRY combination moves to the nucleus where it interacts with the promoter to activate two other genes, *tim* (timeless) and *per* (period). Their messenger ribonucleic acids (mRNAs) move into the cytoplasm, are translated into the protein TIM and PER, that slowly accumulate and combine. When sufficiently abundant, PER–TIM feedback into the nucleus to inhibit *tim* and *per*. As PER–TIM slowly degrades the *clk* and *cry* genes are turned on and the cycle repeats. Delays in the loop include post-translational modifications in PER and CRY that determine how long it takes for the proteins to degrade. In the mouse, the protein FBXL3 is necessary for degradation of clock proteins and without it being functional CRY degradation slows, transcription of *clk* is repressed and the circadian cycle is lengthened.

In mammals the pineal gland acts as the 'hand of the clock' transducing neuronal input into the rhythmic synthesis and release of melatonin thereby tuning the body to the temporal environment. It has been tempting to believe that melatonin might also serve to modulate synchronicity of malaria parasites (Garcia *et al*., 2001). Melatonin secretion by the pineal gland triggers a cascade initiated by activation of phospholipase C, production of inositol 1,4,5-triphosphate ($InsP_3$) and then a release of calcium stores from the endoplasmic reticulum. One study claims that *P. chabaudi*, with its 24-h asexual cycle maturation, is controlled by melatonin by stimulating the transition from trophozoite to schizont (Hotta *et al*., 2000). Since the data were obtained from only a single asexual cycle and with no change in parasitemia there is need for data over several cycles of growth (i.e. at least four or five cycles) before it can be assured that such effects establish synchronicity. Inhibition of melatonin production by surgical ablation of the pineal gland appeared to result in desynchronization of *P. chabaudi*. Similar effects of melatonin were seen with *in vitro* cultures of *P. falciparum*. In a subsequent study, Budu *et al*. (2007) claimed that *N*-acetyl-*N*-formyl-5-methoxykymuramine, a product of melatonin degradation, also acted to synchronize *P. chabaudi* and *P. falciparum* and that the effect was abrogated by the melatonin antagonist, luzindole. The authors suggested melatonin causes a release of calcium from the parasite endoplasmic reticulum although the melatonin receptor itself was not identified.

In *P. falciparum* cultures, exposure to elevated temperature increased the adherence of ring-infected red cells to CD36 (but not ICAM-1) '. . . due to increased expression of PfEMP1 [*P. falciparum* erythrocyte surface

protein-1]' (Udomsangpetch *et al*., 2002), however, heating was not related to enhanced transcription. These investigators explained the apparent discrepancy, 'febrile temperatures increased trafficking of pre-formed PfEMP1', however, it is also likely that there was exposure of cryptic adhesive residues of band 3 protein (Winograd *et al*., 2005).

In a recent study of genome wide expression of *P. falciparum* cultured at 37 °C and 41 °C, heat shock did not increase expression of PfEMP1. However, there was increased expression of two heat shock proteins, HSP70 and HSP90, as well as members of the fatty acyl coenzyme A synthetase family and two phospholipases and 5 *var* transcripts, whereas five enzymes of the GPI biosynthesis pathway were down-regulated (Oakley *et al*., 2007). A notable transcriptional change was seen with proteins containing the PEXEL motif (see p. 208) suggesting that heat shock affected processes associated with re-modeling of the infected red cells. A consistent finding is that elevated temperatures had a deleterious effect on parasite survival, with ring-stage parasites being less susceptible (Kwiatkowski, 1989; Long *et al*., 2001; Sherman personal communication). The thermolabile target(s) remain(s) unknown.

It should be noted that no technique for cell-cycle synchronization has been achieved in any species of *Plasmodium*. Synchronous parasite development produced either by *in vitro* treatments or naturally (i.e. fever) results from selection of parasites of a limited age range, not by blocking the transition to a specific phase in the cell cycle (Naughton and Bell, 2007). Identifying how the asexual or gametocyte developmental rhythms are maintained, which environmental stimuli entrain periodicity, and whether plasmodial 'clock genes' exist, remain fertile ground for future investigations.

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CHAPTER 26

Transcriptomes, Proteomes and Data Mining

Contents

A decade ago, Su and Wellems (1998) wrote, '... progress has been made in the genetics and genomics of malaria parasites. Numerous genes have been cloned and millions of base pairs of the parasite deoxyribonucleic acid (DNA) have been deposited in public databases; genetic maps of the *Plasmodium falciparum* 14 chromosomes and hundreds of linkage markers have been reported; yet despite these advances, we still know very little about the function of most genes!'

Although genomics, the study of an entire genome, had its earliest successes in the late 1970s its pace accelerated in the 1990s. In 1977, Sanger sequenced the genome of the bacteriophage MS2, and by 1995 the genome of a free-living bacterium *Hemophilus influenzae* was sequenced by scientists at The Institute for Genome Research (TIGR). Since that time, complete genomes of many more eukaryotic organisms—including the yeast *Saccharomyces*, the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila*, the malaria mosquito vector *Anopheles gambiae* as well as malaria parasites (*P. falciparum*, *P. yoelii*), and ourselves, *Homo sapiens*—have been sequenced. Knowledge of these genomes and measures of their expression, termed functional genomics, have enabled malariologists to monitor gene activity of the parasite and its hosts. The goal of functional genomics is not simply to provide a catalogue of all the genes and information about their functions but to understand how the components work together during interactions

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of *Plasmodium* spp. with the host as well as when the parasite is under drug pressure. Knowing which genes are associated with particular biochemical pathways and the various stages of the life cycle could provide a wealth of drug targets as well as a biochemical basis for pathogenicity and the *raison d'être* for obligate parasitism.

1. TRANSCRIPTOMES

In transcriptomics, a branch of genomics, the pattern of gene expression is determined by measuring levels of transcription (i.e. messenger ribonucleic acid (mRNA) abundance). The earliest descriptions of *Plasmodium* spp. gene expression date back to the 1970s. Initially, the methods used involved translation of mRNA using cell-free protein synthesizing systems. In 1971, Cook and co-workers at the Walter Reed Army Institute of Research (WRAIR) isolated ribosomes from *P. knowlesi* and used these to develop a cell-free protein synthesizing system (Cook *et al*., 1971). Because of the relative insensitivity of their system (presumably due to ribonuclease activity) protein synthesis was only partially inhibited by puromycin and cycloheximide. Later, working at the National Institute for Medical Research (NIMR, London, United Kingdom) with the 'ribosomologist' R. A. Cox and then at University of California at Riverside (UCR, Riverside, California), I was able to show using both *P. knowlesi* and *P. lophurae* ribosomes and a pH 5 fraction from reticulocytes that cell-free protein synthesis was blocked by cycloheximide and puromycin, potent inhibitors of eukaryotic systems, but streptomycin and chloramphenicol, inhibitors of prokaryotic protein synthesis were without effect (Sherman and Jones, 1976, 1977).

At NIMR I was encouraged by James (Jim) Williamson (1918–1993) to test whether the cell-free protein synthesizing system might provide clues as to the mechanism of action of known anti-malarials. Williamson, who was my mentor during my sabbatical leave at the NIMR, began his long career in chemotherapy with E. M. Lourie at the Liverpool School of Tropical Medicine (LSTM, Liverpool, United Kingdom) (1945) from which he received his doctor of philosophy (PhD, 1949). After a sojourn at the West African Institute for Trypanosomiasis Research in Nigeria (1954–1960) he joined NIMR in 1960 where he remained until his retirement (1983). As his colleague K. Neil Brown wrote, 'Jim had those essential prerequisites for truly original and productive research namely a wide and critical knowledge of the literature, a scant regard for most current received opinion, a disregard for artificial boundaries to scientific thought and action and an irreverent sense of humor. He was convinced of the central role of chemotherapy in the control of parasitic diseases. Jim's curiosity about people and their ways was endless . . . his conversation

was laced with good natured and humorous observation.' Frequently, Jim and I spent lunch together at a local pub where Jim's enthusiasm for travel, art, theatre and science was revealed. To know him was to be enriched not only scientifically but also in a myriad of other ways.

Unfortunately, the *P. knowlesi* cell-free protein synthesizing system was unable to predict chemotherapeutic effectiveness of several antimalarials and the protein (or proteins) being synthesized were not identified. Following this work Eggitt *et al.* (1979) using *P. knowlesi* developed a cell-free protein synthesizing system in which the products made *in vitro* were compared with that made *in vivo* by precipitation using an antiserum. Many parasite-synthesized polypeptides were found and there was a major one of 50–55 kDa. Later, Wallach and Kilejian (1982) employed a similar method for the synthesis of the methionine-containing proteins of *P. lophurae* and found that transfer ribonucleic acid (tRNA) was critical for *in vitro* translation of mRNA.

By 1983, ribosomal genes had been isolated (Langsley *et al.*, 1983) and there were partial (Ellis *et al.*, 1983) and complete sequences of the circumsporozoite surface protein (*CSP*) gene (Ozaki *et al.*, 1983) as well as a *P. falciparum* surface antigen gene (Kemp *et al.*, 1983). Kilejian, believing that the histidine-rich protein (HRP) of *P. lophurae* could serve as a protective vaccine (Kilejian, 1978) used its published sequence (Ravetch *et al.*, 1984) to identify the HRP sequences by hybridization with a synthetic oligonucleotide probe and the *HRP* gene was cloned and expressed in a cell-free system (Kilejian *et al.*, 1985; reviewed by Sharma, 1988). The lophurae HRP, however, turned out not to be a protective vaccine. See Sherman (1981) for a full discussion of the controversy.

Although these methods (i.e. gene cloning and expression) have been (and continue to be) useful in identifying and characterizing genes that express a particular protein, to do this with an entire *Plasmodium* genome would be impossible. To examine the functional role of individual genes and their relationship to other genes, high-density deoxyribonucleic acid (DNA) microarrays of open reading frames (ORFs) were developed in the Stanford University (Palo Alto, California) laboratories of Ronald W. Davis and Patrick O. Brown using the yeast *Saccharomyces cerevisiae* (Lashkari *et al.*, 1997). In the generation of DNA microarrays, called 'chips', stretches of oligonucleotides (= genomic deoxyribonucleic acid, DNA) are either directly synthesized and spotted on a glass slide or, using photolithographic chemistry, are synthesized *in situ* on a glass slide. After isolation of mRNA, it is labeled (usually with a fluorescent tag) and then allowed to hybridize to the array. Using a fluorescence scanner it is possible to quantitatively estimate the degree of fluorescence for each spot and to calculate transcript abundance. Using this approach a functional analysis of the more than 6,000 ORFs of the yeast genome was carried out (DeRisi *et al.*, 1997; Winzeler and Davis, 1997).

The use of chip technology (microarrays) for the study of the *Plasmodium* transcriptome began in the late 1990s in the laboratories of Brown and Davis and depended greatly on their very talented graduate students and post-doctoral students, particularly Joseph De Risi and Elizabeth Winzeler.

Elizabeth Winzeler (1962–) grew up in Reno (Nevada), attended Lewis and Clark College in Portland (Oregon) and was an art major (although a premed curriculum was taken just to show that she was not doing art because she was academically challenged). Upon completing a bachelor of arts (BA) degree (1984) she was still a bit confused about what about what she wanted to do after graduation so she went to work as a computer programer for the United States Bureau of Labor Statistics in Washington, DC for several years and took art classes in the evening with the idea of applying to a masters of fine arts program. Ultimately, Elizabeth did apply to several art schools, however, all rejected her. It was then, at 25 years of age, that she decided to seriously try science. First, she applied to a masters program at Oregon State University (Corvallis, Oregon) and obtained a master of science (MS) degree in biophysics (1990), and then transferred to the Developmental Biology Department at Stanford University where she worked on *Caulobacter* under the guidance of Lucy Shapiro receiving her PhD in 1996. When the time came to do a post-doctorate, Elizabeth told Lucy that she was interested in parasitology; she in turn told Elizabeth that parasitology was fine, but she really thought the laboratory of Ronald Davis (who was at the time in the Biochemistry Department at Stanford) would best suit her interests and temperament. Shapiro suggested that Elizabeth would like working for Ron because of his personality and because he too was a creative soul. This turned out to be excellent advice.

In 1996, Winzeler joined the Davis laboratory, the year the complete sequencing the genome of the *Saccharomyces cerevisiae* was achieved. Elizabeth told Davis that she was interested in his work on *Arabidopsis* but he said that since the yeast genome had just been sequenced there might be some opportunities using this organism. Being an opportunist, Elizabeth decided to work with yeast. Having a background in both computer science and molecular biology turned out to be fortuitous. During her time at Stanford she led a project to create knockout strains for the entire yeast genome, helped with some of the first yeast microarray gene expression experiments and developed methods for studying allelic variation and DNA replication on a global scale. This was a very exciting time in the Stanford Biochemistry department—a time that many thought was comparable to the advent of recombinant

DNA technology. Because the genome of yeast had just been obtained and other genomes' sequences were nearing completion everyone in the Davis laboratory was excited about what the future offered and the potential of what one could do with tools like microarrays, robots, sequencers and bioinformatics.

Although Elizabeth liked working with the members of the yeast community, she wanted to work on an organism with more medical relevance and with more potential for discovery. As a result of her father and mother being anthropologists, Elizabeth had spent 18 months in the Northern Malayan state of Kelantan when she was very young and when her father was working on his dissertation at the University of Chicago (Chicago, Illinois). *P. vivax* was endemic in the region in the late 1960s and Elizabeth had suffered with malaria while there. Ron, aware of Elizabeth's interest in tropical disease, invited her to attend a small World Health Organization (WHO, Geneva, Switzerland) advisory meeting on parasite genomics, where she presented some of her yeast work and illustrated how it could be applied to malaria research. At this small meeting, she met Louis Miller, Thomas Wellems, Daniel Carucci, Stephen Hoffman, David Roos and Chris Newbold, who all encouraged her to try malaria research.

