A Passion for Parasites

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I knew nothing and had thought nothing about parasites until 1971. In fact, if you had asked me before then, I might have commented that parasites were rather disgusting. I had been at the Johns Hopkins School of Medicine for three years, and I was on the lookout for a new project. In 1971, I came across a paper in the *Journal of Molecular Biology* by Larry Simpson, a classmate of mine in graduate school. Larry’s paper described a remarkable DNA structure known as kinetoplast DNA (kDNA), isolated from a parasite. kDNA, the mitochondrial genome of trypanosomatids, is a DNA network composed of several thousand interlocked DNA rings. Almost nothing was known about it. I was looking for a project on DNA replication, and I wanted it to be both challenging and important. I had no doubt that working with kDNA would be a challenge, as I would be exploring uncharted territory. I was also sure that the project would be important when I learned that parasites with kDNA threaten huge populations in underdeveloped tropical countries. Looking again at Larry’s paper, I found the electron micrographs of the kDNA networks to be rather beautiful. I decided to take a chance on kDNA. Little did I know then that I would devote the next forty years of my life to studying kDNA replication.

I arrived at Johns Hopkins Medical School in August 1968 as an assistant professor. I had obtained a Ph.D. degree in biochemistry with Lyman Craig at Rockefeller University and done my postdoctoral research with Arthur Kornberg at Stanford University. I had tremendous momentum to begin experiments as soon as I arrived at Hopkins, but I felt like I was running into a brick wall. My laboratory was filled with old and broken-down equipment, and there were no startup funds in those days. My chair, Al Lehninger, told me that I could get $10,000 from the institutional research grant committee. I had already written a National Institutes of Health (NIH) grant application, which was funded for three years with $28,800/year in direct costs. Despite what seemed like a slow start, in a few months, I was up and running. I had attracted a couple of excellent graduate students (Paul Weigel and Mark Challberg), and in those early days, we published papers on *Escherichia coli* DNA replication, DNA polymerases, and a method for identifying short sequences at the 3’ termini of DNA. This was enough to get my NIH grant renewed, but it bothered me that these projects were too much like what I had done at Stanford. I was looking for something new, something I could call my own.

Learning about kDNA

Since Al Lehninger was a leader in the field of mitochondria and bioenergetics, he had recruited several faculty members in the department who studied mitochondria in various systems, so I thought I was in a good place to study mitochondrial DNA replication. I spent a few months working on chicken liver mitochondrial DNA, but I got nowhere. Therefore, I decided that I should work on a microorganism that could provide enough material to do the biochemical studies that I favored. My turning point came in 1971 when I saw a paper in *Journal of Molecular Biology* by Larry Simpson and his soon-to-be wife, Agda (1). Larry and I had been graduate students together at Rockefeller, and he started at UCLA at the same time that I started at Hopkins. I knew that he had been working on parasites in Bill Trager’s laboratory at Rockefeller, but I had no
idea what he had studied. In his Journal of Molecular Biology paper, I learned for the first time that he was working on kinetoplast DNA (kDNA), a subject unfamiliar to me, but from this paper, I learned that kDNA was a remarkable mitochondrial genome from the parasite *Leishmania tarentolae*, whose normal host is a lizard. (Other species of *Leishmania*, such as *Leishmania donovani*, infect humans and cause serious diseases.) *Leishmania*, trypanosomes, and their relatives are classified as trypanosomatids, and all have kDNA. kDNA is in the form of a planar network containing several thousand interlocked DNA rings called minicircles (0.85 kb in *L. tarentolae*) that are heterogeneous in sequence. The network also contains several dozen interlocked maxicircles. Discovered by Piet Borst in Amsterdam, maxicircles range from 20 to 40 kb in different species (2). Networks look amazing when viewed by EM (Fig. 1). The giant network, spread out as in shown in Fig. 1, is comparable in size to the entire cell, but when the network is inside the matrix of the cell’s single mitochondrion, it is condensed into a highly organized disk-shaped structure (Fig. 2A).

I was excited by Larry’s paper, and over the next few weeks, I decided that I would tackle the replication mechanism of the kDNA network. At that time, I had no idea how this replication process could occur, but it seemed likely that its study would reveal something new about DNA replication in general. Also, because there are no DNA networks in human cells, there was the possibility that kDNA and its replication machinery could be potential drug targets for human diseases caused by trypanosomatids (sleeping sickness, Chagas disease, leishmaniasis). There were many other fascinating questions that entered my mind: why is kDNA in the form of a network? What does the network do for the cell? Are its gene products similar to those of more conventional mitochondria? However, I decided to limit our studies to replication.

**Brief Summary of the Function of kDNA**

The information in this paragraph had not been discovered when I started working on kDNA. I present it here because knowing it in advance may facilitate the reader’s understanding of some of our findings or at least explain why kDNA is a network. kDNA maxicircles have a function similar to mitochondrial DNAs in higher organisms. As in yeast and mammals, they encode ribosomal RNAs and proteins involved in energy transduction, such as subunits of NADH dehydrogenase, cytochrome oxidase, and ATP synthase. What is unique about trypanosomes is their mechanism of mitochondrial gene expression. The
maxicircle transcripts are cryptic and need to be edited before they can be used as mRNAs. Editing involves addition or deletion of uridylyl residues at internal sites in the sequence, thus creating an open reading frame. In some mRNAs, 50% of the sequence consists of uridylyl residues incorporated by editing. In others, editing is quantitatively much less (e.g. AG edited to AUG, creating a start codon). The templates for editing are minicircle-encoded guide RNAs that bind to the maxicircle transcripts by base pair-
ing (including GU base pairs). Editing occurs in a multienzyme pathway involving an editing endonuclease, uridylylate exonuclease (for U deletion), terminal uridylylate transferase (for U addition), and RNA ligase. Editing, which starts at the 3'-end of the transcript, often creates a binding site for the next guide RNA. Why do the minicircles and maxicircles have to be linked to the network? Minicircles are heterogeneous, and different minicircles are required for editing different maxicircle transcripts. If a critical class of minicircles is lost, the cell will die. Being organized into a network may preserve the minicircle repertoire by a mechanism that will be discussed (3). This bizarre and cumbersome mechanism of gene expression, abandoned by later branching organisms, contributes to one’s disbelief in intelligent design.

