Perspectives

Biological time is fractal: Early events reverberate over a life time

DAVID LLOYD
Microbiology, BIOSI 1, Main Building, Cardiff University, PO Box 915, Cardiff CF10 3TL Wales, UK
**Corresponding author (Email, Lloydd@cardiff.ac.uk)*

1. Introduction

Before 1958, the year I began my University career, it was not widely-realised that organisms do not perform in a steady state. Ideas about homeostasis, the self-balancing of living processes, dominated physiology and biochemistry. The discovery of instabilities in photosynthetic reactions and of oscillations of NADH by Duysens and Amesz in 1957 were regarded as somewhat unusual curiosities – we now realise that steady states hardly exist in biological systems and that oscillations, rhythms and clocks are absolutely necessary for the regulated functioning of organisms.

I began by studying bioenergetics at a time when remarkable new insights into aerobic ATP production were being made, for instance in Philadelphia, Baltimore, New York, Madison, Amsterdam and Stockholm. These well-financed and highly effective groups made huge inroads into the detailed understanding of the structure and functions of mitochondrial membranes, effectively building on earlier work at UK – based laboratories (e.g. at Sheffield, Cambridge and Oxford). The heart of the problem, how a membrane makes ATP, was however, to remain a mystery for another 10 years, and even then its solution was so ground-breaking that the chemiosmotic principles required for detailed mechanistic explanation were widely resisted until 1978, the year that the iconoclastic and independent Peter Mitchell was awarded the Nobel Prize (Prebble and Weber 2002; Morange 2007). This was an extremely competitive area, and when the final world-wide consensus was established, the thrill of the chase was never quite to be the same again. Yet there was much left to be done. Almost all of the work had centred on experiments with suspensions of isolated mitochondria, and the functioning of these organelles within the intact cell had been neglected. An especially conspicuous lacuna was the dynamic functioning and energetic efficiency of the mitochondrion within a unicellular organism, tissue or organ.

One of the major shortcomings of the traditional biochemical approach was the blurring of the heterogeneous contributions of individual cells during the preparation of a cell-free homogenate. Two solutions to this problem were evident in theory, but each was rather difficult in practice: either work with single cells, or ensure that the performance of the population average was representative of each cell. Single cell biochemistry was extremely limited at that time despite the startling advances in microspectrophotometry and microspectrofluorimetry. Synchronous cell culture, the second option, often suffered the problem of perturbative artefacts. To discover more about the time course of development of mitochondria during the growth and division of cells we chose to study synchronous cultures of the ciliate protozoon, *Tetrahymena pyriformis*. This led us to observe that respiration oscillates in growing organisms and the cycles we observed were more frequent than the cycles of cell division. They were however, much slower than metabolic cycles. Oscillations of this type are now called "ultradian" (figure 4). Thus, whereas the most intensively studied biological rhythms are circadian (with a period of about a day), ultradian rhythms cycle many times in a day. Unlike circadian rhythms that are reset twice daily (at dawn and at dusk) and serve to match the functions of the organism to its changing environment, ultradian rhythms are essential for the co-ordination of intracellular processes.

My own research career has been influenced by a succession of inspirational scientists. Even before leaving school, teachers of a rare quality (Mr Cledwyn Kiff, Chemistry and Mr Frederick Weaver, Biology) indulged my early obsessions with free access to the school laboratories

Keywords. Clocks; mitochondria; oscillations; rhythms; time-scales

and laid a long-lasting understanding of basic quantitative principles as well as precise practical skills. Mr Kiff, who rather resembled the English Prime Minister, Sir Edward Heath, was an excellent chemist who ran a very well-organized and strictly supervised laboratory. He was very good at demonstrating practical procedures (inorganic analysis of "spots" which involved copious use of H_2S from a Kipp's apparatus kept in a very smelly fume chamber, and also basic organic syntheses). His simple but elegant physical chemistry experiments never failed. Although at that time quite early-on in his career, he was never at a loss for an answer, even on the most unfamiliar elements of the Periodic Table. His dictated notes and blackboard calculations have since proved most useful not only for me but for the chemical education of my sons. The depth of his chemical intuitions complemented Mr Weaver's broad interests in the new biology of the 1950's, cell biology, metabolic biochemistry, genetics, ecology and evolution. Mr Weaver too was a strict disciplinarian of the "old-school", but very considerate and kind. Extremely generous with his "free" time he ran a "Biology Society" with lunch-time quizzes, debates and extra-curricular field excursions. As well as understanding the latest advances, he was also an expert in the more traditional biological mould, especially at the identification of pond life, plants, birds and butterflies. In more favoured places and times, both of these schoolmasters might well have risen to the uppermost heights of University academia. Also while still in school, Graham Palmer, five years my senior, who was eventually to become a world-expert on electron transport at Rice University, Houston (via Sheffield and Ann Arbor, Michigan), first told me about Biochemistry (the "Chemistry of the future"). He was already signed-up for an undergraduate place at the Sheffield Department, where H A Krebs had pioneered work on the tricarboxylic acid and urea cycles.

Our Porth County Boy's Grammar School, founded in 1896, near the confluence of the two Rhondda Rivers was, in the 1950's for about 400 of us, the peak academy in the area with a most impressive record of excellent schoolmasters and high-achieving pupils. From the 1840's onwards from an idyllic setting the sinking of hundreds of coal mines had led rapidly to a huge influx of workers and to the production of a ravaged and industrial waste-land. The South Wales coalfield became, by the start of the First World War, the powerhouse of the British Empire, with the coal-ships from Cardiff (at that time the world's largest coal port, 17 miles south) travelling to the four corners of the globe. Colliery disasters, industrial disease and injuries provided a huge incentive for academic success to younger generations, and this may explain the extraordinary export of talent from this area at that time. By 1985, after a year-long miner's strike, hardly any coal was being mined in the Rhondda, and its rural aspect is slowly returning.

2. Sheffield

At the Biochemistry and Microbiology Departments at the University of Sheffield, I was very fortunate to be in the right place at one of the best of its times. First class Chemistry and Microbiology departments, and arguably the best Physical Biochemistry group in the World, in the late 1950's had an inestimably enormous influence on my development and on everything I have done since. As Rod Quayle (an expert on metabolic biochemistry at Oxford, before becoming Head of Microbiology at Sheffield, and then Vice-Chancellor of the University of Bath) once remarked to me, "One never quite escapes one's formative years". One might add it is not sensible to step off the shoulders of giants (figure 1). Almost invidious to mention individuals, but clearly the examples set by G Porter, G Weber, Q H Gibson, V Massey and S R Elsden as leaders and forward-thinkers who would proceed to make fundamental inroads for much of the remainder of the century were truly monumental. Exceptional teachers too, supported this central edifice.

