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EDUCATION

An appreciation of the prescience of Don Gilbert (1930–2011): master of the theory and experimental unravelling of biochemical and cellular oscillatory dynamics

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Abstract

We review Don Gilbert's pioneering seminal contributions that both detailed the mathematical principles and the experimental demonstration of several of the key dynamic characteristics of life. Long before it became evident to the wider biochemical community, Gilbert proposed that cellular growth and replication necessitate autodynamic occurrence of cycles of oscillations that initiate, coordinate and terminate the processes of growth, during which all components are duplicated and become spatially re-organised in the progeny. Initiation and suppression of replication exhibit switch-like characteristics, that is, bifurcations in the values of parameters that separate static and autodynamic behaviour. His limit cycle solutions present models developed in a series of papers reported between 1974 and 1984, and these showed that most or even all of the major facets of the cell division cycle could be accommodated. That the cell division cycle may be timed by a multiple of shorter period (ultradian) rhythms, gave further credence to the central importance of oscillatory phenomena and homeodynamics as evident on multiple time scales (seconds to hours). Further application of the concepts inherent in limit cycle operation as hypothesised by Gilbert more than 50 years ago are now validated as being applicable to oscillatory transcript, metabolite and enzyme levels, cellular differentiation, senescence, cancerous states and cell death. Now, we reiterate especially for students and young colleagues, that these early achievements were even more exceptional, as his own lifetime's work on modelling was continued with experimental work in parallel with his predictions of the major current enterprises of biological research.

Keywords: cell division cycle dynamics; cell differentiation and oncogenesis; cellular ageing and senescence; limit cycle oscillations; temporal controls; ultradian rhythms of enzyme amounts and activities

Movement: Change in space of part or all of the organism.
"The basic characteristics of living organisms"

(F. Weaver 03.09.1954)

In summary, we propose that ultradian rhythms serve as the dynamic signature and co-ordinators of life. Metabolism is a self-orchestrated and choreographed chemical "dance". Ultradian rhythms in an organism cue other partners in that dance and the next steps appear.

(Yates and Yates, 2008)

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Abbreviations: ATRA, all-*trans* retinoic acid; CHO, Chinese Hamster Ovary cells; EGF, epidermal growth factor; FAD, flavin adenine dinucleotide; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; H-ras, protein encoded by human homologue of the HRAS oncogene of Rat Sarcoma virus; HL 60, human leukaemia cell line; HMBA, hexamethylene bisacetamide; ISOTT, International Society on Oxygen Transport to Tissues; L cells, intestinal entero-endocrine cells; MEL cells, mouse erythroleukemia cells; N-ras, protein encoded by the oncogene NRAS; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; PT1B, protein tyrosine phosphatase 2B; Ras/Raf, signal transduction cascade

Introduction

Don Gilbert was the second of four sons born (1930) in West Ham, London where he was raised. At the age of seven he missed schooling as he was hospitalised for diphtheria and scarlet fever. Of his latter childhood he remembered being on holiday in Wiltshire when World War II was declared, oblivious at the time to its full meaning. He also remembered being back home in London on one Saturday lunchtime and his elder brother dragged him into the garden shed as bombs rained down. He was not evacuated and remembered spending many hours in air raid shelters during the war sheltering from the bombing. It was not a childhood that typically would have led a path to University and achieving a Bachelor of Science Degree but this he did, supported by his wife, as an external part-time student of the University of London. It was about this time (circa 1955) that Don began work as a technical assistant (Figure 1) in the Chester Beatty (Institute of Cancer Research) in London and 3 years later he was promoted to Scientific Staff as a research biochemist (Figure 2), and also studied for his PhD, which was submitted in 1962 and was entitled "Studies in the Chemistry of Xanthine Oxidase".

None of his family had previously demonstrated an interest in study or science, so one can only assume it was an interest developed by his time spent in hospital in those early years. His understanding and move toward technology developed during his national service but this was with regards to communications rather than science. The interest in technology remained with him and came into use later during the development of information technology.