How I Became a Parasitologist

Before I tell the story of our findings on kDNA replication, I want to describe how I expanded my knowledge about trypanosomes and other parasites. I had never had a course in parasitology, and in my early years working on kDNA, I never even went to a parasitology meeting. I did attend meetings on mitochondrial DNA and met a few other scientists working on kDNA. I was very fortunate to have the opportunity (via a Fogarty fellowship) to spend the summer of 1980 in Nairobi, Kenya, at the International Laboratory for Research on Animal Diseases (ILRAD). This well funded laboratory had groups working on two parasitic diseases of cattle, bovine trypanosomiasis and East Coast fever. I joined a strong group (headed by Dick Williams) working on the molecular biology of antigenic variation in Trypanosoma brucei, a very popular research subject at that time. There was one other visiting American scientist working in our group, John Donelson (University of Iowa), who was also starting a career in trypanosome biology. I learned a lot at ILRAD, but I still knew little about the wider field of parasitology, such as the biology of Plasmodium (the malaria parasite), Giardia, or Toxoplasma. I had heard that there was a summer course in modern parasitology at the Marine Biological Laboratory (MBL) in Woods Hole, Massachusetts, and I had even considered taking this course myself. Needless to say, I was very pleased when, in the summer of 1984, I was invited to lecture on kDNA replication in that course. On the evening after my lecture, John David, a professor at the Harvard School of Public Health and the founding director of this course about four years earlier, asked me to dinner at his home. After dinner, he amazed me by inviting me to be co-director of the course starting the following summer. My immediate response was, “But I don’t know anything
about parasites other than a few of those that have kDNA.” His unforgettable response was, “Well then, this will be a good opportunity for you to learn.” John had offered the other job as co-director to Alan Sher, an immunologist at NIH who studied parasites. I had met Alan when he presented a seminar at the Johns Hopkins School of Public Health, but I did not know him very well. We arranged to meet at a restaurant in Columbia, Maryland, exactly halfway between NIH and Hopkins. Finding that we were compatible, we decided to accept John’s offer to be course co-directors. We spent most of the evening discussing how we would run the course that lasted an intense ten weeks. There were morning lectures mostly by visiting world experts on every significant parasite known to cause human disease, and there were labs beginning after lunch and running until the early hours of the morning. Alan and I directed this course from 1985 until 1988. It was in Woods Hole that I first learned about the biology of the most important parasites, and I met scientists from around the world who were studying them. It was during these years that I finally began to feel like an authentic parasitologist (of the molecular type) and to be viewed as one by others in the field. This was not the first time that Woods Hole so influenced my career. It was in this same scientific paradise that I first decided to become a scientist. In the summer of 1959, after my junior year in college, I took the invertebrate zoology course at MBL; funds to take this course had been awarded to me for getting the highest grade in embryology the previous year. Although that Woods Hole course was totally non-molecular, it inspired me to change the direction of my future and apply to graduate school rather than medical school, as I had planned to do for years. My parents were somewhat taken aback by this decision, but within a few years, they were very proud. Now back to the story of kDNA replication.

kDNA Network Replication

We showed that DNA networks in log-phase Crithidia fasciculata, a parasite of mosquitoes, could be resolved into three species when centrifuged to equilibrium on a CsCl gradient containing ethidium bromide (4). Sherry Price Challberg (a graduate student in my laboratory) and Dan DiMaio (a medical student doing a summer rotation) had made similar findings in earlier studies on L. tarentolae kDNA (5). However, because of the intrinsic fragility and ease of fragmentation of L. tarentolae networks, only limited conclusions could be drawn from their experiments. We therefore decided to switch to C. fasciculata, with its rock-solid networks. Moreover, this parasite can be grown to high density, in large quantities (150 liters in a fermenter), and in cheap medium, ideal for biochemical experiments. (Of note, Larry Simpson (Fig. 3) had found a wealth of parasites resembling Crithidia in mosquitoes collected near his backyard hot tub in Southern California.)

Form I networks, which band low in the gradient, exclusively contain covalently closed minicircles. Form II networks, which band higher in the gradient, contain only nicked/gapped minicircles, and the in-between networks contain a mixture of both. Pulse labeling a culture with [3H]thymidine labeled only the in-between networks, indicating that they are undergoing replication. During a chase, the radioactivity moved first into Form II networks and then into Form I networks. We measured the molecular weight of each network form, and from these values, we calculated that Form I networks contain ~5000 minicircles, Form II networks have double that number, and replicating networks have intermediate numbers of minicircles. Remarkably, the nicked/gapped minicircles were not localized randomly on the replicating network. Instead, they formed a ring around the network periphery. The ring thickened as replication proceeded. These studies indicated that Form I networks were pre-replication and unit-sized and that Form II networks, being double-sized, were post-replication. Our evolving replication model strongly suggested that there must be a mechanism for cleaving a Form II network into two Form I networks (4).

Our excitement over these results stimulated our desire to learn more about how individual minicircles replicate. Suddenly, at about ten o’clock one evening, an idea came to me. I stayed up the rest of the night thinking about how my idea fit in with the available data. By dawn, I was convinced that I had the answer, and I rushed off to the lab to plan experiments. My idea, which turned out to be correct, was that minicircles do not replicate while attached to the network. Instead, they are released (decatenated) from the network and undergo replication as free molecules, like any plasmid. The two progeny free minicircles then reattach (recatenate) to the network, causing it to grow in size (6). This model avoided all the potential topological problems that would have occurred if replication occurred on minicircles linked to the network. To test this idea, we pulse-labeled cells with [3H]thymidine, and a radioactive peak appeared in a sucrose gradient whose sedimentation velocity was the same as that of a free minicircle. The networks and nuclear DNA fragments sedimented much faster. During a chase, the radioactive peak disappeared, consistent with network reattachment. These and other data (e.g. all of the minicircle θ-structures observed by EM
are free) provided strong evidence that free minicircles are replication intermediates (6). The concept of free minicircles also explained some beautiful data Larry Simpson had published. Using light microscope autoradiography, Larry reported in 1976 that networks from *C. fasciculata* cells pulse-labeled with [³H]thymidine for 3 min were labeled at only two peripheral sites, 180° apart (Fig. 4A, panel a) (7). Cells pulse-labeled with the same precursor for 6–10 min had the label distributed in a ring around the network periphery (Fig. 4A, panel b). In a pulse-chase experiment, he found that the ring remained intact but moved into the network interior (Fig. 4A, panel c). Larry had suggested that the silver grains at the network periphery marked sites of DNA synthesis, but we thought it more likely that they were sites of free minicircle attachment (especially because in the pulse-chase experiments, non-radioactive free minicircles would attach outside the ring of labeled minicircles, causing the apparent inward movement) (8).

We now know that free minicircles reattach adjacent to the antipodal sites, which are two protein assemblies situated on the network periphery, 180° apart. Dan Ray (a professor at UCLA) had the first look at these structures when he demonstrated by immunofluorescence that topoisomerase II (topo II) had a similar localization (9). (Dan had previously worked on replication of single-stranded DNA phages, such as M13, but now had turned his full attention to kDNA replication.) Subsequently, many other replication enzymes and free minicircles were localized to the antipodal sites.