Gregorio Weber (figure 2) was probably the cleverest, deeply and broadly-educated scientist I have known. Medically-qualified from Buenos Aires, he became a self-taught mathematician during his lone and dangerous voyage across the North Atlantic during the height of the Nazi U-boat activities in 1943. Such was the volume of paper he carried with him, he was intensively questioned at his arrival in Britain as a security risk. One can only imagine his un-worldly reaction to interrogation. His deep insights into future developments in biophysics emerge in his 1990 article (Weber 1990) and the continuing symposia series in his honour (currently the seventh in Kauai, in June 2008) indicate his enormous influence.

A preoccupation with biochemical kinetics and thermodynamics, and biological time in general, was triggered in Weber's tutorials and the books and papers he recommended. Especially fascinating for me was the monograph by Martin Kamen (1963) in which characteristic reaction times were assigned to a log scale, rather like pH. The possibilities for measurement of molecular interactions in very fast time domains using fluorescence with the methods Weber employed was revelatory. Weber's imaginative synthesis of new fluorophores and his development of Perrin's fluorescence polarization techniques to measure protein dynamics on a nano-second time scale, continues to ramify and dominate the more recent advances and commercial developments of diagnostic medicine.

"Problems of Life", by von Bertalanffy (1950), as one of Weber's suggested texts opened my eyes to the organism as an open thermodynamic system, with the accompanying idea of the rapid and extensive nature of macromolecular turnover as a necessary component in the "flux of life".

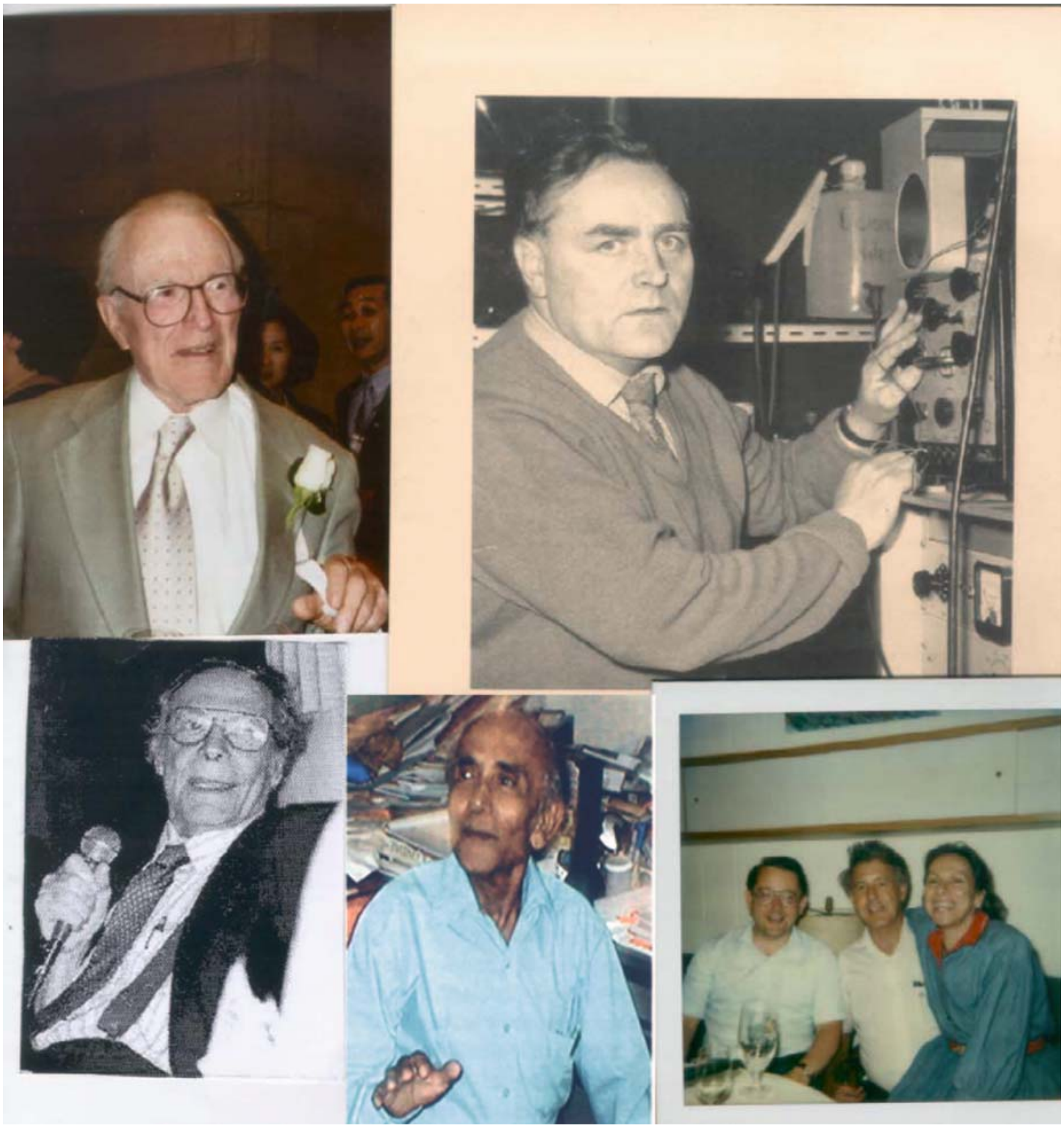
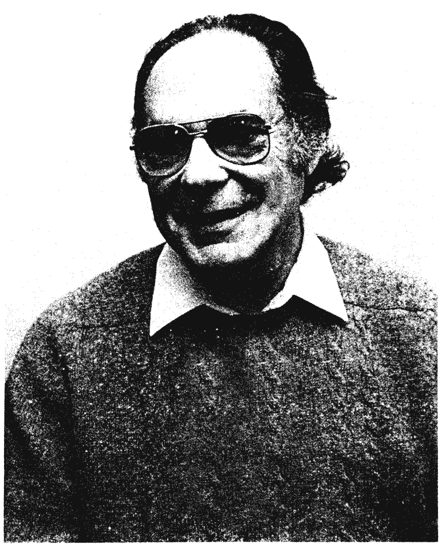


Figure 1. From top left: Britton Chance (90th birthday meeting, Philadelphia 2002), David Hughes (Oxford, 1963); from bottom left: Gregorio Weber (Hawaii, 1996), Pabitra Maitra (60th birthday meeting, 1992), David Lloyd, Woody and Hanna Hastings (Cardiff 1986).