When it came time to find a real job, Winzeler considered microbiology departments and was asked about future directions. She said that she really wanted to work on malaria (even though she had no experience in this). She thought that although malaria parasites had been relatively difficult to work on, the availability of the genome sequence would change this and that soon one would be able to use systematic genome-dependent approaches to predict transcriptional regulatory networks, study evolution, characterize protein complexes and understand drug resistance. This was considered 'overambitious' and she was never invited back for a second interview. (Of course, this was before 'systems biology' had been invented.) Finally, in 1999, she moved to San Diego to join the fledging Genomics Institute of the Novartis Foundation that was being established by Pete Schultz, a well-known chemist from University of California, Berkeley (Berkeley California) who liked Elizabeth's malaria program idea and who also had the foresight to embrace genomic, proteomic and robotic approaches. One year later, she joined the Scripps Research Institute (La Jolla, California) where she is currently an associate professor. To help develop the malaria program she recruited Karine Le Roch, a very talented post-doctoral student to her laboratory and together they published the first comprehensive microarray studies of the *P. falciparum* genome. At present, Winzeler's research encompasses genomics, transcriptional studies, bioinformatics, parasitology, chemical biology and host pathogen interactions, mouse genetics and drug discovery.

Joseph De Risi (1969–), after receiving his bachelor of science (BS) degree from UC Santa Cruz (Santa Cruz, California) (1992) in biochemistry and molecular biology, joined the Brown laboratory intending to study how retroviruses integrate into the host genome. At the time, the Brown laboratory was working on techniques for identifying stretches of DNA passed from parent to offspring using biochemical procedures that separated and purified sequences identical to each parent and then hybridized these to a selected pool of DNA in an array of clones on a filter to produce a map. In 1995, an engineering student, Dari Shalon, developed a simple, low-cost way for printing thousands of long gene fragments onto a glass slide using a robotic microarraying device. Mark Schena, a post-doctoral fellow in the laboratory of Davis and Shalon from the Brown laboratory, used polymerase chain reaction (PCR)-amplified cDNAs from an ordered *Arabidopsis thaliana* cDNA library placed onto the glass slide to study gene expression: mRNA, obtained from the *Arabidopsis* was labeled with a fluorescent dye and hybridized to the array, then the fluorescent signal was measured. Although this microarray technology was efficient for studying gene expression in a large number of clones, its full potential was reached when the complete genomic sequence of the yeast *Saccharomyces* was released (1996) largely due to collaborative efforts by several laboratories under the aegis of Davis. Therefore, not surprisingly, Davis chose to devote a considerable amount of attention and effort by himself and his graduate and post-doctoral students to undertake a functional analysis of the *Saccharomyces* genome. De Risi and a fellow graduate student, Vishy Iyer, developed methods to miniaturize the microarrays and were able to print the first DNA microarrays for the yeast genome. The first result using mismatch scanning was a success and enabled whole gene expression studies for yeast by looking at the pattern of mRNA expression. In the end, DeRisi's thesis became a whole genome analysis of gene expression rather than retrovirology. After receiving the PhD (1997), De Risi moved to University of California, San Francisco (California) (1998) and there began a 'socially driven' project on the biology of *P. falciparum* together with post-doctoral researcher Zbynek Bozdech.

Zbynek Bozdech (1967–), currently an assistant professor at the School of Biological Sciences at Nanyang Technological University (NTU) in Singapore, continues to carry out research on the molecular aspects of the malaria parasite life cycle. Zbynek grew up in Czechoslovakia. In 1990, he received his masters degree in biochemistry from

Charles University in Prague (Prague, Czech Republic). From here he moved to McGill University in Montréal, Canada to initiate his PhD studies. Zbynek completed his doctorate in 1998 on the '*Molecular characterization of ABC [ATP-binding cassette] proteins in* Plasmodium falciparum' and cloned a new ATP-binding cassette protein, however, since it did not seem to have anything to do with resistance, but had a very interesting pattern of intracellular localization, he began to investigate protein trafficking. In 1999, he moved to University of California at San Francisco to work on the proteasome complex in *Trypanosoma brucei* in the laboratory of C. C. Wang as a post-doctoral fellow. However, after 1 year (2000), he settled in the laboratory of DeRisi to explore the transcriptome of *P. falciparum* using the fast-emerging microarray technology. During this work, Zbynek participated in the research that led to assembly of one of the first *P. falciparum* microarrays and to explore the global transcription pattern of the *P. falciparum* intraerythrocytic developmental cycle. In 2004, he moved to the newly formed School of Biological Sciences, Nanyang Technological University (NTU), Singapore, to start his own research group. Presently, Zbynek is exploring several molecular aspects of translation and post-translational regulation associated with the progression of the *P. falciparum* life cycle. In addition, using several technologies of high-throughput genomics and proteomics his group is exploring molecular mechanisms associated with virulence and drug resistance in field strains of *P. falciparum* and *P. vivax*.

A comparison of the transcriptomes derived from patients with the *in vitro* grown 3D7 line detected between 1,872 and 2,988 transcripts in the *in vivo* samples (Daily *et al.*, 2005). There was over-expression of similar genes (28 to 583 for each sample), including those encoding membrane proteins, housekeeping proteins and hypothetical genes. The number of *rifin* and *stevor* genes (see p. 199) was greater in the *in vivo* samples than in the 3D7 line grown *in vitro* DNA and a novel family of surface proteins was discovered. A comparative transcriptional analysis of isogenic clones with distinct antigenic and adhesive phenotypes revealed 262 differentially transcribed genes: 100 in rings, 113 in trophozoites and 49 in schizonts. Genes highly up-regulated in one clone (3D7AH12) included members of the *var* gene family, two glycophorin-binding protein homologues and two new genes. Of some interest was the finding that the dominant numbers of differentially expressed genes were hypothetical genes (Mok *et al.*, 2007). In another study comparing three strains (3D7, HB3 and Dd2) from different geographic origins and with differing drug sensitivity there was strikingly similar gene expression, with differences in expression occurring mainly

in surface antigens. However, there were several transcripts unique to individual strains. Large chromosomal deletions were detected and there were highly polymorphic genes. No changes in gene expression for P-glycoprotein or the chloroquine transporter were detected and this may reflect the fact that *in vitro* grown parasites are not subject to environmental pressure (Llinas *et al*., 2006).

Applying a data-mining algorithm termed 'ontology-based pattern identification' (Zhou *et al*., 2005) for analysis of the transcriptome of sexual development Young *et al*. (2005) found a cluster of 246 genes of which 75% were hypothetical with a high correlation for gametocyte-specific expression patterns. The data support the hypothesis (Lang-Unnasch and Murphy, 1998) that in the transition to the insect vector there is a switch to aerobic metabolism with a down regulation of glycolytic enzymes in gametocytes and transcripts for 15 of 16 mitochondrial tricarboxylic acid (TCA) cycle enzymes are present in gametocytes with pyruvate dehydrogenase (PDH) and MDH transcripts showing gametocyte-specific expression patterns. Since there was also up-regulation of three of six genes for the heme biosynthetic pathway (δ-aminolevulinate synthetase, porphobilogen deaminase and porphyrinogen decarboxylase) it suggests that *de novo* heme biosynthesis may be important in gametocytogenesis. Five putative gametocyte-specific proteases were found, which could be effective drug targets; in addition, the six kinases found could be promising leads for elucidating sexual development signaling pathways.

Microarrays, initially designed to measure gene expression, have been used to determine genetic diversity within *Plasmodium* spp. (Kidgell and Winzeler, 2005) as well as altered transcription by endothelial cells induced by the presence of *P. falciparum*-infected red cells (Chakravorty *et al*., 2007). In this latter study, it was shown that while parasitized red cells are able to mobilize pro-inflammatory and pro-adhesive effects on the endothelium they are also able to suppress gene expression in pathways involved in signal transduction, apoptosis and immune responses. An analysis of parasite adherence showed that differences in receptor use were associated with limited transcriptional differences for both *var* and non-*var* genes (Ralph *et al*., 2005a). No changes in transcription factors were found suggesting that *var* transcription is regulated by histone and chromatin condensation. Silencing of *var* genes was not dependent on antisense RNA but rather mutually exclusive expression of *P. falciparum* erythrocyte surface protein-1 (PfEMP1) proteins resulted from transcriptional silencing of non-expressed genes.

In the *P. berghei* ookinete there was differential gene expression with four genes being involved in ookinete-secreted proteins and two others hypothesized to be involved in signal transduction. Two of the expressed genes—a predicted thioredoxin-like protein and another a small heat shock protein—might contribute to protection of ookinetes during their

passage through the stomach wall. Another with a catalytic domain characteristic of the serine/threonine phosphatase family and a calcium-dependent protein kinase may be involved in parasite invasion of the mid-gut. The *in vivo* functions of these gene products remain to be determined (Raibaud *et al*., 2006).

The interactions between the malaria parasite and its hosts are complex. A SAGE analysis of the salivary gland transcriptome with *P. berghei* showed 57 *An. gambiae* genes differentially expressed (Rosinski-Chupin *et al*., 2007). Of the 37 genes categorized as immune related, four antimicrobial peptides (defensin, Gram-negative binding protein (GNBP), serpin6 and cercropin2) were up-regulated during salivary gland invasion. The cercoropin2 and defensin were expressed throughout the gland whereas GNBP and serpin6 were most abundant in the proximal part of the lateral lobe, which is not concerned by invasion, suggesting a more general response. Seven nuclear genes for mitochondrial proteins had altered expression—four were up-regulated and three down-regulated. A modification of mitochondrial activity is compatible with morphological studies showing an association of sporozoites with mitochondria during parasite passage through the salivary gland cells. Four additional genes involved in sugar metabolism and two others involved in lipid metabolism were differentially expressed, and this may be linked to cell repair after parasite invasion or to the production of metabolites required by the sporozoite. Genes encoding a heat shock protein and a glutathione (GSH) transferase were also expressed. There was little influence on the composition of the saliva and this is in contrast to *P. gallinaceum* infection in *Aedes aegypti* (Rossignol *et al*., 1984). Xu *et al*. (2005) carried out a simultaneous transcription analysis of 4,987 *Anopheles stephensi* mid-guts and *P. berghei* transcripts after different blood-feeding intervals and grouped these into clusters. In the *P. berghei* Pb1 cluster, about 20–40 h after feeding transcripts for metabolic enzymes, replication factors, transcription factors, proteasome and heat shock proteins as well as surface antigens were expressed suggesting stress response to the transition from blood to mosquito. In the Pb2 cluster, three putative heat shock proteins were expressed up to 40 h (when ookinetes invade the epithelium) and also at 14 days (when sporozoites are dispersed in the hemolymph) suggesting a role in motility and invasion. The CSP and thrombospondin-related adhesive protein (TRAP) proteins were transcribed throughout parasite development with a peak at 20 h. The Pb3 cluster contains components of the antioxidant defense system (i.e. thioredoxin and GSH peroxidise), and the Pb4 cluster contained several kinases possibly involved in a signaling cascade. The *Anopheles* As1 cluster was specific for early stages and there were four serine proteases, four aminopeptidases and two maltase transcripts, all presumably involved in early blood meal digestion. The As2 cluster had a hydroxykynurenine transaminase that detoxified 3-hydroxykynurenine to xanthurenic acid (XA)—essential to trigger gametocyte activation. The As3

cluster had expression of serine and nitric oxide (NO) synthase transcripts possibly linked to parasite-induced apoptosis of epithelial cells and immune responses. The As4 cluster was enriched in transcripts implicated in enhanced biosynthetic activity as well as blood digestion and wound healing, whereas the As5 cluster had enriched transcripts for protease transcripts immediately after feeding and then down-regulated prior to 20 h; they were up-regulated again between 4 and 8 days enabling the mosquito to digest the blood meal. In addition, there were peritrophins and chitinases as well as ion pump and transporter transcripts and an aquaporin possibly linked to nutrition uptake and restoration of osmotic balance.

Microarray analysis led to the identification of approximately 200 genes that are transcriptionally active in the salivary gland sporozoites, including around 500 genes that are highly expressed (Le Roch *et al*., 2003). Microarray studies confirmed the expression of rifin, stevor, and var, previously identified in a proteomic analysis of sporozoites (Florens *et al*., 2002). The functional significance of their expression in sporozoites is unclear. Differential gene expression analysis using suppression subtractive hybridization revealed differences in the transcriptome of sporozoites released from the oocyst and those in the salivary gland (Matuschewski *et al*., 2002a). Using this methodology genes encoding a novel thrombospondin-related sporozoite perforin (TRSP), possibly in the rhoptries, and a perforin-like protein localized to the micronemes were identified (Kaiser *et al*., 2004).

In a transcriptomic analysis of the liver stages of *P. yoelii*, it was found that there was abundant expression of CSP and heat shock protein (hsp70) whereas there was little expression of the sporozoite marker TRAP (Wang *et al*., 2004c). In contrast, there was strong expression of blood-stage transcripts such as RESA, MSP-1, MSP-7 and homologues to the *P. falciparum* Pf332 and a sexual-stage-specific kinase. Based on these findings it was concluded that these *in vitro* grown exo-erythrocytic (EE) forms represent 'transition stages between sporozoites and blood stages'.

Karine Le Roch (1971–), presently an assistant professor at UCR, worked on developing functional genomic tools to understand the life cycle progression of the malaria parasite. Karine received her BA in biochemistry (1995) from the University of Paris VI, Paris, France. This was followed by an internship (1995) in a European exchange program (Erasmus) in North Wales University (Bangor, United Kingdom) with M. Doenhoff, where she worked on the development of a vaccine against the blood fluke, *Schistosoma mansoni*. Karine obtained a masters degree in parasitology at the University of Lille II (Lille, France) and Oxford University (Oxford, United Kingdom) (1997) working at the Institute of Molecular Medicine at the John Radcliff Hospital (Oxford, United Kingdom) with A. E. Wakefield on the genetic diversity of *Pneumocystis*

carinii. She completed her PhD (2001) at the University of Paris VI under the supervision of Christian Doerig. Her PhD research concerned the cell cycle regulation of *P. falciparum*, and more specifically on cyclin-dependent kinases. In 2001, as a post-doctoral fellow, she joined the Scripps Research Institute and worked with Elizabeth Winzeler to set up the functional analysis of the *P. falciparum* genome using microarray technology, and in 2004, Karine joined the Genomics Institute of the Novartis Research Foundation where she developed the malaria drug discovery program. Beginning in 2006, Karine has focussed on the ubiquitin/proteasome pathway throughout the life cycle of *P. falciparum*. Using functional genomics approaches such as proteomics, she expects to elucidate critical regulatory networks driving the life cycle progression of the malaria parasite.