Meanwhile, Larry Simpson had gone on to do wonderful work on the molecular biology of *L. tarentolae* mitochondria. In 1978, he reported that the major maxicircle transcripts were 9 S and 12 S species (10), which later turned out to be the smallest ribosomal RNAs known. He mapped these and other maxicircle transcripts. In 1990, Larry published four *Cell* papers, two of which essentially solved the mechanism of RNA editing. I do not believe that any of his competitors were even close to this understanding because they lacked Larry’s skill in computer analysis of nucleic acid sequences. The first of these papers reported the discovery of guide RNAs that serve as templates for uridylicate insertion and deletion. This paper focused on the handful of guide RNAs encoded by maxicircles (11). The second paper reported that minicircles encode most of the guide RNAs encoded by maxicircles (12). With this discovery, we finally learned the function of all of the components of kDNA and that gene expression in trypanosomatid...
mitochondria is dependent on both maxicircles and minicircles. In a related paper, Larry showed that there was an oligo(U) sequence added enzymatically to the 3′ terminus of guide RNAs (13). This tail stabilizes the interaction of the guide RNA with the maxicircle transcript by base pairing. The fourth Cell paper examined the structure of partially edited molecules (14). Subsequently, Larry continued to be a major player in the study of enzymes involved in RNA editing and the complexes formed by these enzymes. He has also published on the evolution of editing (e.g. Ref. 15), and he collaborated with a group to use cryo-EM to determine the structure of L. tarentolae mitochondrial ribosomes (16).

Back to kDNA replication. Steve Hajduk (a postdoctoral fellow in my laboratory) surprisingly found that maxicircles replicate while attached to the network (17). Later, Laura Rocco Carpenter (my graduate student) confirmed that result (18). She also mapped the maxicircle replication origin and showed that, as in minicircles, maxicircle replication is unidirectional.

There is one major dilemma in the model of free minicircle replication: how does network replication progress from the images shown in panel a of Fig. 4A (which show two peripheral zones of labeling 180° apart), to the networks shown in panel b (in which there is a ring of labeling around the whole periphery)? Given that the kinetoplast disk is flanked by two antipodal sites, the only two possibilities I could think of were that 1) the kinetoplast rotated around the vertical axis between fixed antipodal sites (in Fig. 2A, the vertical axis is shown by the vertical bar through the middle of the disk), or 2) the antipodal sites, while remaining 180° apart, moved around the fixed kinetoplast. David Pérez-Morga (a postdoctoral fellow in my laboratory) took up the challenge using an experiment similar to Larry’s, except he analyzed his autoradiograms by EM (Fig. 4B) instead of light microscopy (19). Although use of EM requires much longer exposures, its superior magnification and resolution allow visualization of hundreds of silver grains in comparison with light microscopy, in which only a few dozen grains can be detected. In his experiment, David added radioactive thymidine with very high specific radioactivity to the culture. As it entered the cell and was converted to dTTP, the specific radioactivity of the total pool of dTTP rose with time. Evidence for this rising specific radioactivity was provided by the fact that it took eleven weeks to expose the images in panels a and b of Fig. 4B, whereas it took only 48 h to expose the images in panels c and d. These four images show labeling of kDNA networks in the same experiment, differing only in times of labeling. Note that there is a gradient of density of silver grains on each side of the network in Fig. 4B (panel b). This increase in silver grain density in the 6-min labeled network is due to the rising specific radioactivity of dTTP. David was very lucky in choosing 6-min labeling; if he had gone to 10 min, the gradients might have been obscured.

FIGURE 4. Autoradiography of isolated C. fasciculata kDNA networks that had been metabolically pulse-labeled with [3H]thymidine and then coated with photographic emulsion. A, light microscopy autoradiography of Giemsa-stained networks pulse-labeled for 3 min (panel a), 10 min (panel b), and 10 min followed by a chase with nonradioactive thymidine (panel c). These figures were previously published by Larry Simpson (7, 8). B, EM autoradiography of similar metabolically pulse-labeled networks as in A. The grids were coated with emulsion and developed after eleven weeks (panels a and b) and 40 h (panels c and d). Labeling times were 3 min (panel a), 6 min (panel b), 30 min (panel c), and 60 min (panel d). The emulsion coating on the grid reduced the visibility of the DNA. All networks were from a single labeling experiment. Scale bars = 3 μm. This figure is from Ref. 19.
Because densities are approximately equivalent on opposite sides of the network, this implies a relative rotation between the kDNA and the two antipodal sites where the minicircles attach. We do not yet know whether it is a rotating kinetoplast disk or moving antipodal sites. These data are consistent with both possibilities, although some observations in Ref. 19 slightly favor a rotating kinetoplast. Intuitively, it would seem with both possibilities, although some observations in Ref. 19 attach. We do not yet know whether it is a rotating kinetoplast model.

To ensure that the minicircle repertoire remains complete, it is essential that each minicircle replicates once and only once during a cell cycle. The cell uses an accounting system to distinguish between the unreplicated minicircles and replication progeny. The former are covalently closed; the latter are nicked/gapped and remain so until all minicircles in the network have replicated. It is only after replication is complete (with no more covalently closed minicircles on the now double-sized Form II network) that gap repair and network division take place. The final result is two unit-sized Form I networks that segregate into the two sister cells (see Fig. 2A).

The two papers that I published on network and minicircle replication (4, 6) were the last ones for which I did all the experiments myself and was the sole author. Up to this time, I did my experiments, and the students in my laboratory did theirs, but I got pleasure mainly from my own experiments. After these two papers, I quickly learned how to obtain satisfaction vicariously from the work of graduate students. This was an easy transition for me because I have had some very smart and productive graduate students. After these two papers, I quickly learned how to obtain satisfaction vicariously from the work of graduate students. This was an easy transition for me because I have had some very smart and productive graduate students (see Fig. 5 for a photograph of the Englund laboratory in 1997).

One apparent weakness in the free minicircle model was that there was no precedent for an enzyme that could easily remove and reattach minicircles. Our paper was published about a year before the discovery of topo II, which would ultimately become the obvious choice for catalyzing release and reattachment (20), but before topo II was discovered, there was no simple way to conduct these reactions. One extremely cumbersome possibility that I considered but never mentioned in a publication was that it might be something like the λ-terminase system, involving linearization and recircularization via sticky ends. While I was struggling with this dilemma, by an uncanny stroke of good luck, Leroy Liu, who had discovered T4 topo II only a few months earlier, was being recruited by my department, now chaired by Dan Lane. Leroy generously gave me some of his precious enzyme, and the experiment that followed was striking. For the first time, we were able to observe decatenation of kDNA networks to monomeric minicircles and maxicircles. Joan Marini (an M.D.-Ph.D. student) and Kathy Miller (a graduate student) ran some gels and prepared a few electron micrographs, and, within a week, we had written a paper that we sent to JBC (21). This paper was the only one ever published by my laboratory that was accepted after so little work and in which every experiment was successful. Decatenation of C. fasciculata kDNA has since become a widely used assay for topo II activity. For this purpose, TopoGEN sells kDNA at $199 for 25 μg, but it is easy to isolate yourself (22). C. fasciculata cells are lysed by SDS-proteinase K. Because of their massive size and compact shape, kDNA networks can be separated from nuclear DNA fragments by sedimentation through a sucrose cushion. From a 1-liter culture, the yield is generally 85–250 μg of kDNA networks.