Gibson's pioneering use of stopped-flow and flash photolysis for the study of haemoglobin and cytochrome c oxidase-oxygen kinetics laid the foundations for new methods of study of even more rapid reaction methods (pico-, femto- and atto-second biology) with the advent of pulsed laser technology. Vincent Massey, my final year tutor, although not yet thirty years old was already well on the road to becoming the world leader in research on the reaction mechanisms of flavoprotein catalysis.

3. Cardiff

Back in Cardiff, where I was to spend almost the rest of my working life, my PhD research supervisor A G Callely proved to be exactly right for me, always tempering my wild enthusiasms with a strict old-worldly rigour and attention to detail. A curmudgeonly remark here – Oh that those qualities were more in evidence in our younger colleagues today! He taught me many basic ideas and the one that I have passed



Gregorio Weber

Figure 2. Gregorio Weber.

on is to "treasure your exceptions" (i.e. it is the unexpected results in science that prove most interesting, provided of course that they can be reproduced).

The highly dynamic nature of carbon metabolism became directly evident to me in my own first experiments as a postgraduate on acetate and propionate assimilation using ^{14}C- labelled substrates. Even within the 0.2 s necessary to stop and kill the organisms (*Prototheca zopfii*, a colourless *Chlorella*), a dozen or so metabolic intermediates became radioactive, to show up on the subsequent radioautography of paper chromatograms.

The arrival in 1964 from H A Krebs' Biochemistry Department at Oxford of David E Hughes to Cardiff as the first Professor of Microbiology and Head of the MRC Group for Microbial Structure and Function provided a huge new impetus. Hughes was an expert at spotting young talent. His appointees Julian Wimpenny, Terry Coakley, Al Venables and Alan Griffiths were all to achieve great things, and their continuing support and advice to all at the new Department at all times was invaluable. Indeed all of us were to remain at Cardiff for the entirety of our scientific careers.

Hughes, a charismatic and dynamic leader, was anything but a conventional academic. Essentially a practising scientist, he had left school to become a laboratory technician at the age of fifteen. A succession of jobs with distinguished scientists in colleges of London University preceding a move to the Medical Research Group at Sheffield and then Oxford had seen him at the centre of things in British Biochemistry. His huge experience of science had made him a formidable and iconoclastic figure. His appointment to a Chair (his first academic position) at the age of 50 changed the Cardiff scene completely and provided a worldwide network of contacts with many leading scientists. Hughes avidly embraced everything new; this was the dawn of the new disciplines of molecular genetics and biotechnology. A palpable buzz made Cardiff a most exciting place to be doing microbiology and this small Department, fired by Hughes' infectious enthusiasm, was to produce 30 Professors (Lloyd *et al* 1994).

4. Philadelphia

Hughes' suggestion that I should spend some time with Britton Chance at the Johnson Foundation Biophysics labs at the University of Pennsylvania in Philadelphia was pivotal. Chance liked to listen to a talk every lunch time: sandwiches were prepared for us by Tom, his faithful servant.

We were bombarded by daily lunch-time seminars given by a procession of world-leaders, and a truly-extraordinary atmosphere of dedication to experimental bioenergetics. One of the most impressive aspects of this laboratory was Chance's habitual 15 hour working days and his breadth of interest in diverse biological systems from the electric eel to bio-luminescent bacteria, and in mitochondria from pigeon heart to those from avocado.

Allied to his electronic wizardry (the war years spent at the Massachusets Institute of Technology Radiation Laboratory, developing new methods for radar surveillance of the North Atlantic), and his interests in sailing (helmsman in the US team, 5.5 m class, and a Gold Medal at the Helsinki Olympics, 1952), Chance's interaction with the distinguished weekly roster of visitors made for a unique atmosphere. These visitors almost invariably stayed at least a few days (Mrs Chance owned many of the neighbourhood houses)- long enough to run some experiments. It was at this time that I met Pabitra K Maitra from Calcutta; Pab was Chance's right-hand man! His amazing alacrity and dexterity at taking and quenching samples for fluorometric metabolite assays was already legendary, and several of his papers of the late 1960's have become citation classics. His achievement at obtaining key data and establishing fundamental principles of metabolic control were massive. On his return to the Tata Institute for Fundamental Research at Bombay he generated a whole range of glycolytic mutants of yeast. He became the world leader in the field of the genetic control of sugar utilization. I became a frequent visitor to Pab's lab, and it was at the TIFR in 1982 that I first met Vidya Nanjundiah and learned about cAMP oscillations and their role in pattern formation in *Dictyostelium discoideum.* In due course both Maitra and his long-time co-worker Zita Lobo came to our Cardiff lab as sabbatical visitors. Another young Indian researcher at Chance's Lab, Amal Ghosh, had a few years earlier discovered glycolytic oscillations in whole organism suspensions of yeast. Other friends at Philadelphia who continued to shape my thinking for an entire lifetime included two Englishmen, David Harrison and Ken Pye. Their research on *Klebsiella aerogenes* and yeast respectively, were early contributions still central to our understanding of oscillatory biological behaviour (Chance *et al* 1973). Although at that time I was not engaged in this area of interest, their enthusiasms and passionate commitment resonates still, and I have in some ways taken on their mantles.

5. Back in Cardiff

The Johnson Foundation staff's preoccupation with oscillatory metabolic control mechanisms and non-invasive investigative techniques (spectrophotometry, fluorometery and magnetic resonance methods) has permeated my own research for more than 40 years and an interest in continuous culture and oscillatory dynamics has become a continuing obsession of mine. Our frequent encounters with periodic respiratory activity and enzyme expression in synchronous cultures led naturally to a deeper appreciation of the dynamics of growth and the complexities of temporal organization of living organisms. The continuous and unvarying nature of growth as suggested by the homeostatic paradigm became to us an outdated and oversimplified concept.

Another input came from my annual visits to the new University on the Danish island of Funen, where Hans Degn, became a founder member of the then Odense University (now the University of Southern Denmark). After his return from Philadelphia, he had become a leading authority on O_2 measurements. His use of membrane inlet mass spectrometry to continuously monitor, not only O_2 but also CO_2, was a wonderful advance of great promise. Using this method, Hans was able to demonstrate the Pasteur Effect (the control of glucose utilization by O_2) on-line. This is a technique that we in Cardiff have used to monitor other gases (H_2, CH_4, N_2, N_2O, H_2S) consumed or produced by microbes. The usefulness and broad applicability of this method especially when using the probe devised by Sandor Bohátka at Debrecen in Hungary for continuous minimally-perturbing monitoring of dissolved gas species has even yet to be fully realised by biologists.