In November of 2002, the complete genome of the 3D7 strain of *P. falciparum* was released (Gardner *et al.*, 2002) and subsequently that of *P. yoelii* (Carlton *et al.*, 2002). Partial sequences for *P. berghei*, *P. chabaudi* and *P. vivax* are available and genomic sequencing of other species (i.e. *P. gallinaceum*, *P. knowlesi* and *P. reichenowi*) is in progress (Carlton *et al.*, 2005). In 2002, it was estimated that of the 5,268 proteins encoded by *P. falciparum* genome, 3,208 did not have a significant sequence homology to that of other organisms (Gardner *et al.*, 2002). Three years later, it was estimated that the number of these hypothetical genes numbered 3,548 or 65.6% of the total. This figure is the worst ever recorded for a eukaryotic genome (Birkholtz *et al.*, 2006). In spite of this, monitoring the transcription of the thousands of genes of *Plasmodium* has been possible by the development of microarrays (as well as other techniques such as serial analysis of gene expression (SAGE)) and the use of clustering methods and 'guilt by association' inferences has made it possible to suggest functions for some of these hypothetical genes (Le Roch and Winzeler, 2005).

Attempts have been made to use transcriptomics to define those genes expressed in susceptible versus resistant hosts. The response of myeloid dendritic cells from the spleen of mice infected with *P. yoelii* at two different time points post-infection revealed 694 differentially regulated transcripts with 327 being induced and 367 repressed upon infection (Carapau *et al.*, 2007). The response of the dendritic cell was not only different from non-parasitic pathogens but also from other intracellular parasites, such as *Leishmania* and *Toxoplasma*. *P. yoelii* infection induced expression of cytokine genes such as interleukin (IL)-6, IL-10 and interferon (IFN)-γ as well as three immune-related genes, *fos*, *msr* and *c4b*. Co-stimulatory molecules such as CD80, CD83 and CD86 were down-regulated. The genes for tumor necrosis factor

(TNF)-α and ICAM-1 were not regulated. Several genes involved in prostaglandin synthesis and signaling (i.e. *cox2/ptgs2* (cyclo-oxygenase 2) and *ep4* (a prostaglandin (PG)E2 receptor) and prostacyclin synthase) suggest that *P. yoelii* activates a signaling pathway in the dendritic cell. In support of PGE2 contributing to an increase in the second messenger, incubation of bone marrow-derived dendritic cells with *P. yoelii*-infected red cells induced increases in intracellular cyclic adenosine monophosphate (cAMP) and PGE2 also increased IL-6 gene expression. These investigators speculated that '*Plasmodium*-derived GPI and hemozoin contribute to IL-6 secretion . . . as they induce activation of toll-like receptor (TLR)-2 (or -4) and -9, respectively, which triggers IL-6 production' (as was shown by Krishnegowda *et al.*, 2005 and Coban *et al.*, 2005).

Using a single 'combination microarray' with the *P. berghei* ANKA experimental model for cerebral malaria in susceptible C57BL/6 and resistant (BALB/c) mice it was found that the parasites display a unique transcriptional signature in each tissue and the lung serves as the reservoir for metabolically active parasites (Lovegrove *et al.*, 2006). (This situation is in contrast to that found in human cerebral malaria.) In this analysis many IFN-inducible genes were expressed lending support to the importance of timing and magnitude of interferon responses in mediating the outcome. Indeed the authors claimed, 'Differences in early innate immune responses by IFN-regulated genes could contribute to innate resistance in BALB/c mice and may be prognostic of outcome.' In a subsequent study, most of the differentially expressed genes identified in this mouse model were associated with immune-related gene ontology categories (Lovegrove *et al.*, 2007). Examination of patterns of transcriptional regulation within the set of identified genes implicated a central role for both IFN-regulated processes and apoptosis in the pathogenesis of cerebral malaria.

Although functional genomics provides ample opportunities for speculation as to what specific genes do, it is not always clear which of the activated genes are crucial for protective immunity or those that have little to do with the immune response or are involved in tissue injury. Valuable as gene expression data can be in narrowing down the possibilities on their own, they cannot elucidate which genes are crucial for host defense and which promote invasion, survival and pathogenesis. To accomplish this, low-throughput, hypothesis-driven experimental approaches will be needed to learn which genes are important and which are not.

2. PROTEOMES

Global proteomics describes the analysis of the combined set of all protein products encoded by a genome. It can determine not only expression but also sub-cellular localization and post-translational modifications of

proteins (reviewed by Johnson and Yates, 2005; Johnson *et al*., 2004; Sims and Hyde, 2006). Global proteomic profiles that delineate proteins according to the *Plasmodium* life cycle have been obtained and could provide grist for the drug designer's mill. Two general strategies have been useful for profiling protein expression: (1) two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of a cell lysate, followed by excision of the spots after staining, digestion and then direct peptide sequencing identification of peptides using MALDI-TOF and (2) multi-dimensional protein identification technology (MudPIT), which involves *en masse* protein digestion followed by two-dimensional high-performance liquid chromatography coupled directly to a tandem mass spectrometer.

Florens *et al*. (2002) using MudPIT, confidently identified 2,415 parasite proteins in four stages (sporozoite, gametocyte, trophozoite and merozoite) in the 3D7 line of *P. falciparum* and sorted them into 10 functional categories. Unexpectedly, variant proteins (e.g. PfEMP1 and rifins) characterized as surface antigens of the infected red cell were also expressed at a high degree in sporozoites. It was suggested that this might be part of a survival strategy of the non-replicating sporozoite as it traverses the blood stream to the liver. Lasonder *et al*. (2002) carried out a similar study with another line (NF54) and divided the sexual stages into gametocytes and gametes and the asexual blood stages were considered as a single group. They identified 1,289 proteins, 714 of which were present in asexual blood stages, 931 in gametocytes and 645 in gametes. Five hundred and seventy-five proteins were unique to the sexual stages and included a subset of proteins with domains suggestive of cell–cell interactions.

A similar MudPIT proteomic analysis was taken with *P. berghei* where it was possible to have a complete life cycle and here the data were divided into housekeeping genes and those that were altered in response to the shift from mosquito to mouse, and whether they were involved in invasion, asexual multiplication or sexual development (Hall *et al*., 2005). Using this approach, 1,836 of the over 5,000 gene products encoded in the *P. berghei* genome were identified with high confidence and their expression profile throughout the life cycle determined. Of the 733 gametocyte proteins, 436 were also found in gametocytes, ookinetes and asexual blood stages suggesting these to be 'housekeeping' proteins. Seventy-two were found in both asexual stages and gametocytes and could reflect life within the erythrocyte. One hundred and twenty-seven were unique to gametocytes and therefore function in gametocytogenesis or gametogenesis. Seventy-six were shared with the ookinete and these could represent proteins involved in sexual development that persist into this next stage of development. Since just half of the proteins were detected solely in one stage of the life cycle this indicates the high degree of specialization at the molecular level necessary for accomplishing the metabolic and morphologic transitions. Indeed, when the male and female gametocytes of *P. berghei*

were separated, 305 proteins were found to be associated with the male, 170 to the female, and 69 proteins were common to both (Khan *et al*., 2005).

What is the relationship between the transcriptome and proteome? Analysis of the mRNA transcripts using microarray data and protein abundance levels using MudPIT technology for seven different stages in the life cycle showed discrepancies between mRNA and protein abundance for many genes (i.e. discrepancies between mRNA and protein abundance are due to a time shift between the detection of the transcript and its cognate protein) (Le Roch *et al*., 2004). On average, transcripts were 2.5 times more abundant when proteins were detected in asexual parasites. This was not the case for genes detected in gametocytes suggesting that in these stages the discrepancies were due to post-translational regulation phenomena. Using a statistical method for comparing unrelated data (i.e. Spearman rank correlation) a comparison was made between transcriptome and proteome data sets. This comparison revealed that in many cases the transcriptome of a particular stage correlated better with the proteome of the following stage rather than with the proteome of the same stage suggesting a delay between maximal accumulation of an mRNA transcript and its cognate protein. For example, genes implicated in cell cycle regulation and cell division during EE schizogony were often detected at the transcriptional level in sporozoites. Correlating mRNA and protein expression profiles for individual genes revealed that families of functionally related genes had similar patterns of mRNA and protein accumulation. Indeed, almost all genes involved in glycolysis had low correlations between mRNA and protein expression profiles for asexual stages but, when a time shift was applied by shifting the mRNA stage forwards, the correlation coefficient improved significantly for all but one gene, encoding aldolase—an enzyme known to have a function in motility and invasion (see pp. 9, 315) as well as in glycolysis (Johnson and Yates, 2005).

Jane Carlton (1967–) is currently associate professor in the Department of Medical Parasitology at New York University (NYU, New York, New York). As a child growing up in Edinburgh, United Kingdom, and the surrounding Scottish countryside, she became fascinated with the diversity of the natural world, which later led to a keen interest in genetics and how the inheritance of genes shape the phenotypes of organisms. Graduating with a bachelor of science (BSc) (Hons) in genetics from Edinburgh University (1990), she joined David Walliker's malaria genetics group for her PhD studies. Walliker, a member of Geoffrey Beale's Institute of Animal Genetics at Edinburgh University (Edinburgh, United Kingdom), was one of the first researchers to develop genetic studies of malaria parasites, focusing on the inheritance of loci in rodent malaria crosses. After receiving her

PhD in 1995, she spent a further 2 years as a post-doctoral researcher in Edinburgh analyzing genetic crosses in *P. chabaudi* that differed in their resistance to chloroquine and mefloquine, before relocating to the University of Florida (Gainesville, Florida) to begin studies with John Dame. Dame and colleagues were among the first to realize the potential of expressed sequence tag (EST; short approximately 500 nucleotide 'tags' generated from individual clones of a cDNA library) sequencing projects for gene discovery in *P. falciparum*. While at Florida (1997–2000), Carlton undertook the first large-scale EST and genome survey sequences (GSS; similar to ESTs but generated from genomic DNA library clones) sequencing projects of two other malaria species, *P. vivax* and *P. berghei*. A brief stay as a visiting scientist at the National Center for Biotechnology Information, National Institutes of Health (2000–2001) was followed by an appointment to the faculty of TIGR. Here, Carlton helped co-faculty member, Malcolm Gardner, complete the project to sequence the genome of *P. falciparum*, ironically the 3D7 clone used by Walliker in the first genetic cross of this species, as well as leading the project to sequence the genome of the first rodent malaria species to be sequenced, *P. yoelii yoelii*. Roles in other sequencing projects of eukayrotic parasites followed, including those of *Theileria parva*, the rodent malaria parasites *P. chabaudi* and *P. berghei*, and more recently the sexually transmitted pathogen *Trichomonas vaginalis*. After 5 years at TIGR, Carlton left to follow a more academic career path at the NYU School of Medicine. Here she is completing the project to sequence the genome of *P. vivax*, the second human malaria genome to be sequenced and the most common malaria species outside Africa.

Le Roch *et al*. (2003) and Bozdech *et al*. (2003) were the first to use DNA chips to analyze gene expression in *P. falciparum* across the entire parasite life cycle and across short time intervals within the asexual intraerythrocytic cycle. The first study analyzed total ribonucleic acid (RNA) from sporozoites, merozoites, gametocytes and six stages in the asexual cycle. Using gene expression clustering followed by temporal patterns of expression with genes of known function or cellular processes it was possible to provide functional clues for more than 60% of the genes that the malaria genome project had identified as lacking in sequence similarity to other organisms and called 'hypothetical'. In the second study, a cascade of gene expression was found and 75% of the genes were activated only once during an asexual cycle with activation correlated to time-specific processes. The wave of transcription began as rings grew into early trophozoites. Some 950 genes appeared to be involved in general or housekeeping functions and were induced with broad expression profiles. In the transition from trophozoite to schizont, 1,050 genes

were maximally transcribed and in the mid-to-late schizonts a further 550 genes were induced. Bozdech *et al*. (2003) concluded, 'The lack of continuous chromosomal domains with common expression characteristics suggests the genes are regulated individually. . . [and in a] fundamentally different mode of regulation from other eukaryotes. This . . . implies disruption of a key transcriptional regulator . . . may have profound inhibitory properties.' Indeed, in a later study by Le Roch *et al*. (2004) a correlation analysis revealed that regulation occurs predominantly at the transcriptional level and there is a time shift between a gene's transcription and the synthesis of the cognate protein.

Placental infected red cells bind to chondroitin sulphate A (CSA) and preferentially transcribe the *var2csa* gene, a member of the PfEMP1 family (see p. 196). The *var2csa* gene has been shown to be up-regulated in CSA-binding placental parasites and encodes domains that bind CSA *in vitro*. The proteome of placental malaria parasites was distinct (Fried *et al*., 2007). In total, 2,938 proteins were detected in placental isolates and 2,161 in children. Twenty-six hypothetical proteins were detected in the former but not in the latter. Of the 26, 15 did not bind to CSA. Of the remaining 11, seven hypothetical proteins were exclusively associated with placental parasites. All contained transmembrane domains, four contained a predicted signal sequence and two had PEXEL sequences suggesting translocation across the parasitophorous vacuolar membrane (PVM). PfI1785w was specifically expressed, however, antisera against this protein did not react with live or fixed placental infected red cells. In a genome-wide analysis of the human response to chronic PM, genes were identified and then the corresponding proteins localized to placental cryosections (Muehlenbachs *et al*., 2007). B-cell-related genes were among the most highly up-regulated transcripts. The B-cell chemoattractant CXCL13 was up-regulated by more than 1,000-fold, and B-cell-activating factor was also detected. Both proteins were expressed by intervillous macrophages. Ig L and H chain transcription increased significantly, and heavy depositions of IgG3 and IgM were observed in intervillous spaces. Genes for the Fc binding proteins, FcgammaRIa, FcgammaRIIIa and C1q were significantly up-regulated, and the proteins localized to intervillous macrophages. Birth weight was inversely correlated with transcript levels of CXCL13, IgG H chain and IgM H chain. The results suggest that B cells and macrophages contribute to chronic PM in a process resembling lymphoid neogenesis.