During the next few years, we worked out many details of the mechanism of kDNA minicircle replication. James Ntambi (a graduate student) mapped the replication origin and, together with Terry Shapiro (an M.D.-Ph.D. doing postdoctoral research; now a professor of pharmacology at Hopkins) and Kathy Ryan (a graduate student), discovered a remnant of an RNA primer on the leading strand (23, 24). Kathy demonstrated that replication was unidirectional (25) and, with Terry, discovered a knotted free minicircle (its role in replication, if any, is not yet known) (26). Pete Kitchin (a postdoctoral fellow) showed that Okazaki fragments are not joined until replication of the minicircle is complete (27). Terry evaluated the effect of drugs on targeting topo II (28). In collaboration with Nick Cozzarelli (Nick and I were postdoctoral fellows together at Stanford), Carol Rauch (an M.D.-Ph.D. student) studied the topology of kDNA and how it changes during replication (29–31). At about the same time, some students in Dan Ray’s laboratory independently performed similar experiments, and they reached similar conclusions.

**Studies on T. brucei**

After about 2000, as segments of the T. brucei genome sequence emerged, we began to direct more of our attention to this parasite. Although T. brucei may lack some of the conveniences of C. fasciculata, such as simple large-
scale growth, we did purify a *T. brucei* enzyme to homo-
genosity (32). Furthermore, knowledge of the genome
sequence (the complete sequence was published in 2005),
as well as certain genetic techniques (e.g. gene knock-outs
and RNAi), made *T. brucei* a more favorable object of
study. *T. brucei* kDNA replication differs in one major
way compared with *C. fasciculata*. *T. brucei* has free
minicircles and antipodal sites like *C. fasciculata*, but
rather than having the kDNA disk rotate relative to the
antipodal sites, the antipodal sites in *T. brucei* remain
positioned at the network poles (this mechanism is termed
polar replication) (33–35). Thus, the network replication
intermediates are elongated with nicked/gapped circles at
both ends (Fig. 2C). Lys Guilbride (a postdoctoral fellow)
examined four different trypanosomatids, including *Try-
panosoma cruzi*, *L. tarentolae*, *Leishmania major*, and
*Phytomonas davidii*, as well as *T. brucei* and *C. fasciculata*
(36). All had network replication intermediates resem-
bling those of *C. fasciculata*. Thus, *T. brucei* is the only
known example of polar type replication (see model in Fig.
2C).

**Some Fruitful Collaborations on *C. fasciculata* and
*T. brucei***

Working together with Dave Ward at Yale University
(Dave and I were graduate students together at Rocke-
feller), we used fluorescence microscopy for the first time,
REFLECTIONS: A Passion for Parasites

doing both FISH and immunofluorescence on C. fasciculata. Not only did Martin Ferguson (Dave’s graduate student), Al Torri, and David Pérez-Morga (both postdoctoral fellows in my laboratory) produce two very nice papers (33, 37), but our laboratory became addicted to these techniques; we acquired our own microscope and used it for many subsequent publications.

While examining some restriction digests of L. tarentolae kDNA, Joan Marini discovered that some kDNA fragments migrated much more slowly on polyacrylamide gels than expected from their size (38), whereas on agarose gels, the same fragments migrated normally according to their size. We thought that this behavior might be due to some unusual conformation of the fragments. While thinking about how we could investigate this, I saw a paper in PNAS by Don Crothers at Yale describing a technique called electric dichroism, which was sensitive to DNA conformation (39). I wrote to Don and asked if he would be interested in examining our unusual kDNA fragments. He expressed interest and asked Steve Levene (a graduate student in his laboratory) to work with us. Electric dichroism confirmed that the kDNA fragment was more compact than a conventional fragment. We proposed that the kDNA had a bend within its sequence. In a clever experiment, the Crothers lab mapped the location of the bend and showed that the associated sequence was four runs of 5–6 A in phase with the helical repeat of ~10.5 bp (40). Phasing positions the small bends associated with each A run on the same side of the helix, adding up to form a significant curvature. Bent DNA is found on virtually all DNA in all species, where it is thought to facilitate binding of proteins that augment DNA bending. The most extremely bent molecule I am aware of is a C. fasciculata kDNA fragment (42). With the help of Kathy Gann (a postdoctoral fellow in my laboratory), Pete Kitchin cloned this 200-bp fragment with 18 A runs, 16 of which are on the same strand, and most are phased with the helical repeat. Jack Griffith (University of North Carolina) prepared marvelous electron micrographs of this and other bent fragments. The linear 200-bp fragment looked like a circle, and this micrograph appeared on the cover of Cell (43). About fifteen years ago (circa 2000), I was at the Parasitology Gordon Conference held at a small prep school in New Hampshire. The Biopolymers Gordon Conference was ongoing at the same site, and I noticed that they had a session on bent DNA. I spoke to several scientists working on this subject, including some from the Crothers laboratory, and it was exciting to find out that people were still studying and discovering new things about bent DNA more than ten years after our first paper reporting the discovery of bent DNA.

We also collaborated with C.C. Wang at the University of California, San Francisco. C.C.’s postdoctoral fellow, Ziyin Li, had been working on a T. brucei mitochondrial proteasome. RNAi knockdown of this proteasome had a remarkable effect on the kinetoplast (the condensed form of the kDNA network) (Fig. 2A), causing it to grow enormously in size, sometimes even larger than the nucleus. Because C.C.’s laboratory had never worked on kDNA, he invited us to collaborate with them on this project. We were excited to do so, and the results were extremely interesting. After considerable work, Ziyin and C.C., together with Shawn Motyka and Megan Lindsay (now Megan Lindsay Povelones; two graduate students from my laboratory), postulated the existence of a positive regulator of both minicircle and maxicircle replication (44). The idea was that the proteasome normally degrades this regulator. However, if the proteasome is depleted by RNAi, the positive regulator persists, and replication proceeds out of control, resulting in production of massive kinetoplasts. In parallel and seemingly unrelated studies, Beiyu Liu (a postdoctoral fellow) found that the TbpPIF2 helicase (the T. brucei genome encodes eight PIF helicases; discussed below) regulated the level of maxicircles, and it had properties expected for the positive regulator of maxicircle replication (45). RNAi depletion of TbpPIF2 produced networks with no maxicircles, whereas overexpression of TbpPIF2 produced networks overloaded with maxicircles (up 3–6-fold). These treatments had little effect on minicircles. Thus, there must be separate regulators for maxicircles and minicircles. Providing more evidence for the role of TbpPIF2 in this mechanism, Beiyu Liu found that RNAi depletion of the proteasome elevated the level of TbpPIF2, as expected. This mechanism represents a novel way of regulating kDNA replication, possibly by changing the ratio of maxicircles to minicircles.