A visit to our Cardiff Department by Dr. J.W. (Woody) Hastings (figure 3) in 1985, was also to provide an enormous stimulus to two aspects of my work, the measurement of very low (nM) O_2 using bioluminescent bacteria, and the world of circadian rhythms. The following summer I spent three wonderful months at his laboratory in the Harvard BioLabs building where I was to be exposed to another centre of world-class cutting-edge research. The 'Taylortron' – Walter Taylor's automated bioluminometer specially designed for the study of the biological rhythms of luminous dinoflagellates [e.g. *Lingulodinium* (*Gonyaulax) polyedra*] was at that time the centre-piece of Woody's lab, but it was also a place to learn the latest molecular biological techniques (Hastings 2001). Several of the young post-docs there have contributed much to the field of circadian chronobiology. Perhaps most worthy of special mention is Takao Kondo who was extremely kind to me and to a Postgraduate student, Helen Jenkins, in providing us with the detailed plans and circuitry to measure *Chlamydomonas reinhardtii* rhythms of phototaxis. I had this device built back in Cardiff (by Bill O'Neil and Norman Williams), and it was used by several research and honours students in our work. For instance, Helen was able to demonstrate in this unicellular alga that underlying the dominant circa 24 h rhythm there was a persistent ultradian clock output that showed a 55 min periodicity, especially when organisms had been cultured continuously under intense light.


Figure 3. J W Hastings.

Our initial foray into work with synchronous cultures came after a visit by Geoffrey Turner (now Professor of Genetics, Sheffield University) to Erick Zeuthen in Copenhagen where he learned how to obtain such cultures (*Tetrahymena pyriformis*) using the heat-shock method. Geoff went on to show that chloramphenicol disrupts the mitochondrial-nucleo-cytoplasmic control circuitry

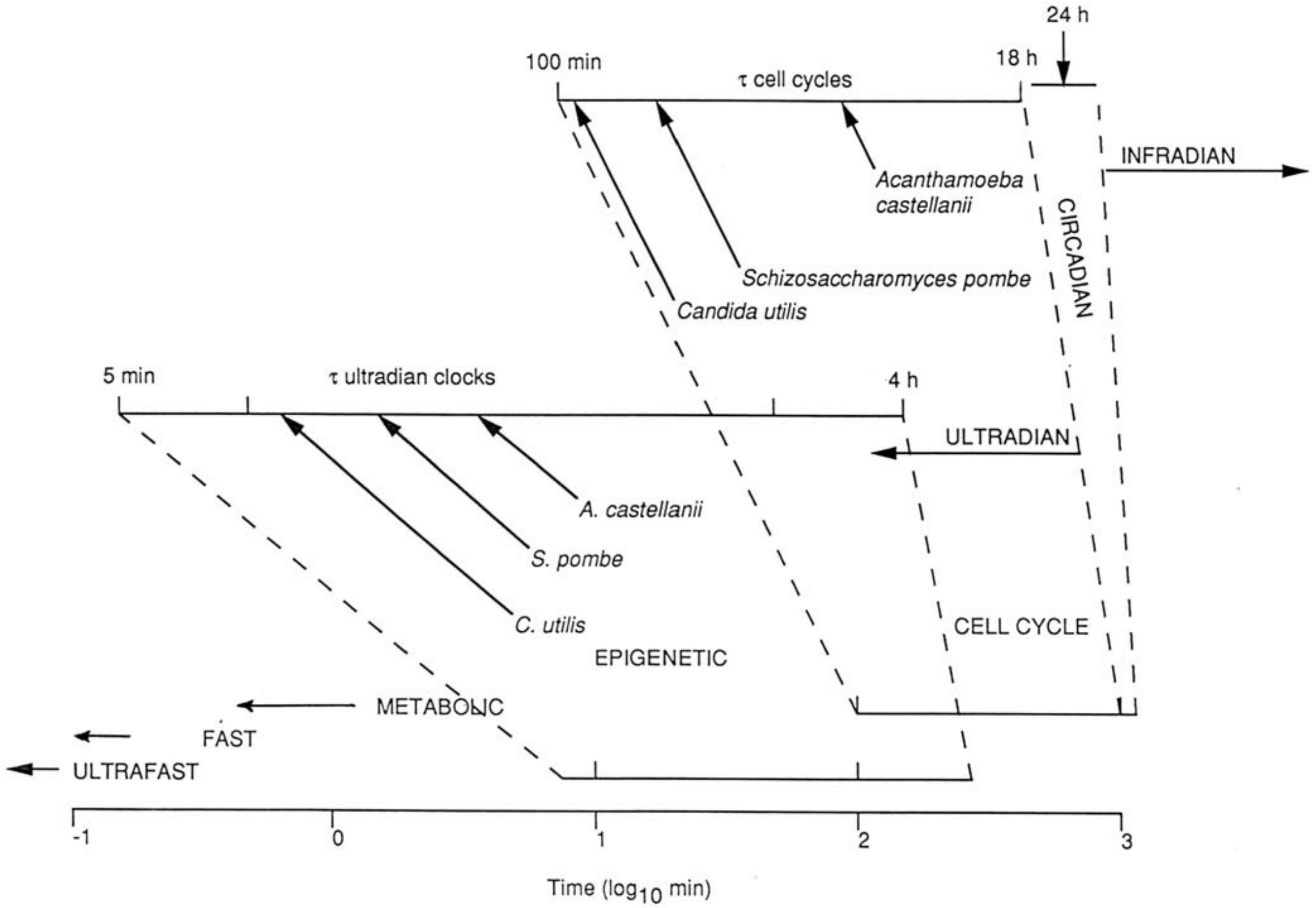


Figure 4. A heterarchy of time scales.