Don developed diabetes in his early adult hood but tried to keep fit by practicing the martial arts introduced to him by his brother who had spent time out in Japan during the war. Don went on to reach a black belt in Aikido and a brown belt in Judo, and enjoyed teaching moves to his children, nieces and nephews. He also recalled how once, after practice, in a pub a rather large fellow came up to his petite teacher (Sensei Yamada) and bet him a fiver (a not insignificant amount of money at the time) that the Sensei could force him to open his clenched fist, after looking carefully for a while Yamada pulled out a five-pound note from his pocket—the man opened his fist! Don often referred to this moment, of learning how to solve what appears to be insurmountable problems but not always using the most obvious or direct route.

Figure 1 Gilbert: the young scientist in his first research position. This photograph was taken on the roof of the Chester Beatty (Institute of Cancer Research) with a background of London landmarks. (Courtesy of Kay Gilbert.)

Figure 2 Don and his initial colleagues at the Chester Beatty Research Institute of the Institute of Cancer Research in London. Photo taken shortly after the author's first publication on temporal organisation in cells and his concept on the nature of cancer. (l to r) the late Dr. C. L. Leese, Don Gilbert, Dr. R. Yasin, the late Prof. F. Bergel, John Biffin. (Courtesy of Kay Gilbert)

Don's work was always a major part of his life but working at Chester Beatty in the early stages appeared to have additional side benefits. Not only did it provide a great place to see fireworks over London but it also became the back drop for encouraging his children to never smoke (the tarred lung in the labs being the clincher). He told his children that he did not work on the many animals kept at the Institute but brought one of the mice to his home to live as a pet with the family. His love for photography grew at this time and overlapped with his love of his work; photographs of family and of cell development often hung side by side in his tiny dark room at home.

It was a blow when Don was made redundant from Chester Beatty and it took nearly 2 years before finding and starting another job in the early 1970s, this time at the South African Institute of Medical Research. In 1972 leaving the United Kingdom to go to South Africa was difficult for him, leaving his friends and much of his family behind him. Don was certainly glad to be working again and settled in Johannesburg, keeping in touch with old friends and colleagues but also making new and good friends. One friend in particular spent a long holiday driving Don and family down the garden route, to Cape Town and back through the desert to Johannesburg. The trip resulted in many beautiful photographs and memories, and one in particular was turning up late for a hotel dinner,

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dusty and tired and getting steered into a corner where the more salubriously dressed guests, accompanying the then prime minister, could not see them. During his time in South Africa he moved from the Institute to the University of Witwatersrand and although he continued with this research he also found satisfaction in encouraging his research students to success.

When Don retired from Witwatersrand he returned to the United Kingdom and continued, as best he could, with his research and many publications. During his semi-retirement, few things exasperated Don in his private life however the exceptions were, first and foremost, losing his sight. The slow loss of sight created obstacles for Don to continue with his work. Even finding a folder on the computer was difficult despite the enlarged screen but he carried on researching, publishing and trying to complete his book. One of his other frustrations in his work was trying to use Microsoft software and he had many rants stating that it would not do what he wanted it to do. Following his typical approach that he took to most things in getting where he wanted to go, Don developed his own programmes and generally put Microsoft to one side. The last frustration though could not be overcome, and this arose from listening to the not so good West Ham football club results on the radio.

Don did not engage particularly in idle chat. Don's passion for this work euqalled his passion for his family and friends and he continued throughout retirement, latterly linking the life, movement, ageing and death of cells. In his life, faith and work Don did not feel bound by existing rubrics, in educating he encouraged his students "to think outside the box", to be free thinking and unconventional but also to follow where the evidence led. Equally he also believed in vigorously investigating and weighing up the outcomes and in his research, he believed he had proposed a new view of cancer and supplied supporting data for it (Gilbert, 1995a).