Proteomic approaches have also been used to study drug effects and resistance (Cooper and Carucci, 2004) as well as to characterize isolates with differing adhesive properties (Wu and Craig, 2006). In a proof of principle that proteomics can dissect out the specific and non-specific effects of antimalarial drugs, Makanga *et al*. (2005) used pharmacological levels of drugs to study the components of CoArtem (artemeter, ARM

plus lumefantrine (LUM)). A three-fold reduction of glycolytic enzymes was seen with ARM but with LUM there was an up-regulation of these enzymes. ARM resulted in the down-regulation of enzymes involved in protein and nucleic acid metabolism (adenosine diphosphate (ADP)-ribosylation factor and *S*-adenosyl-*L*-homocysteine hydrolase) and amino acid biosynthesis (malonyl coenzyme A (CoA)-acyl carrier protein transacylase precursor). Kidgell and Winzeler (2006) suggest that microarrays as well as full genome sequencing may advance our understanding of the genetic and molecular processes that facilitate the emergence of drug resistance as well as aiding in monitoring resistance.

Korir and Galinski (2006) used proteomic analysis to show the schizont-infected cell agglutinin (SICA) of *P. knowlesi*—the antigen that provided the first evidence for antigenic variation in malaria (Brown and Brown, 1965)—shared common sequences with PfEMP1 molecules (see p. 191).

Several proteomic studies have targeted organelles. Using MudPIT technology for the analysis of the immunoprecipitated proteins of Maurer's clefts a novel subtelomeric gene family encoding 11 transmembrane proteins was identified (Vincensini *et al*., 2005). Similar subproteome studies have been carried out with the parasitophorous vacuole (PV, Nyalwidhe and Lingelbach, 2006), the rhoptry (Sam-Yellowe *et al*., 2004) as well as proteins exported to the surface of the red cell (Florens *et al*., 2004).

3. DATA MINING

Chip technology, applied to fully sequenced as well as partial sequences of genomes, has enabled genome-wide analyzes of the levels of transcripts and proteins as well as measuring polymorphisms (i.e. small nucleotide polymorphisms, SNPs). In addition, using this high-throughput method (and others) it has been possible to genotype fresh isolates and to compare these with lines that have been maintained in culture (Carret *et al*., 2005). Expression analysis using ESTs, serial analysis of gene expression (SAGE), as well the availability of chip readers and multi-dimensional chromatographic separation of peptides coupled with detection by mass spectrometry as well as genome-scale protein expression (Vedadi *et al*., 2007) now provide a profusion of data sets that have to be organized and integrated to more fully understand the biology of *Plasmodium* spp., interactions of the plasmodia with their hosts, as well as the effects of environmental perturbations (Llinas and del Portillo, 2005).

To put order into this 'abundance of riches' and to allow a functional analysis all *Plasmodium*, data have been stored and organized in the PlasmoDB database (http://plasmodb.org; last accessed 16 July 2008).

The PlasmoDB Internet resource which began in 2000 (see Bahl *et al*., 2002, 2003; Kissinger and Roos, 2004; Kissinger *et al*., 2002; Whetzel *et al*., 2005) provides genomic sequences of malaria parasites and gene annotations contributed by sequencing centers, principally TIGR and the Wellcome Sanger Institute, and its goal is to provide visitors to the site with the ability to mine integrated data sets. PlasmoDB allows one to view features and functions of orthologous genes permitting inferences of gene and protein properties in different *Plasmodium* spp. New data sets provide predictions and clues as to how the components work together. Experimental evidence for protein–protein interactions is also provided and there are links to the Malaria Research and Reference Reagent Resource Center (MR4) for available reagents. A new PlasmoDB (v. 5) provides full access to the complete genomes of *P. falciparum*, *P. vivax* and *P. yoelii*, along with gene models, annotation, Basic Local Alignment Search Tool (BLAST, a program for database searching) hits, orthologue results, expression data and search functions for querying and integrating results across species (Stoeckert *et al*., 2006).

David S. Roos (1956–), the E. Otis Kendall Professor of Biology at the University of Pennsylvania, was born in Boston, MA to an artist and musician mother, and a steroid biochemist father and grew up in Madison (Wisconsin) and Hanover (New Hampshire). A youth spent mostly in the mountains, forests and rivers of northern New England ensured ample exposure to biology (and the occasional parasitic infection). At one time an avid mathematics and logic enthusiast, he worked as a computer programer for several years before choosing to pursue a fine arts degree at Harvard University (Cambridge, Massachusetts). These interests were ultimately overshadowed, however, by thesis research in the laboratories of Morris Karnovsky and Richard Davidson, which provided an introduction to cell biology and genetics. As a doctoral student with Purnell Choppin at the Rockefeller University (New York), Roos explored various aspects of membrane biochemistry in animal cells and enveloped viruses, earning a PhD in 1984. Parasitology was increasingly in evidence in those years, due to high-profile studies by Piet Borst and George Cross on the molecular basis of antigenic variation in trypanosomes, the characterization of hydrogenosomes by Miklos Muller, the development of *in vitro P. falciparum* culture by William Trager and malaria vaccine studies by the Nussenzweig group at NYU. A post-doctoral stint with Robert Schimke at Stanford University provided the opportunity to learn modern techniques in molecular genetics, in the context of studying drug resistance mechanisms in mammalian cells. Infectious diseases retained their appeal, however, in part due to the tradition of applying

a diverse range of techniques to solve a specific biological problem. Seeking a research topic that was intellectually interesting, scientifically tractable and potentially relevant to human disease, library research revealed many exciting opportunities in virology, bacteriology and parasitology. In particular, the pioneering work of Elmer Pfefferkorn, demonstrating the feasibility of genetics in *Toxoplasma gondii*, suggested that it might be possible to employ this organism as a model for studying various problems relevant to *P. falciparum* and other apicomplexan parasites. Encouraged by John Boothroyd, Jack Remington, C. C. Wang and others, this became the focus of the Roos laboratory. Remarkably enough, jobs (and financial support) were available for this untested project, and Roos moved to the University of Pennsylvania in 1989, where he has remained ever since except for a sabbatical in 1998 at Walter and Eliza Hall Institute (WEHI) with Alan Cowman and others.

The Roos laboratory's research integrates diverse disciplines, from molecular cell biology and pharmacology, to computer science, infectious diseases and international public health. Current interests focus on protozoan parasites, including *Toxoplasma* and *Plasmodium*. Research in the Roos laboratory has yielded genetic tools for the molecular dissection of parasite pathogenesis and drug resistance mechanisms, new insights into the cell biology and evolution of sub-cellular organelles, and computational databases that make genomic-scale datasets available to researchers worldwide. Work specifically related to malaria biology includes the development of antifolate-based resistance markers, elucidation of the mechanistic basis of antifolate resistance, characterization of the diversity of purine (and other) metabolic parasites in apicomplexan parasites, characterization of apicoplast targeting pathways, development of the *Plasmodium* genome database (PlasmoDB.org, a component of the broader ApiDB project), providing an integrated resource for accessing genomic-scale datasets related to malaria parasites. In 2001, Roos founded the Genomics Institute at the University of Pennsylvania, and directed this enterprise for its first 6 years. In addition to computational and wet-laboratory research, he currently directs the ApiDB (Apicomplexa Data Base) Pathogen Bioinformatics Resource Center, University of Pennsylrania, Philadelphia.

Two examples of the power of data mining of the transcriptome: (1) Bozdech and Ginsburg (2004, 2005) were able to identify genes encoding anti-oxidant defense elements during the intraerythrocytic asexual developmental cycle. In most cases there was a clear co-ordination of the participating genes, however, there were also some 'outstanding deviations where gene products that utilized GSH or thioredoxin are transcribed before the genes coding for elements that control the levels of

those substrates are transcribed; (2) analysis of the pentose phosphate pathway (PPP) and its metabolic links (see Chapter 10, p. 89).

Assistance in the use of bioinformatics approaches is available and instruction pertaining to the use of malaria related resources is offered by the Malaria Research and Reference Reagent Resource Center (MR4-http://malaria.mr4.org; last accessed) and the World Health Organization/Special Program for Research and Training in Tropical Diseases (WHO/TDR, Geneva, Switzerland) (http://www.who.int/tdr/; last accessed 16 July 2008); workshops have been offered at several international meetings. Finally, 'it is important to keep in mind that computational analysis is accessible to everyone . . . you can do it!' (Kissinger and Roos, 2004).

The Malaria Parasite Metabolic Pathway (MPMP) is a website (http://sites.huji.ac.il/malaria; last accessed 16 July 2008) that contains over 120 maps that encompasses not only classical biochemical pathways but next to each entry there is a 48-h clock that depicts stage-dependent transcription of a particular gene in *P. falciparum*. At the clock's zero hour, merozoites invade and at 12 o'clock the 48-h cycle terminates. In the middle of the clock appears the hour of maximal transcript level. Clicking on the clock links to a transcriptomic database (http://malaria.ucsf.edu; last accessed 16 July 2008). The enzymes are also linked to the International Union of Biochemistry and Molecular Biology website (http://www.iubmb.org; last accessed 16 July 2008) where there is a detailed chemistry of the enzymatic reaction. Unlike other databases MPMP has been developed, curated and updated manually (Ginsburg, 2006).

Other data mining tools have been described (see Birkholtz *et al*., 2006; Date and Stoeckert, 2006; Fraunholz, 2005; Li *et al*., 2003; Watanabe *et al*., 2007) and these will provide for a better functional synthesis of *Plasmodium* and its relationship to other Apicomplexans such as *Toxoplasma*, *Cryptosporidium* and their hosts.

CHAPTER 27

Mosquito Interactions

Contents

Investigations into the biochemistry of the mosquito stages of malaria have lagged behind those of the asexual stages in the blood mainly because of the inherent inaccessibility of these forms. However, in recent years advances in transcriptomics and proteomics as well as tools such as transfection 'knockouts' and green-fluorescent protein-expressing parasites, *in vitro* cultures, and the use of rodent malaria models has allowed a fuller understanding of the functional attributes of these stages.

1. SEXUAL STAGES IN THE BLOOD

Gametocyte or sex cell formation (= gametocytogenesis) has been primarily investigated in *Plasmodium falciparum*, several rodent malarias including *P. berghei*, *P. yoelii* and *P. vinckei* and *P. gallinaceum* (Alano and Billker, 2005; Alano and Carter, 1990; Baton and Ranford-Cartwright, 2005b; Carter and Graves, 1988; Janse and Waters, 2004; Sinden, 1998). In *P. falciparum*, gametocyte development has been divided into five stages (I–V) covering a period of 7–8 days from merozoite invasion of a red cell to mature sexual development within that same red cell. Although blood-stage schizonts of *P. falciparum* are committed to differentiate into either gametocytes or to develop into asexually reproducing schizonts as early as the trophozoite stage (approximately 24–30 h after

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merozoite invasion), the sexual stages cannot be distinguished from asexual stages either by light or electron microscopy. However, prior to any sign of morphologic change, early gametocytes produce two specific proteins, Pfg27, an abundant (around 10% of the gametocyte cytosolic proteins) phosphoprotein and Pfs16 a parasitophorous vacuolar membrane (PVM) protein (Young *et al*., 2005). Since a knockout of the gene for Pfg27 abolished gametocyte maturation, it appears to be critical for gametocyte development but precisely how is not known. In a transcriptome analysis of gametocytogenesis, two novel early gametocyte-specific genes—*pfeg2* and *pfeg4*—were found in stage I and II gametocytes. The former persists throughout sexual development, whereas the latter is maximally expressed in stage II gametocytes (Silvestrini *et al*., 2005). The function of these gametocyte-specific proteins remains unknown. Sex-specific morphologic features become apparent by stage II: a cortex is laid down resulting in triple membrane pellicle consisting of an outer plasma membrane and a closely juxtaposed inner membrane complex (IMC) underlain by a layer of microtubules. This gives the gametocytes the characteristic crescent shape first seen by Laveran (see p. 3) and now used for microscopic diagnosis of *P. falciparum* infections.

Stage-I–III gametocytes synthesize ribosomes and endoplasmic reticulum and there is expression of Pfs48/45 in addition to Pfg27 and Pfs16 (Young *et al*., 2005). Approximately at day 4, with the formation of stage III gametocytes, there is α-tubulin expression in male gametocytes, whereas Pf377—the only specific marker for the osmiophilic bodies—starts to be produced preferentially in the female gametocyte. At stage IV, as a result of complete encirclement by the subpellicular microtubules, the gametocytes assume a symmetrical shape and with a decrease in ribosomes, endoplasmic reticulum, Golgi and mitochondria in male gametocytes it can be differentiated from the female gametocyte (Alano and Carter, 1990; Carter and Graves, 1988; Sinden, 1983). From stage IV onwards, small osmiophilic bodies become apparent by electron microscopy; these are more numerous in stage-IV and -V female gametocytes and may play a role in liberation of the gametocyte from the red cell; in addition, female gametocytes, unlike male gametocytes, have mitochondria with more cristae. These stages also show expression of Pfs 25/Pfs 28 and Pfs 77 with down-regulation of genes involved in glycolysis, protein synthesis and hemoglobin catabolism (plasmepsins) (Young *et al*., 2005) consistent with the metabolism of these stages being relatively slower in comparison to other stages.

Although stage-V gametocytes remain in this quiescent state for several days, and their ability to undergo gametocytogenesis declines with a half-life of 2–3 days, they remain highly sensitive to environmental stimuli. At stage V, in both male and female gametocytes there is a breakdown of the subpellicular complex resulting in a smoothing of the

gametocyte edges and a curved appearance, a character more pronounced in female gametocytes. Immature stages I–IV sequester in the bone marrow and spleen capillaries (Alano, 2007). Stages I and II, produce *P. falciparum* erythrocyte surface protein-1 (PfEMP1), have knobs and bind to CD36, whereas stages III to IV lack knobs, have a reduced binding to CD36 and are reported to bind *via* a host-related ligand (i.e. band 3 protein; Rogers *et al*., 2000). Based on receptor binding and sensitivity to trypsin, the adhesive phenotype of stages I and II appears to be similar to that of asexual stages. However, despite this no gametocyte cytoadhesion ligand has been identified.

A proteomic analysis found five proteins containing lectin-like *Limulus* clotting factor C (LCCL) motifs in gametocytes and three of these were associated with, but not exposed on, the surface of stage III–V gametocytes. Targeted disruption studies (see below) suggest that their function lies not in gametocyte maturation but in the maturation of the oocyst.