to produce organisms with large numbers of very small mitochondria (Turner and Lloyd 1971). His entry into Denmark was not as troublesome as had been the case when he was arrested at the Philadelphia customs for carrying "a drug" (chloramphenicol). Chance had personally to secure his release on that occasion. Robert Poole used size selection of *Schizosaccharomyces pombe* (Poole *et al* 1973) to uncover the oscillatory respiration of this fission yeast and to produce cell cycle maps. At this time we did not realise that the phenomenon we observed was the most easily observed output of the ultradian clock. Clive Edwards, Carol Phillips and Jalil Kader showed similar oscillatory phenomena in *Crithidia fasciculata*, *Tetrahymena pyriformis* and *Candida utilis* respectively, and went on to measure cellular adenylates. Clive Woffendin and Alan Griffiths showed that the ultradian clock in *Dictyostelium discoideum* has a cycle time of 60 min. Their work was highly controversial at a time when cellular growth was thought to be a smoothly increasing function. Publication was difficult, as certain authorities in the field were not to be easily persuaded that the changes we observed were not the consequence of poorly controlled temperature conditions or sources of experimental perturbation. Neither was it realized (despite the prescient work of another life-long colleague and friend, the late V N Luzikov of Moscow State University) that hydrolytic disassembly of macromolecules is an essential prerequisite for growth (Luzikov 2002). Our assertions that massive intracellular turnover plays a key role, and that half-lives of enzymes are to be measured in minutes (rather than hours or days) were dismissed as fairy tales. At that time the work of Goodwin (1963), Gilbert (1974) and Brodsky (1975) was highly influential to my appreciation of the oscillatory nature of the temporal organization of living organisms. One leading guru was eventually gracious enough to send me a letter of apology for his disbelief of our results, although only a decade later, by which time the roles of proteases, the proteosome and ubiquitination in 'destructive creation' had become clear to all. Abdul Chagla and Steven W Edwards (now Head of Biological Sciences, Liverpool University) also became involved in this work, the former with his supervisor, Dr Alan Griffiths, inventing a very gentle, quick and simple centrifugal size-selection procedure for synchrony of *Acanthamoeba castellanii,* and the latter contributing through a series of heroic experiments to confirm the temperature-compensated nature of its respiratory oscillation. Thereby, the function of the oscillation was established, it was a basic timekeeper, a biological clock, most evidently the one that times the cell division cycle. It should be stressed here that many periodic outputs that had been observed previously as oscillations (often the consequences of sloppy control or sometimes highly damped signalling systems), or rhythms (more sustained), are not biological clocks. For instance the cell division cycle (often mistakenly called a "cell cycle clock") is clearly not temperature-compensated. Thus growth rates are highly temperature-dependent, and shorter division times depend on a diminished number of component ultradian clock cycles. This device we referred to as "the epigenetic clock", because its functioning was independent of nuclear events (e.g. DNA replication). In fact what we had discovered was the Ultradian Clock, only the second class of biological clock to be described (Edwards and Lloyd 1978, 1980; Lloyd *et al* 1982). The first (the Circadian clock) had been discovered exactly a quarter of a millennium earlier. The pronounced temperature dependence of all the other metabolic oscillations so extensively researched and documented (e.g. the glycolytic oscillations and the cAMP oscillations in *Dictyostelium*) excluded them from being "ticks of the biological clock". I remember that Chance was well aware of this even in 1967. A Meeting organized by Dr Maurice Stupfel in Paris on ultradian rhythms in 1984 opened eyes to the wider significance of this field. There I met Gunter Hildebrandt from Marburg, who had spent a life-time studying the "harmonic organization" of human physiology, Peretz Lavie from the sleep laboaratory at Haifa, and Lee Edmunds working on *Euglena gracilis*. Stupfel had himself obtained highly impressive time series data on the respiratory rhythms of small mammals and birds. He was a most influential figure who contributed a great deal to my interest in this field; he became an excellent co-author (Lloyd and Stupfel 1991).

6. Tübingen

A short stay at Wolfgang Engelman's lab in Tübingen provided further insights into ultradian rhythms in *Thallassomyxa australis* (Heather Silyn-Roberts), *Desmodium gyrans* (Bob Lewis and M K Chandrashekaran), as well as *Oxalis glycine*; Shekar, Bob and Heather have all spent sabbaticals at Cardiff. The 40 foot Cardiff tides were a revelation to Shekar, and he was able to study a Chlorella-containing planarian worm, *Convoluta roscoffensis,* at its most northerly habitat. In 1986, Bünning the distinguished emeritus Professor, was still very much a presence in Tübingen, where he had pioneered much of the basics of chronobiology and photoperiodism, not only in plants, but also in animals and unicellular organisms. His excursions to Java led to his recognition that circannual rhythmicity in non-tropical plants could have evolved by selection from the great variety of periods exhibited by plants living in the tropics. He also pointed out that "a demonstration of a similar kind is not possible concerning the evolution of circadian rhythmicity" (Bünning 1977).

7. In Cardiff again

A visit to Cardiff by Ernest Rossi provided the stimulus for our co-editing a book (Lloyd and Rossi 1993). It was amazing to me that a hypnotherapist should have found the rhythmic organization of Acanthamoeba interesting in the context of his work, but he explained to me that the 90 min human rhythm, so evident in the alternating episodes of REM and non-REM sleep (Aserinsky and Kleitman's basic-rest-activity cycle, BRAC) in fact continues during the waking hours. Thus the susceptibility of human subjects to hypnosis also shows this rhythm. The most easy observable indication of the 90 min ultradian is the nasal cycle (as monitored by the alternating velocity of air-flow through the left and right nostril). Hemispherical laterality of brain activity can also be followed using this technique. Latest developments confirming that ultradian timekeeping constitutes an essential organizational framework for biological function throughout the living world from amoeba to human physiology is to be found in a second co-edited volume (Lloyd and Rossi 2008).

Preparation of synchronous cultures of *Saccharomyces cerevisiae* by a size-selection method was never so easy as it was for the fission yeast, due to the highly asymmetrical mode of its cellular proliferation mechanism, and serious studies of the Ultradian Clock in this organism awaited the seminal experiments of Hiroshi Kuriyama in Tsukuba City, Japan, in the early 1990's. In the meantime, Ghassan El'Khayat (now Professor of Food Science at Damascus University), had produced excellent new data on the fission yeast, *Schizosaccharomyces pombe,* and Fred Kippert had in his MSc Thesis described the use of many of the available mutants to pinpoint clock control of the cell division cycle by the ultradian clock to the *wee1* protein kinase region of the cellular network.

Two visits to the Tsukuba lab were to prove pivotal in the future direction of our Cardiff work: there I was able to show that nitrosonium cations (NO^+) severly disturb the 40 min rhythm in *S. cerevisiae*. Continuing collaboration was ensured by the return of Dougie Murray on a Royal Society return fellowship to a post-doctoral position at Cardiff. He established temperature compensation of the period of the respiratory oscillation in the spontaneously synchronized yeast system in continuous culture, thereby confirming it to be an Ultradian Clock output. While I was on sabbatical in Australia, and with the help of a second post-doc, Manfred Beckmann, Dougie set up the Kuriyama system on home ground. Eshanta Salgado, from Sri Lanka, discovered that the 40 min Ultradian Clock of yeast is slowed by Li^+, and that monoamine oxidase type A inhibitors also perturb the system. These observations suggest the presence of signalling pathways in common with those of circadian control. This continuous culture equipment was used to great advantage by Marc Roussel, a six month sabbatical visitor from Lethbridge, Alberta, to run a 3 month experiment with the direct membrane inlet mass spectrometer probe providing measurements of dissolved O_2, CO_2 and H_2S every 15s (Roussel and Lloyd 2007). The resulting time series is perhaps the best experimental evidence for the operation of a chaotic attractor in a biological system (figure 5). This was a finding of enormous significance for our studies, as previous modelling studies by A. L. Lloyd (Lloyd and Lloyd 1993, 1995) with the help of Evgenii Volkov (Russian Academy of Sciences, Moscow) and Lars Folke Olsen (U. Southern Denmark, Odense) had pointed to the complex dynamics of interaction between the cell division cycle oscillator and the Ultradian Clock.