Early work

Don's earliest publication was of work from 1958 at the Chester Beatty Research Institute, Institute of Cancer Research at the Royal Cancer Hospital in London. This concerned a metalo-flavoprotein, xanthine oxidase, an enzyme he showed to have anti-tumour and other effects in mice that he helped purify from bovine milk. This important advance was achieved in the group headed by Professor A. Haddow. Working also part-time with soluble nucleotides and nucleotide-amino acid compounds from yeast, he completed his PhD in 1962, already having published two papers in Nature (Gilbert and Yemm, 1958; Haddow et al., 1958).

By this time Don had become an expert at the most advanced enzyme purification and assay procedures for redox enzymes and had collaborations at the biochemistry department in Sheffield University. Mechanisms of reactions of oxidative enzymes were probed there by Massey et al. (1960), soon to become the leading world expert on flavoproteins. Quentin H. Gibson with L. Milnes in the Biochemistry Departmental workshop (Gibson and Milnes, 1964), had devised and built instruments to study the rapid kinetics of enzyme reactions, and their use had resulted in seminal insights. Don's first solo paper (Gilbert, 1963a) was his third one in Nature, that most prestigious of journals, and was followed in successive years by another there (Gilbert, 1963b), and then by two more in the Biochemical Journal (Gilbert and Bergel, 1964, Gilbert, 1964), thereby helping completion of a long-running series of 10 contributions on xanthine oxidase by the Chester Beatty team. His 1964 paper, detailing an improved method for the purification of xanthine oxidase, using bovine buttermilk obtained from the National Institute for Research in Dairying at Shinfield near Reading, was with F. Bergel who had worked for more than a decade with R. C. Bray, a well-known expert on flavin enzymes (Bergel and Bray, 1959).

With Dr. M. McCabe of the Department of Biochemistry at the Institute of the Chest, Brompton Hospital, also in Fulwood Road, new measurements of the diffusion coefficient of oxygen (McCabe and Gilbert, 1965), through actively respiring tissues of various types are clearly of considerable importance, but still present many complications. From the work of Krogh (1918) until the present time, a very wide range of values for oxygen diffusion coefficients have been reported ranging from 1.1×10^{-4} to 4×10^{-8}cm^2/s. Because of these uncertainties it was decided to revaluate using a technique essentially similar to that described by Longmuir and Bourke (1960), but with the difference that the measurements were made a single slice of tissue at a time, thereby avoiding the complication of "edge" effects. The International Society on Oxygen Transport to Tissues (ISOTT) had its 47th meeting last summer (2019) at Albuquerque, New Mexico, to debate the newest methods of investigation. The paramount importance of the efficiencies of transport of oxygen from blood capillaries to cells in situ in tissues in animal models and humans (under both physiological and pathophysiological states), cannot be overestimated, and advances are regularly published in "Advances in Experimental Medicine and Biology".

Gilbert's major contributions: cellular oscillatory organisation, reorganisation and disorganisation

In the mid-196s only a very few investigators appreciated the detailed underlying kinetics of the forces and fluxes driving the microscopically evident ceaseless motion of the "cytosol", or the forces responsible for astonishing

Figure 3 Don at his desk. (Courtesy of Kay Gilbert)

transformations as observed by more extended microscopic observation of living cells and organisms.

In 1968, Don began to provide answers mainly at his desk (Figure 3), but was increasingly backed by his own ideas, observations and measurements from experiments on the growth of cultured mammalian cell lines. These novel approaches were received only in passing at the time, and hence not widely cited. Ahead of their time, they are now more widely appreciated: even so still not emphasised in textbooks or undergraduate lectures. The application of basic principles of the dynamic behaviour of physical systems to living organisms, and incorporating ideas from newly emerging electrical circuit theory and analogue computing was in its infancy and being considered by only a few exceptional investigators (Goodwin, 1963; Higgins, 1967; Chance et al., 1973).