A comparative transcriptome time course in the gametocyte-producing clone 3D7 and the gametocyte-less F12 clone identified 'remarkably few differences in gene expression . . . between these parasite clones' (Silvestrini *et al*., 2005), however a cluster of 117 genes was up-regulated in 3D7 and their profiles correlated with the gametocyte-specific genes *pfg16* and *pfg27*. Inspection of the proteomic data for 80 of the 117 genes revealed that 18 genes were gametocyte specific and, in six out of eight, expression was controlled at the transcript level. These results support the findings of Le Roch *et al*. (2003) where only five genes were found to overlap with those up-regulated in stages III and IV. Such findings are consistent with the changes in metabolism and cytoadhesion seen in the early gametocyte stages and distinct from later stages.

From stage II onwards, gametocytes are insensitive to antimalarials (i.e. chloroquine, atovaquone and pyrimethamine) that act on asexual stages. In contrast, primaquine is active against late-stage gametocytes but less active against younger-stage gametocytes and may be due to differences in mitochondrial structure and function (see also Young *et al*., 2005).

2. GAMETOGENESIS AND OOKINETE FORMATION

Ingestion of blood-containing mature (stage V) gametocytes by a female mosquito exposes the gametocytes to unique environmental stimuli in the blood meal in the mid-gut. Three stimuli to which they respond are a fall in temperature as the parasitized red cells leave the vertebrate blood stream, a rise in the extracellular pH and a rise in the concentration of mosquito-derived xanthurenic acid (XA). In addition, glucose and physiological concentrations of NaCl and HCO^{3-} are required for gametocyte activation *in vitro* (see p. 41 and Alano, 2007; Alano and Billker, 2005; Carter and Graves, 1988; Janse and Waters, 2004; Talman *et al*., 2004).

The first involvement of **Pietro Alano (1959–)** in experimental life sciences was during biology degree studies with Gianni Dehò and Gianpiero Sironi at the University of Milan (Milan, Italy), where he was attracted by the regulatory interactions between a helper and a satellite bacteriophage (P2 and P4) with their *Escherichia coli* host (bachelor of science, BS; 1983). This interest continued in his subsequent doctor of philosophy (PhD) studies (obtained in 1986), which brought him to investigate the genetics of the phage–host interplay underlying the lysis-lysogeny of bacteriophage lambda, with Flora Banuett and Ira Herskowitz at the University of California at San Francisco (California). It was only after his PhD, though, that his attraction for genetic mechanisms of developmental decisions and interplay between a host and a 'guest' organism found a focus in the developing field of parasite molecular biology.

In 1986, at the biology of parasitism course (Marine Biological Laboratory (MBL) Woods Hole, Massachusetts) he met Richard Carter and David Walliker, and their discussions on how *P. falciparum* was able to develop alternatively as asexual stages or gametocytes in the human blood stream moved in a few months from the beaches of Cape Cod to the hut (a former veterinary stable) that hosted the newly established laboratory of Richard Carter in the Kings' Buildings at Edinburgh University (Edinburgh, United Kingdom). Here in the unique scientific and intellectual environment represented at the end of the 1980s by Geoffrey Beale, John Scaife, Janna McBride, Richard Carter and David Walliker Pietro first learned malaria biology and carried out his post-doctoral work, identifying some of the first described gametocyte-specific genes of the human malaria parasite. The opportunity to return to Italy, where malaria research was, at the beginning of the 1990s, limited to a few, albeit significant, laboratories, was given by Clara Frontali and her group at the Istituto Superiore di Sanità (Rome, Italy). A few years before, Clara's background in physics had merged with molecular biology expertise in the farsighted approach of addressing fundamental questions on the genome structure of *P. berghei*, research that led, in 1985, to the first description of telomeric sequences of the malaria parasite.

From 1991 to the present, Pietro's research on the molecular biology of *P. falciparum* development is continuing at Istituto Superiore di Sanità, with his collaborators, and benefiting of the interactions with Marta Ponzi and Tomasino Pace, and Elisabetta Pizzi's expertise in computational biology. Although the original questions on the commitment of malaria parasites to sexual development have not been fully answered, some of its mechanisms have become clearer in recent years. Meanwhile, additional genes and proteins expressed in

P. falciparum gametocytes have been identified and characterized, and their regulation investigated in Rome, and through collaborations (that in some cases have turned into real friendships) with malaria researchers around the world. Besides fundamental biology, an increasing interest has also been dedicated to how knowledge generated on *Plasmodium* sexual differentiation could provide tools capable of interfering with parasite transmission and the spread of malaria. After 2000, questions on parasite development began to be asked at the genome-wide level, made accessible by the Malaria Genome Sequence Consortium. Since that time, parasite culture flasks and Percoll gradients have been increasingly accompanied in Pietro's laboratory by multi-sheet transcriptome and proteome datasets, along the fascinating route of studying the fundamental biology of *Plasmodium*.

Exflagellation was first seen by Laveran in 1880. Laveran was under the impression that he was witnessing the extrusion of flagella, 'I was astonished to observe that at the periphery of this body was a series of fine transparent filaments that moved actively ... the filaments, flagella, detached themselves from the spheres out of which they came and swam off.' In actuality what he saw was the release of male or microgametes a phenomenon appreciated by MacCullum (1898). In the blood meal, and often within 15 min of ingestion, the male gametocyte emerges from the blood cell. The male gametocyte then undergoes three rounds of deoxyribonucleic acid (DNA) replication and mitosis and within 15 s of induction eight flagella are assembled within the cytoplasm. Each flagellum is attached to one haploid nucleus and when microgametes are finally expelled from the surface it is called exflagellation.

Heat shock proteins and chaperones may be critical for the regulation of exflagellation and the essential role of XA suggests that specific receptors are either activated or inhibitors sequestered but what these are remains unknown. Other signaling pathways activated during microgametogenesis include cGMP, calmodulin and phospholipase C-mediated conversion of inositol 1,4,5-triphosphate ($InsP_3$) that results in a release of calcium ions from the endoplasmic reticulum, triggering a redistribution of Pf155 in the parasitophorous vacuole (PV), expression of P25/P28 and activation of CDPK4 that serves to regulate DNA replication and polymerization of the microtubules of the mitotic apparatus as well as the flagellar axoneme of the microgametes (summarized in Sinden, 2004).

Robert Sinden (1943–), as the son of a farmer, 'inevitably' studied zoology, at Kings College, Durham University (Durham, United Kingdom; bachelor of science, BSc; 1964). A subsequent year working as a

research assistant in the Medical Research Council's Unit for Demyelinating Diseases (Newcastle-upon-Tyne, United Kingdom) engendered a lifelong fascination in research, but also the recognition that a higher degree was essential to personal intellectual freedom. The emerging revolution in molecular genetics prompted a move to the Institute of Animal Genetics, Edinburgh University (Edinburgh, United Kingdom). A practical project on *Paramecium* with Geoffrey Beale during the masters course in genetics led to a PhD project on the synthesis of the surface immobilization antigens using immunoelectron microscopic methods. Beale's young team at the time included Tait, Walliker, Carter and Knowles, a group that reveled in the exciting opportunities science was then providing. Upon completion of the PhD (1969) the question arose, 'Where to go from such a dynamic environment?'

The answer was actually simple. Having worked with some of the country's best geneticists, the opportunity presented itself in 1971 to use the newly developed skills in cell biology in collaboration with the United Kingdom's leading (protozoan) parasitologists P. C. C. Garnham, E. U. Canning and R. Killick-Kendrick at Imperial College London (London, United Kingdom). Working in converted stables of a Victorian mansion, the ultrastructural organization of the mosquito and liver stages of a wide range of malaria parasites was decoded. It was immediately apparent that for significant progress to be made it would be necessary to develop methods to culture the parasites. The rapid development of methods to support gametogenesis and fertilization *in vitro*, initiated a lifelong fascination with the stunning molecular and sub-cellular organization of the malarial parasite during its development in the mosquito bloodmeal. Subsequent studies unequivocally identified the first post-zygotic division of the parasite as meiosis, an observation critical to the rapidly progressing genetic studies stemming from Beale's laboratory in Edinburgh. Exploiting Trager and Jensen's method for the culture of blood-stage parasites and together with Smalley in The Gambia, and Ponnudurai in Nijmegen, reproducible methods were finally developed to culture the sexual stages of *P. falciparum*. Attempts to pioneer cell-biological approaches to malaria research at that time were occasionally frustrating, efforts to seek funding to study the molecular basis for parasite motility and invasion were rejected, with the reviewers comments 'Malaria parasites are non-motile'—a challenging environment! Thereafter Sinden's laboratory diversified to develop methods to culture the liver stages of *P. berghei*, and study their ultrastructural and molecular organization. A highlight of this period was the invitation to participate in the studies with Garnham, Krotoski and Bray to discover the hypnozoite of *P. vivax*. At that time the destructively

competitive nature of research into liver stages, contrasted starkly with the collaborative and forward-thinking group of scientists then engaged in the development of transmission-blocking strategies. This environment focused his future studies on malaria in the mosquito. In collaboration with others, methods were developed for the culture of all the mosquito stages of development of *P. berghei* (the only malaria species for which every life-stage can currently be cultured). Sinden's group then identified XA as the molecular trigger for sexual development in the mosquito vector.

Recently, through the mediation of Dan Carucci in productive collaboration with John Yates laboratory, Sinden used their experience in culture techniques to underpin wide-ranging proteomic analyses of the malarial life cycle. Extensive collaboration with Waters and Janse in at the Center of Infectious Diseases (Leiden, The Netherlands), subsequently led to the widespread application of gene mutation, tagging and knockout studies in these, now high-throughput analyses. This led to the identification and characterization of scores of gene products essential to parasite development in the vector. The movement of the Kafatos laboratory from the EMBL to Imperial College has now established a world-leading collaboration to spearhead the development of understanding of methods for intervention against parasite transmission through endemic populations. The latest studies singularly emphasize how *Plasmodium* has been transformed from an organism, once rarely observed in Giemsa-stained blood films, into a tractable, and dare one suggest 'model-organism' for developmental studies, but as for any such models, hypotheses generated must still be tested directly in the human pathogens. Sinden is presently senior dean and professor of parasite cell biology at Imperial College London.

Although a drop in temperature is required for exflagellation, it alone is insufficient for activation. Indeed although, it was shown that exflagellation, even at the permissive temperatures of 26–32 °C, was triggered *in vitro* only when there was also a rise in pH it was not until 1977 that the experiments of Carter and Nijhout (1977) conclusively showed what was actually occurring (see pp. 41, 306).

Richard Carter joined the National Institutes of Health (NIH, Bethesda, Maryland) laboratory of Louis Miller intending to do a genetic cross with *P. falciparum* using *Aotus* monkeys. Shortly after arrival it became obvious this was going to be very difficult. Apart from anything else, the chances of having two monkeys infected with two different strains of *P. falciparum* carrying gametocytes, let alone infectious gametocytes, on the same day, was extremely low. The only alternative at that time was to try to deep-freeze gametocytes from two infections and then

later bring them back to life together on the same day for a mixed membrane feed. To work out the principles, however, a working model was needed, and *P. gallinaceum* was the best available. Carter never looked back, or considered doing a genetic cross with *P. falciparum* again until almost 10 years later after infectious gametocytes had become available from Trager-Jensen cultures. Working with *P. gallinaceum* gametocytes, the first issue was how to stop them exflagellating spontaneously while trying to freeze them. Building upon the work of Bishop and McConnachie (1956) and following a tip from Miller, Carter 'invented' gametocyte 'suspended animation medium'—just Tris-buffered physiological saline, with glucose and no bicarbonate—it kept them alive and happy and quiescent for at least an entire working day, but ready to begin exflagellation immediately when the right stimulus was given. Then he began to figure out how to determine the relative roles of bicarbonate, CO_2 and pH in the control of exflagellation (again he relied on Ann Bishop' studies showing bicarbonate was the 'only' ion essential for exflagellation in *P. gallinaceum*). Since bicarbonate, CO_2 and pH are inextricably inter-dependent, the usual rule in scientific experimentation of changing one parameter only and measuring the effect, did not apply. Carter worked out the principle of changing each of these one at a time (while of course the other two went on their own ways) and measured exflagellation rates, however, he did not have the technical means to do this properly. When Mary Nijhout joined the laboratory and became interested in the problem they teamed up. Mary located the two essential pieces of equipment to do the experiments, namely Dvorak-Stotler chambers, and a blood gas analyzer from the Clinical Center. Using these they were able to control and measure the gas tensions (O_2 and CO_2) to which the gametocytes were being exposed in the chambers. Now any concentration of bicarbonate could be chosen, and the pH measured. These measurements allowed a determination of how pH, bicarbonate and CO_2 interacted to control the exflagellation and emergence of female gametes in *P. gallinaceum* (Nijhout and Carter, 1978). The conclusions were clean and clear. So long as bicarbonate ions were present over the range of concentrations used (between 15 and 100 mM), exflagellation of *P. gallinaceum* gametocytes took place in a strictly pH-dependent manner. Up to around pH 7.7, nothing happened; above this pH the amount of exflagellation increased to a maximum at pH 8.0. Thereafter it declined rapidly again and did not take place at all above pH 8.5; CO_2 gas tension, as such, was quite irrelevant to the amount of exflagellation. They also confirmed Bishop's original finding (Bishop and McConnachie, 1956) that *in vitro* exflagellation was absolutely dependent upon the presence of bicarbonate ions in at least the concentrations reported. Everything that Carter subsequently did and became interested in concerning gametocytes, and, indeed, transmission-blocking immunity, arose from and

during this diversion from isozymes and genetics and into studies of gametocytogenesis and ookinete formation.

The basis for Laveran's discovery of exflagellation is that an increase in pH to 7.8–8.0 occurs when blood is allowed to equilibrate with air. Although at the time of Carter and Nijhout's experiments there was no absolute proof that bicarbonate/pH was the basis of exflagellation by *P. gallinaceum* gametocytes, neither was there an obvious reason to conclude that it was not or that a mosquito factor was needed. Indeed, much the focus of thinking at the time was away from the possibility of mosquito factors being importantly involved. Nevertheless, Nijhout and Carter suggested that mosquitoes 'might possess vector-specific synergistic or inhibitory substances capable of controlling gametogenesis in malaria parasites independent of the bicarbonate-dependent pH control …' As described earlier (see p. 306), Nijhout proceeded to conduct various experiments until she had proved beyond any doubt that within the blood meal was a factor of mosquito origin that potently stimulated exflagellation of *P. gallinaceum* gametocytes (Nijhout, 1979). Conclusively shown not to be bicarbonate, she called it mosquito exflagellation factor (MEF). It was from that specific and clear discovery that a series of efforts were made over the years to try to identify MEF. A decade later using a high-resolution mass spectrometer, the molecule was purified from mosquito extracts and found to be XA (Billker *et al*., 1998). This successful identification derives absolutely from the discovery of MEF by Mary Nijhout. Indeed, without her experiments there would have been no conclusive basis upon which to know about its existence. The calculated XA in the blood of *Aedes* is 2.5–2.8 μM, a level sufficient for effective microgametogenesis for *P. gallinaceum* and *P. falciparum*, but not *P. yoelii* and *P. berghei* where the required concentration is 9 μM (Arai *et al*., 2001). In all species of *Plasmodium*, XA can completely replace the pH stimulus but with *P. berghei* XA and pH act synergistically.