8. In Baltimore

Access to multi-photon scanning laser microscopy in the Molecular Cardiobiology group of Brian O'Rourke at the Johns Hopkins Medical School in Baltimore provided a powerful extension to our confocal microscopy facilities in Cardiff, where Tony Hayes had employed his considerable expertise to our imaging needs over a period of about 10 years. Miguel Aon and Sonia Cortassa, with whom I had collaborated on theoretical ideas of cellular dynamics since 1998, on their home ground at Chascomus, Argentina were able, shortly after their arrival in Baltimore to provide experimental input for our ambitions to image oscillatory phenomena in *S. cerevisiae*. A really tremendous collaboration has resulted. Considerably bolstered by two visits by Katey Lemar, a PhD student from our Cardiff Laboratory, we were able to measure apoptotic events in *Candida albicans* and respiratory oscillations with a period of minutes in single *S. cerevisiae* (Aon *et al* 2007). Loss of autonomy of the individual yeast occurs when it is surrounded by others in a single layer on the surface of a slide. Within a very few minutes after the commencement of perfusion by aerated buffer containing glucose, the entire population falls spontaneously into

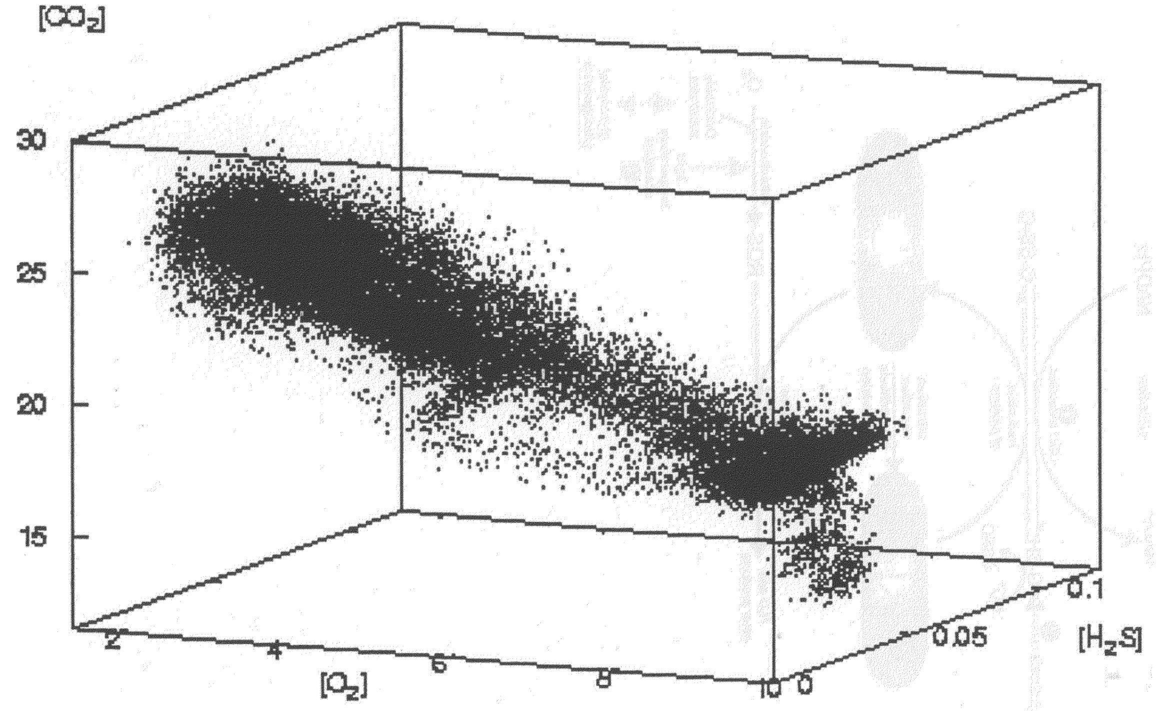


Figure 5. The metabolic attractor of *Saccharomyces cerevisiae* growing in a spontaneously synchronous continuous culture. Relative O_2, CO_2 and H_2S signals measured in the liquid phase are plotted using data points obtained at 15s time intervals over a period of 3 months. Monitoring was by a membrane inlet probe fitted to a Hiden quadrupole mass spectrometer (Roussel and Lloyd 2007).

a synchronized state, in which regular pulsation of the intracellular NADH pools (period 2 min) in both mitochondria and cytosolic compartments can be observed as blue fluorescence.

We now know much about the central functions of the 40 min ultradian clock in yeast. It acts as the central co-ordinator for eukaryotic metabolism, transcription and biogenesis of membranes and organelles, as well as timing nuclear events and the cell division cycle. Its core mechanism is a redox switch (Chance *et al* 2004, Lloyd and Murray 2006). It provides the time-base for the synchronization and convergence of events and processes within, not only unicellular organisms, but also in higher animal cells. It is highly-conserved and probably universal. It is an ancestral system; circadian rhythmicity is probably generated from it by an as yet uncharacterised mechanism (Lloyd 1998). Alternative possibilities include newly-understood principles emerging from studies of the dynamics of complex networks that involve metabolic and transcriptional interactions (Lloyd and Murray 2005, 2007). Cell-cell interactions (figure 6) and the development of multicellularity are dependent on the higher frequency respiratory oscillations that develop from ion fluxes (H^+, K^+, Ca^{2+}) across mitochondrial membranes and the interactions between these organelles within and between cells. Ultradian rhythms have recently been described as “the basic signature of life” (Yates and Yates 2008) on account of their ubiquity and the diversity of their functions (table 1).

Coherent operation of the whole panoply of reactions, metabolic pathways, transcriptional and translational processes, as well as membrane assembly and the operation of control systems within the living cell requires massive parallel processing synchronized to a time-line. Analogies with the complexity of computer construction help our understanding of these requirements. But of course comparisons are inadequate for the fathoming of the living organism where the scale of complexity far outstrips that of any man-made device.