Necessary properties of the maintenance of cellular energy generation and the capacity for survival as well as orderly replication for the continued existence of the species required a novel theoretical basis and the formulation of physiologically realistic and useful models. This leads to the conclusion that ultradian rhythmic processes on many time scales (milliseconds to hours and days) underpin the life of the cell (Goodwin, 1963; Brodsky, 1964, 1975; Gilbert, 1966; Reich and Sel'kov, 1975; Poole and Lloyd, 1973; Stupfel and Plétan, 1983; Schultz and Lavie, 1983; Lloyd and Stupfel, 1991; Stupfel et al., 1995; Guzman et al., 2017).

Many years previously there were experiments indicating the involvement of dithiol-disulphide reactions in redox maintenance in rabbit skeletal muscle (Rapkine, 1931), and eventually Mano (1977) described the involvement of glutathione in cyclic protein synthesis in developing sea urchin embryos. Klevecz (1969) studied the synthesis of lactate dehydrogenase in the cell cycle of synchronised Chinese Hamster Ovary (CHO) cells and described oscillatory synthesis such that the half-life of this enzyme was 2–2.5 h in a cell cycle time of 11.5–12 h.

Resting cells exhibiting quiescence were considered to reflect a steady state, in which "homoeostasis" results in balanced synthesis and degradation of cellular constituents in a controlled process of slow turnover. Replication requires the autodynamic occurrence of a cycle of oscillation that initiates, coordinates and terminates a process of growth, during which all components are duplicated and become spatially re-organised in the progeny. Initiation and suppression of replication represent switch-like characteristics, that is, bifurcations in the values of parameters that separate static and autodynamic behaviour (Goodwin, 1976).

Sel'kov (1970) published a mathematical analysis of the behaviour of the dithiol-disuphide transformation as a pair of differential equations yielding limit cycle solutions. This model was developed very much further in a series of papers reported between 1974 and 1982 by Gilbert showing that most or even all of the major facets of the cell division cycle could be accommodated. The demonstration that growth involves very highly dynamic turnover (synthesis and degradation) of many, or even most, of the cellular coenzymes, metabolites and proteins, on time scales of minutes to hours was revelatory.

This is also so for enzymes, not only in a fission yeast (Poole and Lloyd, 1973), but in human embryo kidney epithelial (HaK) cells and fibroblasts from hamster kidneys (BHK) cells (Gilbert, 1974a,b; Gilbert and Tsilimigras, 1981), a wide variety of mammalian tissues and cells, (reviewed in Brodsky, 1975, Brodsky and Nechaeva, 1988), *Crithidia fasciculata*, a trypanosome, (Edwards et al., 1975), *Tetrahymena pyriformis* (Lloyd et al., 1978) and *Acanthamoeba castellanii* (Edwards and Lloyd, 1978, 1980; Lloyd et al., 1982b).

All these experiments required a reappraisal of the "homoeostatic stability" of the living state: Yates (1994) has previously advocated that the term "homeodynamic" be used to indicate the generally unexpected transience of physiological lifetimes of molecular species, and Lloyd et al. (2000) suggested that the term "homeostatic" should only be used where strict limits of control are measured.

The advantages of the adaptability of structure and function in yeast were exploited very productively by V. N. Luzikov and his team between the late 1960s until Valentin's untimely death in 2007. They showed that the stable expression of the respiratory components, and the activities of the entire mitochondrial cytochrome chain, were dependent on the continued energy demands of the cell. Moreover, arrest of energetic demand led to immediate and rapid active degradation by proteolytic enzymes. Now, Luzikov's highly original ideas about the necessity for the rapid turnover of mitochondrial organelles, membranes, and especially mitochondrial respiratory chain components is well accepted. After a lifetime's work on yeast: ("selection by performance criteria" and "stabilisation by functioning": Luzikov et al., 1970; Luzikov, 1985, 2009), controls of mitochondrial fission and fusion, during the cell cycle, and also mitoautophagy, are all "hot topics" of inquiry (Lemasters, 2005).