It has been claimed (but not proven) that the female or macrogametocyte escapes from the enclosing red cell through a combination of enzymatic and mechanical forces (Alano and Billker, 2005). In *P. gallinaceum*, 3 or 4 min into the process, the surrounding red cell cytoplasm at a certain point, collapses around the now rounded up gametocyte within, and the gametocyte together with the host erythrocyte nucleus are held closely together. At the same time, the membrane of the host cell now beads and bubbles out from the collapsing cell in streamers. Around 1–2 min later, the host cell nucleus drifts away and the female gamete is left completely free of all host cell membranes or other host cell debris, floating free, a perfect sphere with little lumps on its surface (due to projecting malaria pigment) (description in Nijhout and Carter, 1978). Presumably, similar processes occur in mammalian malarias.

Disappearance of the osmiophilic bodies correlates with the breakdown of the red cell membrane but a biochemical link between the two is lacking. The macrogametocyte is pre-adapted to differentiate into the zygote once it is released from the red cell. However, the most abundant messenger ribonucleic acids (mRNAs) that encode proteins essential for mid-gut invasion are kept translationally silent in the macrogametocyte of *P. berghei* due to the presence of a stored DDX6 class ribonucleic acid (RNA) helicase named DOZI (development of zygote inhibited) complexed to mRNAs in cytoplasmic bodies. Translation of these mRNAs is initiated only after activation during mosquito ingestion and results in macrogamete formation. When there is a loss of DOZI, the parasite is unable to store and stabilize the mRNAs, specific proteins are not made, and normal zygote development does not occur (Hall *et al*., 2005; Mair *et al*., 2006). The mechanism of post-transcriptional silencing appears to be used by more genes than those in the female gametocyte. Pace *et al*. (2006) have described *SET*, a single copy gene that encodes a protein involved in chromatin dynamics in both asexual stages and male gametocytes. *SET* expression is regulated through the use of distinct stage-specific promoters. *SET* inhibits the activity of a PP2A phosphatase, resides mainly in the nucleus and strongly accumulates in male gametocytes. This stockpiling may contribute to the prompt entry into the mitotic phase by the developmentally arrested male gametocyte once activated. (*SET* also produces mRNA transcripts in asexual parasites with a different size compared with that of gametocytes (Pace *et al*., 1998) and this may account for the differences in the expression of *SET* between sexual and asexual blood stages.

Although there is no evidence of chemotaxis between the male and female gametes, within 1 h fertilization occurs. 'Factors derived from both the vector and the vertebrate host … i.e. antibodies, cytokines, leucocytes, and the biochemical composition of the blood meal all contribute to the success or failure of these events' (Sinden, 2002). Within the bloodmeal bolus each zygote differentiates, over a period of 5–18 h, into a single motile ookinete. More than a century ago, MacCallum (1989) described it, 'The movement is slow and even … with the pointed end forwards. It can move in any direction readily … often it is seen to rotate continually along its long axis. The forwards progression … occurs with considerable force … pushing directly through the obstacle. The ultimate fate and true significance of these forms is difficult to determine.' Incorrectly, he concludes, 'it is reasonable to suppose … it is the much sought resistant stage.'

Differentiation of the ookinete involves three key processes: (1) a change in the surface from a fertilization-receptive macrogamete into an invasive cell capable of resisting immune attack by the mosquito and interaction with the mosquito mid-gut, (2) fabrication of a cortex and apical complex that contain the molecular motor and secretory apparatus to escape the bloodmeal and invade the mid-gut wall and (3) meiosis

resulting in four haploid nuclei (Sinden *et al*., 2004). During development into an ookinete the parasite changes the composition of its surface coat and becomes sensitive to complement, however, at the same time complement factors (B, D, C_{3a} and C_5) are destroyed by the proteases secreted by the mosquito gut to digest the blood meal so that no membrane attack complex can be assembled (Grotendorst and Carter, 1987; Grotendorst *et al*., 1986; Sinden, 2004). Zygotes are resistant specifically to the alternative pathway of complement of their own natural vertebrate host but not to others.

The anterior pole of the ookinete is covered by an apical complex that is more elaborate than either the merozoite or sporozoite in that it has a rigid collar that forms a protrusible apical prominence. Chitinase has been localized to the collar. The micronemes contain circumsporozoite protein (circumsporozoite- and thrombospondin-related adhesive protein (TRAP)-related protein (CTRP); a TRAP homologue found in sporozoites but with multiple A domains and a thrombospondin type I repeat) and a TRAP-related protein, von Willebrand factor A domain-related protein (WARP) and secreted ookinete adhesive protein (SOAP). Unlike the other invasive stages, the ookinete lacks rhoptries. Thus the presence of 'rhoptry-associated proteins' described in the ookinete proteome may indicate that their sub-cellular localization was incorrect and a micronemal location is more appropriate. This in turn correlates with the fact that the ookinete does not form a PV in the invaded mid-gut cell. A major switch in ookinete surface proteins occurs 5–7 h after ingestion of the bloodmeal with a new array of GPI-anchored proteins of which P25 and P28 are dominant. How these proteinaceous protectors against proteolysis are transported to the ookinete surface is not known.

A proteomic analysis of the ookinete found 307 proteins to be unique but the cell motor proteins required to move organelles in all life stages were shared whereas one motor protein, actin 3, was unique to the ookinete (Sinden, 2004). The pellicle of the ookinete consists of microtubules and an IMC; an actin–myosin motor is located in the space between the plasma membrane and the outer membrane of the IMC (Kappe *et al*., 2004a). Consistent with this, a subset of the IMC proteins was found only in the ookinete. The inner membrane of the IMC contains large pores and these may allow for trafficking of macromolecules between the cytoplasm, the plasma membrane and the molecular motor. Some 19–36 h after feeding the ookinete moves out of the bolus by a gliding motion driven by the actin–myosin motor. CTRP is secreted onto the ookinete surface following Ca^{2+} signaling. The molecular motor pulls the CTRP backwards and because the extracellular portion of the protein is anchored to a fixed substrate this leads to forward movement much like a tank's caterpillar track. The nature of the substrate employed in gliding is not known but it is likely provided by the parasite itself. As the ookinete

glides forwards P25 and P28 are shed. Parasite losses in the mid-gut are extensive even in susceptible mosquito vectors and only 10% of the macrogametocytes form ookinetes and 50% of the latter show signs of apoptosis both *in vivo* and *in vitro* (Vinetz, 2005).

In *P. gallinaceum*, mid-gut (or plasmodial?) proteolytic enzymes activate a chitinase encoded by PgCHT1, and secreted from the ookinete micronemes as a proenzyme; the chitinase results in the disorganization of the chitin, a (1-4)-β homopolymer of *N*-acetylglucosamine, that acts as a scaffold for the peritrophic matrix. The chitinase is required for passage across it in *A. aegypti*, however, it may not be necessary for transmission of this parasite in *Anopheles gambiae* and *An. stephensi*. A *P. falciparum* chitinase (PfCHT1) has also been found in the genome database; in contrast to PgCHT1, it does not encode a proenzyme (Sinden *et al.*, 2004; Vinetz, 2005).

Thereafter the ookinete encounters the microvillar network and binds to the mid-gut epithelium. Although there are an array of possible receptors on the mid-gut epithelial cells little is known about their significance other than that sialic acid-like moieties are involved in the binding of the ookinete to the isolated mid-gut epithelium and that these interactions are blocked by the expression of the peptide SM1 (PCQRAIFQSCIN) (Ito *et al.*, 2002) or pre-treatment of the epithelium with snake or bee venom phospholipase 2 (Moreira *et al.*, 2002; Zieler *et al.*, 2001).

Having crossed the peritrophic matrix and the microvillar network the ookinetes attach to and then invade the epithelial cell using the gliding motion (described below). It has been suggested that the epithelial receptors could be sulphated glycosaminoglycans (GAGs). CTRP is essential for successful invasion of the mid-gut epithelium and CTRP knockout parasites are non-motile and unable to invade. Within the epithelial cell the ookinete secretes a subtilisin-like protease SUB2 from the micronemes and P28 is shed from its surface. Ookinetes can migrate from cell to cell in the mid-gut epithelium but the manner by which they exit the cells is unknown (Baton and Ranford-Cartwright, 2005a). Two membrane-attack ookinete perforin-like proteins (MAOPs or PPLPs) are essential for the ookinete to breach the apical membrane of the mid-gut epithelial cell (Ecker *et al.*, 2007). Ookinetes with the gene disrupted attach to the apical tip and invaginate the membrane towards the cytoplasm, however the cell membrane is not ruptured and so cell entry does not occur. As a result parasites do not arrive at the basal lamina and no oocysts are formed (Kadota *et al.*, 2004). In addition, a calcium-dependent protein kinase, CDPK3, of *P. berghei* produced in the ookinete stage may be critical in parasite transmission to the mosquito vector since targeted disruption of the *CDPK3* gene decreased ookinete ability to infect the mosquito mid-gut by nearly two orders of magnitude. An *in vitro* migration assay showed that these ookinetes lack the ability to migrate through an artificial gel, suggesting that this defect caused their failure to access the epithelium. *In vitro* migration assays

also suggested that this motility is induced in the wild type by mobilization of intracellular stored calcium. The results indicate that a signaling pathway involving calcium and CDPK3 regulates ookinete penetration of the layer covering the mid-gut epithelium. Because humans do not possess CDPK family proteins, possibly CDPK3 could be a target for blocking malarial transmission to the mosquito vector (Ishino *et al.*, 2006).

Invasion of the mid-gut cells results in extensive death in the invaded cell and even cells that have simply come in contact with the ookinetes gliding along their surface, show signs of apoptosis, as first noted by MacCallum (1989) '(the ookinetes) can and do dash through the body of a leucocyte, tearing it apart ... passing unharmed they attack red blood corpuscles and leave those in their path as shadows ... this blanching of the corpuscles seems to become possible ... by the merest touch of their anterior pointed ends...' Ookinetes penetrate the basement membrane of the epithelial cell but do not continue through the basal lamina into the hemocoel. The basal lamina contains both laminin and collagen type IV and ookinete surface molecules such as P28, P25, CTRP and SOAP are able to bind to these (Matuschewski, 2006). Although contact-dependent induction of oocyst development can be used for the *in vitro* production of sporozoites by adding ookinetes to Matrigel and cultured insect cells (Warburg and Miller, 1992; Warburg and Schneider, 1993) it is not known which of the ookinete surface molecules (if any) by binding to laminin or collagen or no other molecule (see below) is responsible for arresting ookinete movement or whether this interaction promotes development into the trophic and non-replicative form, the oocyst. Indeed, *in vitro*, transformation of *P. berghei* ookinetes has recently been shown to be a two-step process; initiation of transformation requires sodium bicarbonate, however, for completion of transformation, a suitable pH (7–8) and nutrients but not basal lamina components were required (Carter *et al.*, 2007).

In 'immunologically stressed' mosquitoes some or all of the ookinetes reaching the basal lamina may be subjected to melanization *via* the phenol oxidase cascade (Lehane *et al.*, 2004b). Development underneath the basal lamina is thought to shield the developing oocyst from the mosquito immune system because the basal lamina is 'self' however when unprotected the immune system can attack the developing oocyst. Indeed, melanized oocysts were first reported in non-natural mosquito vectors by Ross as 'black spores' (Ross, 1923).

3. OOCYST AND SPOROZOITE FORMATION

The major biological role of the oocyst is to amplify parasite numbers in the vector (Matuschewski, 2006). Initially the oocyst assumes a spherical shape by loss of its apical complex and disassembly of the microtubules

and the IMC (organelles associated with invasion and locomotion). The single ookinete nucleus undergoes 11 endomitotic divisions so that after 12–18 days 6,000–8,000 haploid nuclei are formed. From day 6 onwards the oocyst cytoplasm is subdivided by multiple clefts that seem to arise from the cisternae of the endoplasmic reticulum. Together the plasma membrane and clefts form a large vesicular structure called a sporoblast covered with circumsporozoite protein (CSP). Sporozoites bud from the sporoblast surface and in the mature oocyst there may be thousands of sporozoites packed in a multi-lobed structure, resembling a pomegranate with its seeds representing sporozoites (Sinden and Matuschewski, 2005).

As the oocyst grows it secretes an amorphous cyst wall, the capsule. The oocyst capsule is bilayered with a thick outer wall derived by a parasite transglutaminase cross-linking mosquito-derived basement membrane components (laminin?) and an inner capsule covered with a dense coat of CSP. It is likely that the nutrients for oocyst development come from the mosquito hemolymph and in *in vitro* cultures of oocysts, exogenous *p*-aminobenzoic acid (pABA) was required (Al-Olayan *et al*., 2002).

Sporozoite differentiation is linked to microtubule organizing centers that control nuclear division and cytoskeletal organization. The first morphological evidence for sporozoite bud formation is the appearance of the IMC and the associated microtubules. Over time the sporozoite bud lengthens by simultaneous extension of the IMC and the plasma membrane and the underlying microtubules to form the triple-layered pellicle. The plasma membrane of the sporozoite is derived from the sporoblast plasma membrane whereas the IMC arises *de novo* from the Golgi apparatus. Gene disruption of PbIMC1a—involved in the synthesis of IMC proteins—resulted in abnormal sporozoite shape with a bulky protrusion (Khater *et al*., 2004). More important than such a cosmetic change was that knockouts of IMC1a lost their capacity to invade the salivary gland.