The multiscillatory state of the respiration of a self-synchronized continuous culture of *S. cerevisiae*, as measured by the direct membrane inlet mass spectrometry probe described earlier shows periods of approximately 13 h, 40 min and 4 min, and as previously mentioned its trajectory can be represented as a chaotic attractor (Roussel and Lloyd 2007). Scale-invariant (fractal) features of this temporal behaviour are indicated by an analytical method known as Relative Dispersional Analysis (RDA) of the time series of the variation in dissolved O_2 levels in the culture. In this, statistical treatment of the experimental data compares the coefficient of variation of oscillation amplitudes over increasing intervals of sampling. Over three decades of time this measure did not change. Power spectral analysis gives an inverse power law relationship with a spectral exponent (β) of 1.95 (Aon *et al* 2008). The behaviour of dissolved CO_2 is more complex, possibly because it is a fermentation product which may feedback on the system. These analyses indicate a fractal temporal coherence of the yeast population over a range of scales from many hours to minutes and perhaps even to shorter time intervals limited by the resolution of the sampling.

A similar conclusion arises from extensive studies on guinea pig ventricular cardiomyocytes using two-photon excitation scanning microscopy to observe and monitor mitochondrial membrane potential and reactive oxygen species by fluorescence. Relative Dispersional and Power Spectral analyses of this system, like those obtained with yeast, showed a broad frequency distribution and evidence for long-term memory of the oscillatory dynamics. The multiple time scales (ms to h) exhibited by both systems suggest multioscillatory behaviour with simultaneously

Table 1. Functions of oscillations, rhythms and clocks

- A. Temporal organization
  - 1. Separation of incompatible processes
  - 2.Co-ordination of events, processes and states (Entrainment, synchronization)
    - a. Intracellular processes
    - b. Co-ordination of tissues or organs
    - c. Environmental matching (tidal, circadian, lunar, annual)
    - d. Predictive functions
- B. Energetic advantage?
- C. Spatio-temporal organization
  - 1. Signalling
  - 2. Developmental processes
    - a. Embryonic
    - b Growth

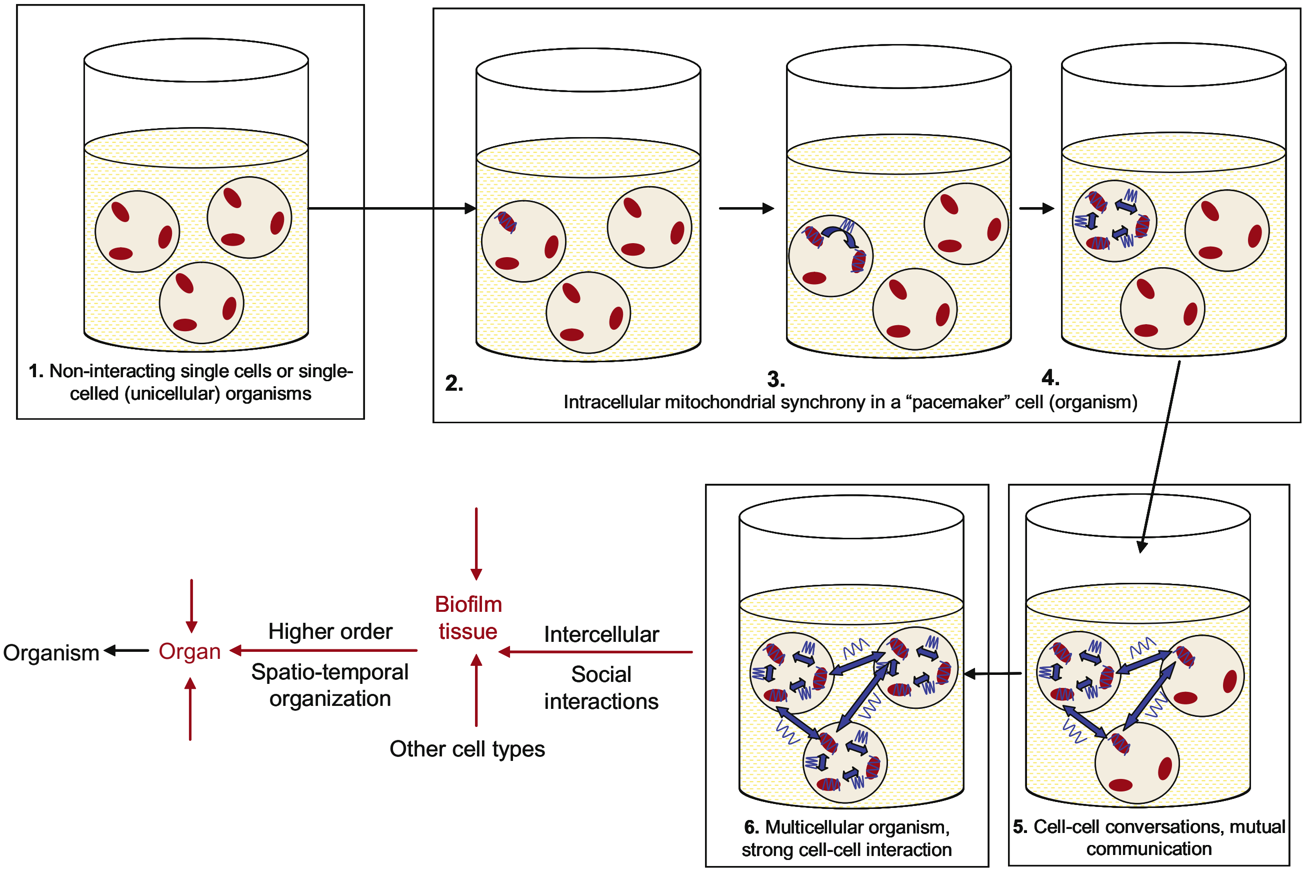


Figure 6. Spontaneous ultradian rhythms with periods of the order of a minute originate from mitochondria, firstly within a single organelle, then in a single organism where all the mitochondria have become synchronized. A messenger substance (e.g. superoxide) diffuses to a neighbouring organism, which resonates with the first organism. The synchrony rapidly spreads through the population, so that the autonomy of the individual is lost. Social interactions by way of intercellular conversations lead to multicellularity. For an on-line movie of this process in yeast, as monitored by fluorescence emission of NAD(P)H, *see* Aon *et al* (2007). (Figure by Dr Victoria Gray.)

frequency- and amplitude-modulated functional responses. Sustained and robust yet flexible integrated performance is characteristic of such scale-free networks and this description of a yeast culture provides new insights into the organization of its systems biology. Natural selection has ensured evolutionary conservation of the core mechanisms of the redox cycling system upon which this intracellular timekeeping and coordination is based.