Proteins Involved in kDNA Replication

Another major effort was to study the proteins involved in kDNA replication. This work was conducted mainly in the laboratories of Dan Ray and Joseph (Yossi) Shlomai (more about him later), as well as in my laboratory (see Fig. 6 for a photograph of the three of us). Dan purified the first one, which was one of the most important. That was the
C. fasciculata mitochondrial topo II (9, 46). Initially, we decided to go after DNA polymerases. Al Torri purified the first DNA polymerase from C. fasciculata, the one with the major activity in mitochondrial extracts (47–49). It was a DNA polymerase (pol)β. This was a totally unexpected finding, as polβ enzymes in all other cells are found only in the nucleus, where they fill gaps as part of base excision repair. This was the first example of a mitochondrial polβ, and it localized in the antipodal sites. Tina Saxowsky (a graduate student) and Michele Klingbeil (a postdoctoral fellow) subsequently found that there were two polβ enzymes encoded by the T. brucei genome, and even more surprisingly, both were mitochondrial although with different localizations (50). The first polβ localized in the antipodal sites and probably functions with DNA ligase kα (51) to close most of the minicircle gaps between Okazaki fragments prior to attachment to the network. The second polβ was in the kinetoplast disk, where it is thought to operate together with DNA ligase kβ (51) to close the final minicircle gaps after replication is complete. (The two ligases were discovered and studied by Dan Ray.) The two polβ enzymes seemed very unlikely to be major mitochondrial replicative enzymes, so Michele searched the T. brucei genome again and found no gene for polγ, the mitochondrial replicative polymerase in other eukaryotes. Instead, she found genes for four candidate polymerases related to bacterial DNA polymerase I (52). TbPOLID was localized, at least transiently, in the antipodal sites (53). TbPOLIB and TbPOLIC were localized in two sites within the kinetoflagellar zone (KFZ; the region between the kinetoplast and the mitochondrial membrane adjacent to the basal body) (52). Because Mark Drew (a graduate student) had previously localized free minicircles and found that they initiated replication in the KFZ (54), it seems likely that TbPOLIB and TbPOLIC and maybe TbPOLID are replicative polymerases. Michele is now studying these POLI-related enzymes in her own laboratory at the University of Massachusetts.

Congjun Li (a postdoctoral fellow) purified a primase from C. fasciculata mitochondria. Congjun and Catharine Johnson (a graduate student) showed that it localized to the proximal face (adjacent to the KFZ) and the distal face of the kinetoplast. Immunofluorescence indicated there was twice as much enzyme in the proximal face (55, 56). We never identified the gene for this enzyme. Jane Hines and Dan Ray (Jane is Dan’s wife) subsequently identified genes for two additional mitochondrial primases (57, 58), and neither one appeared to be Congjun’s enzyme.

Our laboratory also studied mitochondrial helicases related to yeast PIF1 helicase. Other eukaryotes contain one or at most two PIF1 homologs in their mitochondria. T. brucei has eight genes encoding PIF1 homologs, and six of the gene products are in the mitochondrion. Beiyu found that three of these genes are essential (TbPIF1, TbPIF2, and TbPIF8), and TbPIF5 has a phenotype after overexpression. The four have different functions. TbPIF1 functions in minicircle replication (59); TbPIF2 is involved in maxicircle replication (see above for details) (45); and TbPIF5, although not essential in T. brucei and absent in L. major, is involved in removal of RNA primers from Okazaki fragments (60). Jianyang Wang (a postdoctoral fellow) found that TbPIF8, the most divergent of the PIF1
homologs, is essential for growth, but its function is not yet known (61).

About thirty-five years ago, I first met Yossi Shlomai, an Israeli from the Hebrew University of Jerusalem. Yossi had worked as a postdoctoral fellow in Arthur Kornberg’s laboratory at Stanford about ten years after I did. The story about how we met is interesting. Yossi had just accepted a faculty position in the department of parasitology at the Hebrew University, and he thought that it would be appropriate to conduct his future research on a parasite. After suggesting to Yossi that he should work on kDNA, Arthur phoned me to say that Yossi was planning to visit me in Baltimore on his way back to Israel and asked if I would provide him with a stock of our \textit{C. fasciculata} cells. I admit to having been slightly nervous about Yossi becoming my fierce competitor. In fact, I was actually a little intimidated when I saw this man with a fantastic long black beard emerge from the plane. However, it became quickly apparent that Yossi was not intimidating at all, and it did not take us long to become close friends. I was soon extremely happy to have him in the field (sadly, his amazing beard was gone the next time I saw him). For many years, we were co-principal investigators on a grant from the United States-Israel Binational Science Foundation that has provided us with the opportunity to visit each other’s laboratories and collaborate on projects. One of our collaborations involved the TbPIF2 helicase, which, as described above, was especially fascinating (45). The referee was dissatisfied with our attempts to express the recombinant protein and demonstrate helicase activity and insisted that we do a better job in this regard. Beiyu and Nurit Yaffe (a graduate student in Yossi’s laboratory) worked on this project simultaneously, using different approaches in hopes that at least one of them would work. This was a memorable but tense effort for all involved. Beiyu would work all day, and sometime after midnight, as Beiyu was going home and the Israelis were just entering their laboratory, I would telephone Yossi to report on our results and find out how things were going on their end. Fortunately, both Nurit and Beiyu were successful in expressing the TbPIF2 helicase. We added both of their results to our paper, and it was promptly accepted.

Yossi’s major work has been on one protein, UMSBP. It is a 14-kDa binding protein that binds to the universal minicircle sequence (UMS), a conserved 12-bp sequence in the kDNA minicircles of all trypanosomatid species (5’-GGGGTTGGTGTA) (reviewed in Ref. 62). It serves as the origin of replication, and the leading strand initiates within it. In another collaboration sponsored by the Binational Science Foundation, Yossi sent one of his graduate students, Kawther Abu-Elneel, to visit our laboratory for the purpose of determining the intracellular localization of UMSBP. With the help of Derrick Robinson (a postdoctoral fellow) and Mark Drew, Kawther showed by immunofluorescence that UMSBP is localized together with the replicative polymerases TbPOL1B and TbPOL1C in two sites in the KFZ; there is no signal in the nucleus (63). \textit{In vitro}, UMSBP binds only to single-stranded UMS, but it also binds to intact double-stranded kDNA networks, indicating that there is an unusual conformation of double-stranded DNA in the UMS region (64). \textit{C. fasciculata} UMSBP has five CCHC zinc fingers stabilized by disulfide bonds (65). Four of these zinc fingers are required for binding to the UMS sequence, and the protein appears to be regulated by the redox state of the cell (66). Redox controls the oxidation-reduction of the essential disulfide bonds.

In 2001, Yossi sent his graduate student, Neta Milman, to visit our laboratory to learn how to do RNAi. After Neta had been working for several months, Yossi and his wife, Zippi, arrived at Baltimore-Washington International Airport at 3 a.m. on September 11. They stayed that night in a motel at the airport with the intention of boarding an afternoon flight to Boston for the upcoming Woods Hole Molecular Parasitology Meeting (MPM). Prior to the flight, we were to meet in my laboratory at 9 a.m. to discuss Neta’s data. At 8:30 that morning, I picked Neta up at her apartment, and we drove to the laboratory to meet Yossi. On the way, however, we (along with the rest of the world) first heard news on the radio of the attacks on the New York World Trade Center and the Pentagon. My entire laboratory and my wife, Christy, along with Yossi and Zippi, had been scheduled to fly on the same flight to Boston. Because the meeting was cancelled, as well as all flights, Yossi and Zippi stayed at our house for over a week.