Microneme and rhoptry formation are initiated concurrently with sporozoite budding. In addition to these apical organelles associated with invasion, the emergent sporozoite contains an elongate nucleus, mitochondrion, apicoplast, endoplasmic reticulum, a Golgi apparatus, a pellicle and an apical polar ring to which the microtubules are attached. Little is known about most of the biochemical events that govern sporogenesis however it is clear CSP is involved (Kappe *et al*., 2004b). Localization of CSP to the bud sites triggers bud formation. By down-regulation of CSP levels at the time of sporozoite formation, control of sporozoite morphology was possible (Thathy *et al*., 2002). In addition, cytokinesis and sporozoite budding sites were found to be probably dependent on the putative GPI-anchor addition sequence of CSP (Wang *et al*., 2005b), and CSP knockouts, although showing normal oocyst formation, were severely impaired in sporozoite budding.

Although ultrastructural studies showed that sporozoites get through the oocyst capsule *via* small openings, the mechanisms of release are poorly known. Knockouts of the *P. berghei* cysteine protease, egress cysteine protease 1 (ECP1), resulted in sporozoites unable to exit the oocyst (Aly and Matuschewski, 2005). Failure to escape the oocyst resulted in continuous circular movements of the sporozoites that lasted several days. This suggests that sporozoite motility precedes oocyst rupture and that sporozoite motility is a pre-requisite for release from the oocyst. ECP1 is up-regulated in oocyst sporozoites and it is likely that its substrate is a constituent of the inner oocyst membrane. *P. berghei* clones with mutations in the arginine and lysine residues in the region II-plus of CSP were unable to exit the oocyst (Wang *et al*., 2005a). Mechanically released sporozoites appeared to be morphologically indistinguishable from wild type, however, they were non-infectious. It has been suggested that ECP1 acts to cleave CSP but whether the recognition site is the charged residues, or if CSP is cleaved directly, remains to be studied using purified recombinant protein.

Between 10–14 days after ingestion of an infective blood meal sporozoites leave the oocyst and enter the mosquito hemolymph. About 20% of these sporozoites reach the salivary gland most likely through passive transport; however, since they preferentially invade the median and distal lobes it suggests the presence of specific receptors. Despite their morphological similarities, oocyst and salivary gland sporozoites are different phenotypically. Salivary gland sporozoites are highly infectious, display a circular gliding motion and can elicit a strong protective immune response, whereas oocyst sporozoites have a reduced infectivity and are unable to produce protective immune responses. Profiling gene expression of these two sporozoite phenotypes, Matuschewski *et al*. (2002b) found that 30 genes were specifically turned on in infective salivary gland sporozoites. Regrettably, the signals that trigger this differential expression remain unknown. A microarray analysis identified approximately 2,000 genes that are transcriptionally active in salivary gland sporozoites (Sidjanski *et al*., 1997), including 500 that are highly expressed (Le Roch *et al*., 2003). Sporozoite expressed genes are distributed over all chromosomes and do not cluster in transcriptionally active regions save for the multi-gene families *var, rifin and stevor* (Kappe *et al*., 2004b).

There is clear evidence for the involvement of three sporozoite-expressed proteins in the invasion of the salivary glands: CSP, TRAP, MAEBL and four members of the LAP (LCCL/lectin adhesive-like protein family) (Raine *et al*., 2007) involved in sporozoite development. CSP, encoded by a single copy gene, is not found in any other apicomplexan and has been the target of many vaccine studies. CSP, is the major surface protein of the sporozoite, forming a dense coat on the parasite surface. It is presumably secreted by the apical organelles, has a signal peptide, a central domain with many amino acid repeats and a C-terminal

hydrophobic sequence. The conserved motifs are called region I, II-plus and III. Region I consists of the peptide KLKQP and is involved in the attachment of the sporozoite to a variety of host cells. The role of the repeats is unknown and the *C*-terminus encodes a GPI anchor. Region II-plus consists of an 18 amino acid sequence embedded in the thrombospondin type I repeat domain and mediates adhesion of the sporozoite to liver cell and possibly salivary gland heparan sulphate proteoglycans. CSP is involved in sporozoite localization to the mosquito salivary glands (Myung *et al*., 2004; Sidjanski *et al*., 1997). MAEBL localizes to the micronemes and/or surface and is expressed in blood-stage merozoites, late oocysts and sporozoites. Knockouts of MAEBL in *P. berghei* have sporozoites incapable of invading the salivary gland but all other stages appear normal (Kariu *et al*., 2002). Hence, MAEBL appears to be required for entry into salivary glands. MAEBL is newly expressed in salivary gland sporozoites and in a form distinct from what is present in the mid-gut sporozoites or present in erythrocytic stages. Both ligand domains (M1 and M2) were expressed as part of a full-length membrane form of MAEBL in the salivary gland sporozoites in contrast to the other stages that retain only the M2 ligand domain, as part of the membrane form of the protein. Antisera developed against the cysteine-rich regions of the extracellular portion of MAEBL inhibited sporozoite development to exo-erythrocytic forms *in vitro* (Preiser *et al*., 2004).

Photini Sinnis (1960–) went to Dartmouth Medical School (1983–1988) and took a year off between her third and fourth years when she received a Howard Hughes Research Scholarship to work at NIH. At the NIH (1986) she spent one year in the laboratory of Thomas Wellems and when she returned to medical school she did a three month medical rotation in The Gambia where she saw patients with malaria. After that she knew she wanted, in some capacity, to work on this fascinating and devastating disease. After completing a medical residency at Columbia Presbyterian Hospital in New York (1990) she did a post-doctoral fellowship in the laboratory of Victor Nussenzweig (1991–1998) where she worked on the pre-erythrocytic stages of *Plasmodium*; an area that she continues to work on as an independent investigator.

TRAP is found in the micronemes and on the surface of the sporozoite and is involved in gliding (Kappe *et al*., 2004b). TRAP contains two extracellular domains that bind to cellular receptors; the transmembrane A-domain has a metal ion-dependent adhesive site (MIDAS) able to interact with putative sporozoite receptors such as laminin and collagen

and the thrombospondin type I repeat domain most likely binds to heparin sulphate proteoglycans. In addition, there is a cytoplasmic tail of TRAP that connects to the actin–myosin motor (see below). *P. berghei* knockouts of TRAP show normal oocyst development, however, the free sporozoites are immobile and do not infect the salivary gland or liver cells (Sultan *et al*., 2001). A thrombospondin-related sporozoite protein (TRSP) that exhibits a single thrombospondin type I repeat in its putative extracellular *N*-terminal region is highly conserved among *Plasmodium* spp. Using targeted gene disruption in *P. berghei*, lack of TRSP had no effect on the asexual blood-stage cycle, parasite transmission to the mosquito, sporozoite development and infection of mosquito salivary glands. However, analysis of TRSP knockout sporozoites *in vitro* and *in vivo* indicated that this protein has a significant role in hepatocyte entry and, therefore, liver infection (Labaied *et al*., 2007a).

Sporozoite gliding is essential for invasion and is accomplished by an actin–myosin motor located in the cortical space between the plasma membrane and the outer membrane of the IMC (Kappe *et al*., 2004a). Myosin is tethered to the IMC and the actin is apposed to the plasma membrane where it interacts with the cytoplasmic tail of TRAP *via* fructose 1,6-bisphosphate aldolase (Bosch *et al*., 2007). Aldolase is located on the surface of some micronemes and in the cytoplasm of resting sporozoites. Upon sporozoite activation the TRAP-containing micronemes fuse with the sporozoite membrane carrying along aldolase and allowing access to the cortical space where the motor can be engaged. As the molecular motor pulls TRAP backwards the sporozoite glides forwards.

Aldolase, a glycolytic enzyme has been shown to have a non-glycolytic function in the invasive stages of *Plasmodium*. Indeed, a transcriptome analysis has shown that, unlike other glycolytic enzymes that are down-regulated in late schizonts and invasive merozoites, aldolase expression is increased. Because the activity of the molecular motor needs adenosine triphosphate (ATP), glycolysis is required. It has been suggested that the bridging and catalytic functions of enzyme can be mediated by different subunits of the tetrameric aldolase. Indeed it was shown that the *C*-terminus of TRAP enters the active site of aldolase and mimics the substrate (Bosch *et al*., 2007).

Six LAP genes have been identified in the *Plasmodium* genome. LAP1 has been detected in all life stages analysed. LAPs 2, 4 and 6 are highly expressed in the sexual and ookinete stages. In *P. falciparum* gametocytes LAPs 1, 2 and 4 were detected on the parasite surface, in the PV, in vesicles secreted into the PV and in the parasite cytoplasm. In *P. berghei* PbLAP1 is essential for sporozoite formation in *P. berghei* and PfLAP 1 and 4 are essential for infectivity of the salivary gland. The inheritance of PbLAP 1, 2, 4 and 6 from the female gametocyte is critical to oocyst maturation and sporozoite formation and essential for mosquito

transmission. The absence of a PbLAP function in the gametocyte to ookinete period of differentiation ultimately results in lethality (Raine *et al*., 2007).

4. JOURNEY TO THE LIVER

'Liver infection by *Plasmodium* is the first obligatory step of infection. It is also clinically silent and lasts a week in the human infection . . . allowing enough time for development of novel chemotherapeutic and immunologic interventions' (Mota and Rodriguez, 2004).

Malaria-infected female mosquitoes probe the skin of the host and deposit saliva containing vasodilators, anticoagulants and sporozoites. Mosquitoes may inject as few as 20–200 sporozoites into the skin. Sporozoites are released from the skin into the blood stream in a trickle lasting for hours after the mosquito bite (Amino *et al*., 2006; Jin *et al*., 2007; Kebaier and Vanderberg, 2006; Sidjanski *et al*., 1997; Vanderberg and Frevert, 2004; Yamauchi *et al*., 2007). Thus in contrast to the rapid loss of infectivity seen *in vitro* when sporozoites are held at 37 °C and in physiological saline there are factors in the skin that act to preserve sporozoite infectivity for hours.

After gliding along the surface of the skin cells, the sporozoites stop next to a capillary endothelial cell. These endothelial cells contain heparan sulphate proteoglycans on their basement membrane and it is presumed this is recognized by the sporozoite CSP. Recently a phospholipase (PL/UIS10) capable of damaging cell membranes was found in a subtractive library from salivary gland sporozoites (Matuschewski *et al*., 2002b) and localized to the surface of *P. berghei* sporozoites (Bhanot *et al*., 2005). Mutants lacking this enzyme show a decrease in infectivity when delivered through the bite of a mosquito but not when directly injected into the blood. Phospholipase-deficient sporozoites infect host cells with the same efficiency as wild-type parasites suggesting that this enzyme is not involved in host cell invasion but serves for migration through the skin cells and to allow sporozoites to penetrate the endothelial cell (Bhanot *et al*., 2005).

Once in the blood vessels, the sporozoites do not adhere to proteoglycans on the vascular endothelial surface until they enter a sinusoid of the liver. To infect the liver hepatocytes the parasites must cross a sinusoidal barrier composed of special endothelial cells and Kupffer cells (the resident macrophages). Kupffer cells, by virtue of their highly exposed fixed position in the sinusoidal lumen and the presence of sporozoite receptors, provide access to the space of Disse and act as a portal of entry for the sporozoite into the liver hepatocyte (Frevert *et al*., 2006). It is clear from a study where two different Kupffer cell-deficient murine models were compared, that is mice with a genetic defect in CSPF-1 expression and

clodronate-treated mice, that Kupffer cells served as a gateway to the liver (Baer *et al*., 2005, 2007b; Frevert *et al*., 2006).

The sporozoites glide along the sinusoid most likely by binding to extracellular matrix proteoglycans, which are produced by stellate cells and thought to reach from the space of Disse into the sinusoidal lumen (Frevert and Nardin, 2005; Pradel *et al*., 2002). Eventually, the parasites bind to a Kupffer cell by a dual interaction involving small proteoglycans called syndecans, and the low-density lipoprotein receptor-related protein (LRP-1). CSP binding to this receptor complex induces a signaling cascade that raises the intracellular level of cAMP and $InsP_3$ to inhibit the respiratory burst of the Kupffer cell (Usynin *et al*., 2007). By avoiding Kupffer cell activation and by blocking the assembly of the nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase in response to secondary stimuli known to induce a respiratory burst, the sporozoites are able to safely pass through these professional phagocytes and to develop subsequently in neighboring hepatocytes.

The increase in the cAMP level also modulates cytokine secretion in murine Kupffer cells towards an overall anti-inflammatory profile (Frevert, personal communication). Stimulation of *P. yoelii* sporozoite-exposed Kupffer cells with LPS or interferon (IFN)-γ revealed a down-modulation tumor necrosis factor (TNF)-α, interleukin (IL)-6 and MCP-1 and up-regulation of IL-10. The shift of the cytokine profile required parasite viability and contact with Kupffer cells, but not invasion. Further, Kupffer cells purified from *P. yoelii*-infected mice responded in a corresponding manner to subsequent *in vitro* stimulation with LPS. Although sporozoite-exposed Kupffer cells exhibited signs of apoptotic death such as membrane blebbing and nuclear condensation and fragmentation, sporozoites remained intact and appeared to develop to early exo-erythrocytic (EE) forms in Kupffer cell cultures. Steers *et al*. (2005) found that sporozoites down-modulate expression of major histocompatibility complex (MHC) class I molecules and up-regulate the production of the anti-inflammatory cytokine IL-10 in murine Kupffer cells. The data indicate that sporozoites possess mechanisms to render Kupffer cells insensitive to pro-inflammatory stimuli and eventually are able to eliminate these macrophages by forcing them into programmed cell death. In addition, in the sporozoite the PEXEL motif is used to introduce CSP into the hepatocyte cytoplasm and a nuclear signal allows CSP entry into the host cell nucleus where it out competes with nuclear factor-kappa B (NFkB) nuclear import (Singh *et al*., 2007). The result: down-regulation in the expression of many genes controlled by NFkB, including those involved in inflammation. Thus, the presence of CSP in the hepatocyte acts to enhance parasite growth and development.

Sporozoites remain infectious to hepatocytes for at least 2 h *in vitro* when co-cultured with Kupffer cells and continue to invade these

macrophages for at least 6 h *in vitro* (Pradel and Frevert, 2001). Why this roundabout way of infecting the sporozoite target cell, the hepatocyte? Frevert *et al*. (2006) speculate that '*Plasmodium* spp. infecting mammals have evolved to gain access to a nutritionally richer and conceivably safer environment, hidden behind a sinusoidal barrier and shielded from immune cells that continuously patrol the liver and that they access that niche using macrophages of the mononuclear macrophages as do the evolutionarily related avian and reptilian species that carry out much of their EE development in Kupffer cells and other macrophages'.