Loss of coherence under stress (or at the onset of pathological degeneration) is indicated by loss of long-range correlations that presage cell death. The best-substantiated examples of this come from studies of heart rhythm (West 1999). Yeast provides another case where temporal disorganization leads to death by necrosis or apoptosis (Aon *et al* 2008). Thus, increased randomness of oscillatory performance (uncorrelated white noise), random-walk behaviour (Brownian noise), narrowing of the frequency spectrum on the appearance of highly periodic dynamics dominating one time-scale are all characteristic of dynamic disfunction that could be important diagnostics of disease states.

9. Conclusion

As in our model systems, the temporal organization of one's life-story is subject to a coherence that is at present beyond our full comprehension. One thing leads to another, and although little seems to be planned, it is predictable that our career trajectory is largely determined by the outside influences of greater minds (both of teachers and students). As role-models, Chance (now 95 and still prodigiously research-active) and Hastings (80 years old and still with a small team of researchers) are exemplary. Gregorio Weber, my earliest biophysics mentor passed away in 1997. His legacy continues to change the face of physical biochemistry and medicine. For eclectic vision and generosity of spirit, I would single out David Hughes, a truly remarkable Head of Microbiology at Cardiff for 18 years from 1964.

Acknowledgments

To my exemplary teachers, enthusiastic, inspiring postgraduates/postdoctoral associates and a host of collaborators. Those mentioned here are only those engaged in work on biological rhythms. Other projects, especially on organisms of medical or industrial significance, have been necessary to finance this rather arcane, but perhaps not misguided obsession.

References

Aon M A, Cortassa S, Lemar K M, Hayes A J and Lloyd D 2007 Single and cell population respiratory oscillations in yeast: a 2-photon scanning laser microscopy study; *FEBS Lett.* **581** 8–14

Aon M A, Roussel M R, Cortassa S, O'Rourke B, Murray D B, Beckmann M and Lloyd D 2008 The scale-free organisation of yeast and heart; Biophys. J. (submitted)

Bertallanffy L Von 1950 The theory of open systems in physics and biology; *Science* **111** 23–29

Brodsky V Y 1975 Protein synthesis rhythm; *J. Theor. Biol.* **55** 167–200

Bünning E 1997 Fifty years of research in the wake of Wilhelm Pfeffer; *Ann. Rev. Plant Physiol.* **28** 1–22

Chance B 2004 Mitochondrial redox state, monitoring, discovery and deployment in tissues; *Methods Enzymol.* **386** 361–370

Chance B, Pye E K, Ghosh Ad and Hess B (eds) 1973 *Biological and biochemical* oscillations (New York: Academic Press)

Edwards S W and Lloyd D 1978 Oscillations of respiration and adenine nucleotides in synchronous cultures of *Acanthamoeba castellanii*: mitochondrial respiratory control *in vivo; J. Gen. Microbiol* **108** 197–204

Edwards S W and Lloyd D 1980 Oscillations in protein and RNA during synchronous growth of *Acanthamoeba castellanii*: evidence for periodic turnover of macromolecules; *FEBS Lett.* **109** 21–26

Gilbert D 1974 The nature of the cell cycle and the control of cell proliferation; *Biosystems* **5** 197–206

Goodwin B C 1963 *Temporal organization in cells* (London: Academic Press)

Hastings J W 2001 50 years of fun; *J. Biol. Rhythms* **16** 5–18

Kamen M D 1963 *Primary processes in photosynthesis* (New York: Academic Press)

Lloyd A L and Lloyd D 1993 Hypothesis: the central oscillations of the circadian clock is a controlled chaotic attractor; *BioSystems* **29** 77–85

Lloyd A L and Lloyd D 1995 Chaos: Its significance and detection in biology; *Biol. Rhythm Res.* **26** 233–252

Lloyd D 1998 Circadian and Ultradian clock controlled rhythms in unicellular microorganisms; *Adv. Microb. Physiol.* **39** 292–339

Lloyd D and Murray D B 2005 Ultradian metronome: timekeeper for orchestration of cellular coherence; *Trends Biochem. Sci.* **30** 373–377

Lloyd D and Murray D B 2006 The temporal architecture of eukaryotic growth; *FEBS Lett.* **580** 2830–2835

Lloyd D and Murray D B 2007 Redox rhythmicity: clocks at the core of temporal coherence; *BioEssays* **29** 465–473

Lloyd D and Rossi E L (eds) 1993 *Ultradian rhythms in life processes: an Inquiry into fundamental processes of chronobiology and psychobiology* (London: Academic Press)

Lloyd D and Rossi E L (eds) 2008 *Ultradian rhythms from molecules to mind: a new vision of life* (Berlin: Springer-Verlag)

Lloyd D and Stupfel M 1991 The occurrence and functions of ultradian rhythms; *Biol. Rev.* **66** 275–299

Lloyd D, Edwards S W and Fry J C 1982 Temperature-compensated oscillations in respiration and cellular protein content in synchronous cultures of *Acanthamoeba castellanii; Proc. Natl. Acad. Sci. USA* **79** 3785–3788

Lloyd D, Venables W A and Evans B (eds) 1994 *Microbiology, past, present and future* (Bioline Cardiff University)

Luzikov V N 2002 Quality control: proteins and organelles; *Biochem (Moscow)* **67** 171–183

Morange M 2007 What history tells us XI. The complex history of the chemiosmotic theory; *J. Biosci.* **32** 1245–1250

Poole R K, Lloyd D and Kemp R 1973 Respiratory oscillations and heat production in synchronously dividing cultures of the fission yeast *Schizosaccharomyces pombe* 972h$^-$; *J.Gen. Microbiol.* **77** 209–220

Prebble J and Weber B 2002 *Wandering in the gardens of the mind: Peter Mitchell and the making of Glynn* (Oxford University Press)

Roussel M and Lloyd D 2007 Observation of a chaotic multioscillatory metabolic attractor by real-time monitoring of a yeast continuous culture; *FEBS J* **274** 1011–1018

Turner G and Lloyd D 1971 The effect of chloramphenicol on growth and mitochondrial function in the ciliate protozoon *Tetrahymena pyriformis* strain ST; *J. Gen. Microbiol.* **67** 175–188

Weber G 1990 Whither Biophysics?; *Ann. Rev. Biophys. Biophys.Chem.* **19** 1–6

West BJ 1999 *Physiology, promiscuity and prophesy at the millennium: a tale of tails* (Singapore: World Scientific) pp 162–193

Yates F E and Yates L B 2008 Ultradian rhythms as the dynamic signature of life; in *Ultradian Rhythms from Molecules to Mind: a New Vision of Life* (eds) D Lloyd and EL Rossi (Berlin: Springer-Verlag) (in press)

*e*Publication: 31 January 2008