When Neta finally made it back to Israel, she used her newly learned technique of RNAi and showed that knockdown of UMSBP inhibited not only kDNA replication but also kDNA segregation and even had effects on the nucleus (67). Thus, UMSBP apparently has much broader actions than originally thought. Yossi’s laboratory has recently shown that UMSBP binds to kDNA networks in another way when the basic DNA-binding proteins CfKAP3 and CfKAP4 are present (68). UMSBP binds to the KAP protein via protein-protein interactions and then binds the DNA to make it more susceptible to topo II. This may be relevant to minicircle release by mitochondrial topo II.

Although the three groups have identified 30 – 40 proteins involved in replication, it is likely that there may be as
many as 150 proteins involved in replication and maintenance of kDNA. One reason for the abundance of proteins is that some of them, such as the DNA polymerases and the PIF1 homologs, are present in multiple activities, with each one having a separate function. The reason could be that the enzymes have not evolved sufficiently to be streamlined so that they can handle multiple functions. In contrast, mitochondrial topo II is a product of a single-copy nuclear gene, and this enzyme performs all the topological activities needed to replicate and maintain a kDNA network. One enzyme, in this case, does it all. A second reason for the proposed abundance of enzymes is that kDNA replication is incredibly complex. Most of the enzymes are positioned in specific locations surrounding the kinetoplast, such as within the antipodal sites, KFZ, and the kinetoplast itself. Thus, the kinetoplast is closely associated with its own replication machine, and if the disk rotates, this machine must have moving parts. Catharine Johnson found that the localization of replication proteins depends on the cell cycle (69). She reported that some antipodal proteins (e.g., topo II) remain in the antipodal sites at the end of the mitochondrial S phase, whereas others disappear (e.g., pol β).

RNA Interference

Elisabetta Ullu and Chris Tschudi at Yale reported RNAi interference in *T. brucei* (36) soon after its initial discovery in *Caenorhabditis elegans*. Zefeng Wang (a graduate student) learned about RNAi when he took the summer course at the MBL in Woods Hole that was run that year by Chris and Elisabetta. He returned to Baltimore excited about the prospect of quickly knocking down the activity of any gene. Zefeng teamed up with Jim Morris (a postdoctoral fellow) and Mark Drew to develop a new vector that simplified RNAi. Using opposing T7 promoters and endogenous T7 RNA polymerase (the cells used for RNAi had been transfected to constitutively express T7 RNA polymerase and the tetracycline repressor), they produced double-stranded RNA from an ~500-bp fragment of the gene of interest. This vector, known as pZJM, was designed so that RNAi could be turned on by addition of tetracycline to the culture medium (70). We have subsequently sent this vector to many laboratories, and it has been widely used. John Donelson created a very similar vector that has also been used widely (71).

One of Zefeng’s first experiments was to knock down the activity of mitochondrial topo II (72). The result was dramatic. After a few days of RNAi, the kinetoplast disappeared! Zefeng then set out to see which step in replication had been blocked, and because nicked/gapped minicircles accumulated, he concluded that it must have been network reattachment that was blocked (72). This fit well with the finding that topo II is in the antipodal sites where minicircle attachment takes place (46). Megan Lindsay Povelones, in collaboration with Keith Gull and his postdoctoral fellow, Eva Gluenz, at the University of Oxford, later showed that depletion of topo II by RNAi also had an effect on networks (73). They became much more porous and appeared to have small holes. Apparently, these holes are normally repaired, but not when topo II is depleted. We do not yet know how minicircles are released for the purpose of replication. Presumably, it is by the same enzyme that attaches them because this is the only mitochondrial topo II encoded in the nuclear genome. The release mechanism could be related to the interaction of UMSBP and CfKAP3 or CfKAP4, mentioned previously (68).

Mark Drew, Jim Morris, Zefeng Wang, and Shawn Motyka developed the first RNAi library in any organism (74, 75). This was a forward genetic technique that allowed us to select or screen for mutations in any function. We found mutants that were defective in kinetoplast replication. Zhixing Zhao (a graduate student) and Eddy Agbo (a postdoctoral fellow) used the library to identify a gene that encodes a protein known as p166 (76). They showed that this protein is part of the tripartite attachment complex (TAC) filaments that link the kinetoplast with the basal body outside the mitochondrion (77). Based on morphological data, TAC had been suggested to be involved in kinetoplast segregation. Our studies showed that p166 was likely in the mitochondrial membrane, and as expected, RNAi of p166 severely blocked segregation of the kinetoplast. p166 was the first molecular component of TAC to be discovered, raising the possibility of a molecular dissection of TAC. David Horn (now at the University of Dundee) has made amazing improvements in screening an RNAi library, and it may soon be possible to discover all the genes in a pathway such as kDNA replication (78).

Antitrypanosomal Drugs and kDNA

One reason we started studying kDNA was because animals do not have DNA networks. Therefore, there was the possibility that kDNA or one of its replication enzymes might serve as a drug target for eradication of infections caused by trypanosomatids. There was some controversy about this idea because bloodstream forms of *T. brucei* can survive without a kinetoplast (these cells are known as dyskinetoplastic (DK)). However, it is not easy to obtain stable DK cells. If one treats an infected mouse with ethidium bromide, most cells become DK in a few hours. If
the cells are passaged, all cells are initially wild type, and if ethidium bromide is added again, the same thing happens. Only after many passages does one have a chance to actually obtain a stable DK strain. The reason is that expression of kDNA maxicircles is required in bloodstream trypanosomes because maxicircles encode the essential A6 subunit of ATP synthase. In bloodstream trypanosomes, this enzyme does not make ATP. Instead, the enzyme runs in reverse, hydrolyzing ATP that is obtained by glycolysis of the abundant blood glucose. This is why the ATP synthase is also called the proton-translocating ATPase. ATP hydrolysis enables development of a proton gradient required for mitochondrial maintenance (79). It so happens that every trypanosome that is stably DK has a particular mutation in the γ-subunit of the ATP synthase that eliminates the need for the A6 subunit (80). Thus, the reason that it is difficult to obtain stable DK cells is that there must be a spontaneous mutation at that site (see Ref. 81 for further discussion of these mechanisms). Because this mutation is so rare, a drug that can knock out kDNA or one of its replication enzymes should be effectively trypanocidal.