In *P. gallinaceum* infections of the chicken it was found that in the liver, the parasites exclusively infect Kupffer cells and remained small, while infected brains contained large EE stages in capillary endothelia (Frevert *et al*., 2007). Additionally, EE stages were found in macrophages and endothelia from other major organs as well. Livers exhibited focal hepatocyte necrosis, Kupffer cell hyperplasia, stellate cell proliferation, inflammatory cell infiltration and granuloma formation, histopathological changes strikingly similar to mammalian species. These data support Frevert's hypothesis that in the course of evolution, the malaria parasite has shifted its EE development cycle from macrophages and endothelia to hepatocytes, but still uses Kupffer cells as a port of entry into the liver.

The parents of **Victor Nussenzweig (1928–)** came to Sao Paulo, Brazil, from Poland in the 1920s as poor immigrants. His father started as a peddler carrying the merchandise on his shoulders and his mother was a housewife who kept a kosher kitchen and took care of the children's education. Victor and his two brothers went to public schools from kindergarten through graduate school.

Victor entered medical school at the University of Sao Paulo (Sao Paulo, Brazil) in 1946 just after the end of the World War II. Like many of his friends, he was impressed with the important role of the Soviet Union in the defeat of Nazi Germany, and was attracted to socialist ideals and Marxist ideology. During the first three years of medical school he was deeply involved in student politics. His future wife, Ruth, was in the same class, and when the two started dating she convinced Victor that science was much more interesting than politics. Since then, their collaboration has been long lasting and fruitful in every sense of the word.

Victor and Ruth's first project was an attempt to reproduce the findings of two Russian scientists, Ruskin and Klueva, who claimed that the extracts of *Trypanosoma cruzi* inhibited the growth of tumors in mice. The chairman of the Department of Parasitology at the Medical School, Samuel Pessoa, provided space, a microscope and a small desk centrifuge although he must have realized that the project was

doomed to failure. Indeed, it was soon found that the tumors that grew in outbred mice were not affected by cruzi extracts. Nevertheless, during these experiments Victor and Ruth fell in love with the wiggling blood stages of the trypanosome that had been isolated from a patient with chronic Chagas's disease.

Soon afterwards, another problem attracted their attention, the possibility of transmission of Chagas disease by blood transfusion. Impressed by Paul de Kruiff's wonderful book 'Microbe Hunters', Ruth and Victor tried to sterilize the blood destined for transfusion by incubating it with Gentian Violet, a dye that had been used to treat *Strongyloides stercoralis* infestations in humans. Once success was achieved, the thrill of the daily experimentation sealed their decision to pursue research careers.

After receiving doctor of medicine (MD) degrees, Ruth and Victor went to Paris with their two young children (1958–1960). Ruth worked at the Collège de France (Paris, France) studying the metabolism of thyroid hormones, and Victor was accepted in the laboratory of Professor Pierre Grabar at the Institute Pasteur (Paris, France) working on antigen processing. He discovered that fragmentation of fibrinogen by plasmin generated the peptides, called C and D, with entirely different antigenic determinants. This was a quite a surprise to Elvin Kabat, a prominent immunologist who at the time was at the Pasteur on a sabbatical leave, and who insisted that proteins were like carbohydrates and had repetitive identical epitopes.

Back in Brazil, Ruth and Victor worked with a prominent Brazilian immunologist, Otto Bier. In 1963, Victor received a Guggenheim Memorial Foundation Fellowship and went to the United States with Ruth and their three children, Michel, Andre and Sonia. Ruth worked with Zoltan Ovary and Victor with Baruj Benacerraf. It was an exciting time, when the structure of immunoglobulins was being solved. Although they tried to go back to Brazil in April of 1964, they were deterred because the military had just overthrown the elected government and started a witch-hunt at the University of Sao Paulo. Therefore, they returned to New York in October. Ruth was appointed assistant professor in the Division of Parasitology at New York University (NYU, New York) Medical School and started working in malaria pre-erythrocytic vaccines. Within a few years, she was appointed chairwoman of a newly created Department of Medical and Molecular Parasitology.

Since 1964, Victor has been in the Department of Pathology of NYU Medical School. Early interests were on the complement system where his main contributions were the discovery of complement receptors in leucocytes, the studies on control of activation of the cascade by C4 binding protein (C4BP) and decay accelerating factor (DAF), and the

demonstration that complement had a central role in the clearance and metabolism of immune complexes.

Again, Ruth changed Victor's career when she discovered in 1967 that mice could be completely protected against malaria infection by immunization with irradiated sporozoites. Their joint quest for the 'protective' antigen led to the discovery of the circumsporozoite surface (CSP) protein, its function in the initial stages of invasion of hepatocytes, the potent inhibitory role of interferon gamma in the development of the liver stages, and to studies demonstrating that a CSP-based human vaccine against *P. falciparum* was possible.

Ruth and Victor have been fortunate in having numerous superb co-workers as well as many talented scientists with whom it has been indeed a privilege to work with who now pursue successfully their own careers all over the world.

When exiting the Kupffer cells the sporozoites enter the space of Disse where they have direct access to hepatocytes. After traversing several hepatocytes, fatally wounding them in the process, the sporozoite invades a hepatocyte where it is enclosed by a PVM (Frevert and Nardin, 2005; Mota and Rodríguez, 2002). A micronemal protein SPECT2, a perforin-like protein is essential for cell passage, but not hepatocyte invasion (Yuda and Ishino, 2004). Knockouts of SPECT2 lack the ability to enter the intact liver. In addition infection by *P. falciparum* and *P. yoelii* sporozoites has been shown to be dependent on CD81 and cholesterol-dependent tetraspanin-enriched domains on the hepatocyte surface (Silvie *et al.*, 2003, 2006, 2007) and, antibodies against CD81 block *P. falciparum* infections in human hepatocytes *in vitro*. How CD81 functions in sporozoite invasion remains unknown but it appears not to act as a receptor (Silvie *et al.*, 2003). To complicate matters further *P. berghei* sporozoites, unlike *P. falciparum* (NF54 line) and *P. yoelii*, use pathways for the invasion of HepG2, HuH7 and HeLa cells without involvement of cholesterol or CD81 whereas both CD81 and cholesterol were required for infection of mouse hepatoma cells. Whether CD81 and cholesterol or alternate invasion pathways are involved in *P. berghei* sporozoites invading hepatocytes of their natural host, the African tree rats, *Grammomys*, remains to be determined (Silvie *et al.*, 2007).

Ute Frevert (1951–) received her early education in Germany. After gymnasium (high school), she took a 2-year training course at a school for technical assistants and took the exam to become a radiation and laboratory medicine technician. After that, she went to veterinary school and graduated from the School of Veterinary Medicine at the Freie Universität Berlin, Berlin, Germany (1981). In 1982, she

completed her PhD at the Department of Pathology, School of Medicine, Freie Universität Berlin. Under the direction of Professor Sigurd Blümcke she completed her dissertation '*Electron microscopic examination of fetal lung development in vitro*.' and, in 1991, defended her habilitation at the Institute of Veterinary Biochemistry, School of Veterinary Medicine, Freie Universität, Berlin, Germany, with a thesis '*Trypanosoma congolense*: functions of the variant antigens and cellular transport processes.' In 1991, during a sabbatical in the laboratory of Victor Nussenzweig she contributed to a paper on the recognition of the liver by the circumsporozoite protein and was hooked to malaria. When Ruth Nussenzweig offered her a faculty position in her department, she gladly accepted.

Since then, her focus has been on the cell biology of malaria in the mammalian liver. Her interests include sporozoite targeting to the liver, molecular communication between sporozoites and Kupffer cells, EE development in hepatocytes, mode of release and fate in the body of hepatic merozoites, and the comparative biology of the exo-erythrocytic phase of mammalian and avian malaria infections. Establishment of the methodology to observe fluorescent parasites in various organs of live animals has provided a window into the complex life cycle of *Plasmodium* in the mammalian host.

Why do sporozoites not invade the first hepatocyte they encounter and instead wound several before settling down? Mota and Rodriguez (2004) hypothesize that hepatocyte traversal activates sporozoites for infection and increases the susceptibility of host hepatocytes. Mota and Rodriguez (2002) have shown that sporozoite secretions are induced by migration through cells and sporozoites can also be activated by incubation with Ca^{2+} and host lysates; when this was done sporozoite infectivity was increased. Wounding of host cells, they claim, induces secretions of host cell factors such as hepatocyte growth factor, which renders hepatocytes more suitable for the early development of the parasite by inhibiting apoptosis but do not affect invasion per se. In contrast, Kumar *et al*. (2007) showed that in their travel from the skin to the liver the sporozoites are exposed to shifts in K^+ concentrations; when *P. berghei* and *P. yoelii* sporozoites were exposed to intracellular concentrations of K^+ (i.e. 142 mM) infectivity for hepatocytes was increased eight to 10-fold and four to five fold, respectively. Enhanced infectivity was time dependent, increased between 15–35 min of incubation, was independent of SPECT and enhanced transcription seemed not to be involved.

Within the PV the parasite spends a significant period of its intracellular residence (2–3 days in rodent malarias and 5–7 days in human malarias) de-differentiating into a trophozoite. Interestingly, this transformation of

sporozoites can occur without host cells if they are exposed to 37 °C and serum *in vitro* (Kaiser *et al*., 2003). However, completion of this transformation and further growth does requires a host cell and for this a PV is critical. The PV is largely accessible to exogenous molecules suggesting that there are open pores present in the PVM. It is assumed that these pores allow for the transport of low-molecular-weight nutrients, growth-regulating molecules, signaling compounds from the hepatocyte into the parasite, as well as removal of parasite waste products. The PVM is cholesterol enriched and this is likely derived from the host membrane components because *Plasmodium* cannot synthesize sterols *de novo* (see p. 218). Since only host endoplasmic reticulum (and not mitochondria or Golgi) are concentrated around the PV enclosing *P. berghei* it suggests that the host cell endoplasmic reticulum may play a role in providing lipids (Bano *et al*., 2007). It is not known whether sporozoites are able to modify the porosity of the PVM.

Two parasite proteins UIS3 (up-regulated in infectious sporozoites 3) and UIS4 have been shown to be essential for development of *P. berghei* in the liver. UIS4, a small integral protein, is expressed in the micronemes, released after hepatocyte invasion and localizes to the PVM throughout development (Meis and Verhave, 1988; Mikolajczak and Kappe, 2006). UIS3 is likely to be an integral protein of the PVM. Since it binds to liver fatty acid (FA)-binding protein it is tempting to suggest that UIS3 may play a role in the acquisition of FAs from the hepatocyte and contribute to the rapid (2–7 day) and enormous growth seen in liver stages (Mikolajczak *et al*., 2007). Sporozoites containing a knockout of UIS4 invade hepatocytes, form a PV, transform into trophozoites but show growth defects in the cultured hepatoma cells and in mouse livers. Similar growth deficiencies occur with UIS3 knockouts. Despite an absence of a precise function for these two liver-stage growth proteins they have been used to develop an attenuated vaccine, that in mice immunized with UIS3 or UIS4 knockout sporozoites were completely protected against infection with wild-type parasites (Mueller *et al*., 2005; Jobe *et al*., 2007; Tarun *et al*., 2007). Other sporozoite proteins (P52) described in *P. yoelii* and *P. falciparum* (and P36 in *P. berghei*) as a GPI-anchored protein may also be involved in hepatocyte invasion. P52/P36 knockout sporozoites were able to invade hepatocytes but unlike UIS3 and UIS4 did not form a PVM and were arrested in growth similar to but more severe than radiation attenuated sporozoites; these also conferred protection against sporozoite challenge (Douradinha *et al*., 2007; Labaied *et al*., 2007b; van Dijk *et al*., 2005) and may serve as the basis for a protective vaccine. However, important challenges remain (Frevert and Nardin, 2005). What will be the longevity of immunity? Can sterile parasites free of mosquito debris be prepared and will there be efficient methods for cryopreservation of the sporozoites? What will be the route of immunization and how many times will an individual have to be vaccinated?

One of the enigmas associated with the EE stages has been: How do the hepatic merozoites reach the blood? Recent studies show how this may be accomplished and how phagocytosis by macrophages might be avoided. *In vitro*, *P. berghei* were shown to induce death and detachment of the hepatocytes followed by budding of parasite-filled vesicles (called merosomes) into the lumen of the sinusoid (Sturm and Heussler, 2007; Sturm *et al*., 2006). Simultaneously the parasites actively accumulate intracellular Ca^{2+} and this keeps its level low in the host cell and inhibits the exposure of phosphatidylserine (PS) on the outer leaflet of the hepatocyte plasma membrane so long as the parasites are viable. In this way 'eat me' signals are not provided and the migration and protection of the merosomes is ensured. The *in vivo* studies by Tarun *et al*. (2006) and Baer *et al*. (2007a) conducted using fluorescent *P. yoelii* in mice confirm the work of Sturm *et al*. Baer *et al*. also demonstrated that the majority of merosomes exit the liver intact, adopt a relatively uniform size, survive the passage through the heart undamaged and accumulate in the lungs. Merosomes were absent from blood harvested from the left ventricle and from tail vein blood, indicating that the lungs effectively cleared the blood from all large parasite aggregates. PS was not exposed on the merosome membrane suggesting the infected hepatocyte did not undergo apoptosis prior to merosome release. *Ex vivo* analysis showed that merosomes eventually disintegrate inside pulmonary capillaries thus liberating merozoites into the blood stream. Thus, merosome packaging protects hepatic merozoites from phagocytic attack by sinusoidal Kupffer cells and release into the lung microvasculature enhances the chance of successful erythrocyte invasion. This previously unknown part of the plasmodial life cycle is thought to ensure an effective transition from the liver to the blood phase of the malaria infection.

Although rodent malarias may serve as convenient surrogates for human malarias it is worthwhile emphasizing that they do have their limitations. Findings in model rodent malarias will ultimately need confirmation in human systems. Genome wide expression and proteomic studies of EE developmental stages may reveal unique genes however this will require the efficient isolation of early and late stages—something yet to be achieved.

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