One example is ethidium bromide. This intercalating agent causes efficient loss of kDNA without producing stable DK cells. Terry Shapiro, Rob Jensen (professor of cell biology also at Hopkins), and I recently discovered the mechanism of killing of \textit{T. brucei} by ethidium bromide (still in field use as a drug to cure bovine trypanosomiasis). Arnab Roy Chowdhury (a postdoctoral fellow) and Rahul Bakshi (Terry’s postdoctoral fellow), together with others in our laboratories, showed that ethidium bromide blocks kDNA replication in an unexpected manner (82). This intercalating agent highly supertwists free minicircles, but not network minicircles, an unexpected finding because both are in the same compartment, the mitochondrial matrix. The concentration of free minicircles rose by ~10-fold, suggesting that minicircle release was unimpaired but that replication was blocked. This is not surprising, as supertwisting of free minicircles must prevent the binding of UMSBP and other proteins, thereby stopping replication. Network minicircles presumably do not supertwist because bound proteins prevent intercalation of ethidium bromide. However, we then realized that what actually kills them is a loss of maxicircle gene products (the only one of these required for bloodstream forms is the proton-translocating ATPase). Depending on the turnover of this protein, loss may not occur for several days after blockage of kDNA replication. We found that cells treated with ethidium bromide did not die immediately after drug addition, but all of them were doomed to die a few days after minicircle replication had been blocked. It is as if they carried a time bomb, set to go off several days after drug treatment. Unfortunately, ethidium bromide and related intercalating dyes cannot be administered to humans because these agents are probably mutagenic.

The Future

This is a perfect time to expand work on \textit{T. brucei}. There are fantastic projects just beginning (e.g. Ref. 83), and new techniques offer tremendous power for further basic studies (78). Furthermore, we have made progress on kDNA replication, but much more needs to be done, and three of the four scientists featured in this article (Larry Simpson, Dan Ray, and me) have already retired.

Glycosylphosphatidylinositol Anchors

In this Reflections, I have covered only half of the work in my laboratory for the past forty-five years. We also worked on \textit{T. brucei} variant surface glycoproteins (VSGs). Because there were so many laboratories in the late 1970s that were studying gene rearrangements during antigenic variation, we decided to study the biochemistry of VSGs. The idea of a glycosylphosphatidylinositol (GPI) anchor was just emerging at this time, and we focused most of our attention on that. In studying the biosynthesis of GPIs, we were the first to discover the GPI precursor, glycolipid A. Using a cell-free system containing trypanosome membranes and soluble radioactive precursors, such as UDP-GlcNAc and GDP-mannose, we could produce glycolipid A’, the non-myristoylated form of the GPI anchor precursor. Thus, we were the first to elucidate most of the steps of the GPI biosynthetic pathway. We also found out how the anchor precursor gets myristoylated in a fatty acid-remodeling reaction. In this reaction, which also occurs in our cell-free system, longer fatty acids are sequentially removed from glycolipid A’ and replaced by myristate from a myristoyl-CoA donor. Later, in searching for the source of myristate, we discovered that all fatty acids in \textit{T. brucei} are synthesized by a mechanism that differs from both the eukaryotic type 1 fatty acid synthesis system and the prokaryotic type 2 system. The fatty acids are synthesized \textit{de novo} by microsomal elongases, whereas in other cells, elongases take long chain fatty acids and make them even longer. By around 2004, I downsized my laboratory and maintained the kDNA grant, but not the one for VSGs. Perhaps I am better known for the GPI work, but in this Reflections, I decided to focus on kDNA because it is so unusual. To recognize the contributions of the GPI people in my laboratory, I have listed them all in Table 1 and briefly described their work.
The Birth of Molecular Parasitology

Today, the field of molecular parasitology is booming, very different from when we started working on parasites. At that time, there were few biochemists and molecular biologists studying these organisms, except in one area. People were beginning to work on the incredible phenomenon of antigenic variation in *T. brucei*. In this process, the parasites escape elimination by the host immune system via periodic switching of VSG coats from one to another of different amino acid sequence. We now know that there are over 1000 different VSG genes in the genome, including gene fragments that can recombine to form new VSG genes. Therefore, the potential for escape from immune destruction is enormous. I suggest that the field of molecular parasitology began with the publication of George Cross’ paper on antigenic variation in *T. brucei* published in Parasitology in 1975 (84). (According to this definition, I was actually in this field from the very beginning.) Although George’s paper was not published in a frontline journal and was probably not even widely read at the time, it ultimately had a tremendous impact, and it put the phenomenon of antigenic variation on a solid chemical basis. What he did was clone variants of *T. brucei*, each expressing a single unique VSG, and all were descended from a single cloned variant. He purified VSGs from several variants and showed that they were abundant ~65-kDa proteins with markedly different amino acid compositions. They all reacted specifically with a membrane-impermeable radioactive probe, confirming that they derived from the cell surface. He immunized mice with a purified VSG and showed that they were protected from subsequent challenge by trypanosomes carrying the same, but not different, VSGs. George’s paper not only revealed some basic features of antigenic variation but also provided tools for biochemical (85) and molecular biology (86) studies that followed. These papers made trypanosomes world famous and attracted many scientists to the field of molecular parasitology. Interest in the molecular aspects of trypanosomes led to rapid growth in studies of other protozoan parasites (*e.g.* *L. major*, *T. cruzi*, *Toxoplasma gondii*, *Giardia lamblia*, *Entamoeba histolytica*, *Trichomonas vaginalis*, and *Plasmodium falciparum*; see below). By the time the first MPM was held in Woods Hole in 1989, there were strong groups working on all of these organisms. However, the meeting organizers (Dyann Wirth from the Harvard School of Public Health, Lex Van der Ploeg then at Columbia University, and Jeff Ravetch from Rockefeller University) were so nervous that too few people would come that I received a phone call from Jeff a couple of weeks prior to the meeting to make sure that my laboratory would show up. About 200 people did attend, and the meeting was considered a success. Within a few years, MPM had sellout crowds, and it spawned two more meetings, also held at the MBL. One is Immunoparasitology (with about 200 participants), and the other is Kineto-plastid Molecular Cell Biology (held every other year with

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**TABLE 1**

Projects of students and postdoctoral fellows who worked on GPI anchors and related subjects

Only one reference is listed for each person. Postdoctoral fellows are indicated; all other names are of graduate students. GPI-PLC, GPI-specific phospholipase C.

| Student/postdoctoral fellow | Project |
|-----------------------------|---------|
| Alvaro Acosta-Serrano (postdoctoral fellow) | Procyclins form the surface coat of procyclic trypanosomes. Developed purification of procyclins and characterization by mass spectrometry. Determined the number of procyclin EP repeats in parasites *in vitro* and in the tsetse midgut. In the latter, identified the program of expression and discovered proteolysis of procyclins (94). |
| Jay Bangs | Studied synthesis processing and intracellular transport of GPI-linked and N-glycosylated VSG. Showed that GPI is cotranslationally added to VSG (95). |
| Larry Buxbaum | Studied myristate exchange, an alternative pathway for GPI myristoylation (96). |
| Tamara Doering | Worked on GPI biosynthesis and myristoylation and showed that GPI-glucosamine comes from UDP-GlcNAc. Studied myristate metabolism and demonstrated that GPI myristoylation was inhibited by certain myristate analogs and that some analogs were toxic to *T. brucei* (97). |
| Jenny Stephens Guler | Discovered mitochondrial fatty acid synthesis in *T. brucei* and evaluated its function (98). |
| Dale Hereld | Purified and cloned *T. brucei* GPI-PLC (99). |
| Kuo-Yuan Hwa | Isolated and characterized glycosylation mutants (100). |
| David Jiang | Studied four fatty acyl-CoA synthases and determined their specificity for fatty acid chain length (101). |
| Jessica Krakow | Discovered glycolipid A, the GPI precursor for VSG (102). |
| Soo Hee Lee | Discovered that *T. brucei* makes fatty acids *de novo* using elongases (103). |
| Wayne Masterson (postdoctoral fellow) | Developed cell-free systems for synthesis and myristoylation of GPls. Worked out the mechanisms of both (104). |
| Kojo Mensa-Wilmot (postdoctoral fellow) | Studied developmental regulation of expression of GPI-PLC and developed one-step purification of recombinant GPI-PLC using monoclonal antibodies (105). |
| Ken Milne (postdoctoral fellow) | Discovered a novel *T. brucei* GPI, possibly involved in catabolism (106). |
| Yau Morita | Studied fatty acid remodeling. Discovered fatty acid biosynthesis in *T. brucei* (107). |
| Kimberly Paul (postdoctoral fellow) | Studied fatty acid synthesis in *T. brucei* and the effects of triclosan (108). |
| Jayne Raper (postdoctoral fellow) | Studied GPI myristoylation (109) and other steps in GPI synthesis. |
| Karl Werbovetz (postdoctoral fellow) | Synthesized and evaluated drugs containing myristate analogs (110). |
about 400 participants). The latter was formed in reaction to the fact that the original MPM was becoming dominated by talks on the apicomplexan parasites, malaria, and Toxoplasma. Now, twenty-five years later, in 2014, the field has matured, and the quality of research is very high. One of the most important characteristics of these meetings, and one that reflects the wisdom of the original organizers, is that from the very beginning, virtually all of the talks have been given by graduate students and postdoctoral fellows, not by the principal investigators.

**Malaria**

A special case is the malaria parasite, *P. falciparum*. Progress in malaria research has been frustratingly slow. In the meantime, the parasite has become resistant to many drugs. Also, insecticides that previously killed the mosquito vector have lost their efficacy and, more importantly, been found to be environmentally hazardous. Finally, no effective vaccines are yet available, although it is clear that African children can develop immunity after multiple infections. Ruth Nussenzweig at New York University School of Medicine showed that irradiated sporozoites provided an effective vaccine (87). However, Ruth’s technique is impractical as a vaccine because of insufficient availability of sporozoites (the life cycle stage injected by the mosquito into the human body). There was much excitement when the circumsporozoite protein (the major surface protein on sporozoites analogous to VSG on trypanosomes) was cloned and expressed, but it has not yet lived up to expectations as a vaccine candidate. When I was on the NIH study section (from 1992 to 1996), very few malaria grants were funded because a typical proposal was to clone genes and test the gene products as a candidate vaccine. However, all of these efforts failed to produce an effective vaccine.

A major problem was that malaria was known to be dreadfully difficult to work on. For example, until 1976, when Bill Trager at Rockefeller University worked out the culturing method for malaria (88), it was necessary to obtain these parasites from infected monkeys. Also, there were not yet any genetic techniques that allowed one to study the function of a gene. The latter problem was partially resolved when Tom Wellems (89) and Alan Cowman (90) developed transfection techniques. Major improvements to these techniques would be tremendously helpful, and one may be very close at hand (91). In the last fifteen years, because of both the worsening malaria situation in the field (one million African children die each year from this disease) and increased interest from funding agencies, scientific research on *P. falciparum* has skyrocketed. Now many parasitologists, young and old, are focusing on malaria, and the science is getting much more exciting. This is as it should be, but as for me, I still have a soft spot for *Trypanosoma brucei*.

**My Students and Postdoctoral Fellows**

I could not truly reflect on my scientific life without saying more about my lab offspring. We were generally a happy lab (e.g. see Fig. 5), but as in every family, there were ups and downs. My long-time technician, Viiu Klein, was the lab mother, and she would personally look after students who were having problems, scientific or otherwise. As every family should, we had our rituals, especially the celebration of Thanksgiving. For as long as I can remember, our lab would host a traditional Thanksgiving dinner every November. This was one of our greatest events of the year. The newest member of the lab would come in early and start roasting the turkey under Viiu’s watchful eye. When Viiu retired, Gokben Yildirir, her replacement (who was actually from Turkey), took over responsibility for supervising the roasting. Viiu had already trained Gokben rigorously in the science of turkey roasting. All the other lab members would bring in an array of side dishes they had prepared at home (e.g. stuffing, traditional cranberry sauce, mashed potatoes, and apple cider), but given the nature of our lab, there would also be Chinese dumplings and other international offerings. We always invited the other parasite labs, including those of Terry Shapiro, Barbara Sollner-Webb, and Dennis Grab. At around 1 p.m., when the smell of roasting turkey had permeated the whole department, we gathered for dinner. This was a great tradition.

The saddest time in my lab was when Wayne Masterson died of metastatic melanoma in 1991 at the age of 31. Wayne was a brilliant and exemplary British postdoctoral fellow. He was also very funny (with hilarious imitations of faculty members, especially me). In the first of his two *Cell* papers, Wayne described the cell-free biosynthesis of GPls and identified the biosynthetic intermediates. In his second *Cell* paper, he described the pathway of myristoylation of trypanosome GPls. The VSG GPI anchors of trypanosomes are unusual in that they have myristate as their sole fatty acid. Wayne demonstrated that myristate is incorporated into the GPI in a fatty acid-remodeling reaction in which the two fatty acids originally on phosphatidylinositol are sequentially removed and replaced by myristate. In his second year in my lab, Wayne was diagnosed with melanoma. He returned to Scotland to be near his family and to work in Mike Ferguson’s lab at University of Dundee. We kept in very close contact with him, and we
were devastated when we received the inevitable phone call from Mike telling us that Wayne had died, leaving his wife, Claire, and 3-year-old son, Kyle.

My Mentors

I could not have been more fortunate in my chosen teachers. My graduate studies with Lyman Craig taught me the fundamentals of research and introduced me to the highest scientific standards. Dr. Craig was a very creative scientist. He is the only person I know of who published a Journal of Biological Chemistry paper without a single reference. This was not because he was negligent, but because his work was so original (92). I also thank T. P. King, a junior faculty member in Dr. Craig’s laboratory with whom I discussed my work every day. T. P. taught me the fundamentals of protein chemistry, thus preparing me well for my future work. Dr. Craig encouraged me to seek postdoctoral training with Arthur Kornberg at Stanford.

This was one of the most exciting periods for discoveries in biochemistry and molecular biology, and it is fair to say that Arthur led one of the best biochemistry laboratories of the latter part of the twentieth century. It was fast moving and high pressure, but I loved it (at least I feel that way now). When I left Rockefeller, I could not imagine myself running an independent laboratory. When I left Stanford, after a two-year postdoctoral fellowship, I was very eager (but still a little nervous) to have my own laboratory. My mentors set me up well for my future career, for which I am forever grateful.

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