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Furthermore, that "quantised" cell division times may be controlled by a multiple of higher frequency (ultradian) rhythms in mammalian cells, *A. castellanii* and yeast (Klevecz, 1976; Lloyd et al., 1982a, 1992) gave further credence to the central and key importance of oscillatory phenomena. Much recent work indicates that most coenzymes, metabolites, RNA species, peptides and proteins show oscillatory formation and decay (or "active destruction" on short time scales [minutes to a few hours] [circahoralian or ultradian half-lives]) during cellular growth. Perturbation of redox balances (e.g., by excessive generation of reactive oxygen species) leads to accelerated decline of the capacity for redox balances and consequent generation of energy and cell death (Lloyd et al., 2003; Lemar et al., 2005, 2007).

Research initiated in the laboratories of Brodsky (1964), Ghosh and Chance (1964), Gilbert (1966), Klevecz (1969), Luzikov et al., (1970), Poole and Lloyd (1973), Poole et al. (1973); Satroutdinov et al. (1992), Aon and Cortassa (1994, 1997), and Cortassa et al. (2012), now carried forward by their many collaborators and students (Lloyd et al., 2018), has led to indisputable evidence for oscillations, synchronising timekeepers ("clocks"), and autonomous rhythms throughout biology.

Further application of the concepts inherent in limit cycle and more complex multi-oscillatory dynamic modes of operation were shown to be applicable to cellular enzymology, growth, differentiation, oncology, senescence and cell death. These linked topics preoccupied Don Gilbert over his entire career.

Dynamics of cellular differentiation and oncogenesis

This was a novel outline concept of differentiation and oncogenesis based on the existence of cellular biochemical periodicities. From 1966, Gilbert was considering the functions of isoenzymes in cells of various tissues, and in 1968 he proposed that many properties of a cell are largely determined by the pattern of oscillatory variations in constituent levels resulting from a concerted operation of numerous metabolic control circuits. These ideas were providing interpretation of many observations acquired from experiments in the field of bioenergetics with systems from the electric eel (Maitra et al., 1964) to yeast (Chance et al., 1973) and bacteria (Harrison, 1970; Degn and Harrison, 1971).

Gilbert (1968) went a stage further to suggest that cellular differentiation and oncogenesis are emergent processes requiring alterations in the absolute and relative values of the dynamic parameters governing cellular periodicities and hence changes in the patterns of cellular rhythms. Thus, the actions of oncogenic agents were also interpreted in terms of temporal disturbances. Fertilisation of the ovum could be considered to be a perturbation initiating rhythmic responses in the regulatory circuits, while organogenesis and ageing reflect the approach of cells (and the organism as a whole) towards a new "aperiodic state" as a result of declining (dampening) processes. Such views indicate that malignant transformations may occur spontaneously. Some experimental evidence, indicating periodic isozyme pattern changes in cells, was also presented in support of these concepts. By 1969, previously reported studies involving kinetic analytical methods had indicated that cold shock could induce periodic variations in the lactic dehydrogenase and aldolase isozyme patterns of cultured cells.

Phase plane plots analyses suggested the possibility of coupling between the two isozyme systems (Gilbert, 1969).

Thirty years later, new evidence for the extremely dynamic relationship of the amounts of enzyme proteins and activities of the lactate dehydrogenase isoenzymes (Gilbert and Hammond, 2008) confirmed his theoretical predictions and continuing experimental work that presented excellent evidence "indicating the need for a thorough re-evaluation of our understanding of cell biochemistry". Wheatley (2000) also reviewed his own earlier results on determinations of protein turnover rates, and correctly questioned the evidence for the ubiquity and physiological significance of ultradian rhythms. His main and entirely understandable (and widely-held) opinion was that the enormous energetic cost of rapid degradation of newly synthesised proteins makes such behaviour unlikely, or at least a rare curiosity. However, homeodynamic activity appears to overrule the idea that cellular growth in well-oxygenated conditions is energy-limited (Lloyd and Callely, 1965). In the same special issue of "Cell Biology International" (Guest Editor, V. Y. Brodsky, Brodsky (2000)), a useful collection of contributions further confirmed the growing conviction of the necessary functions of rhythms. However, strict criteria for setting-up adequate sampling regimes, minimisation of disturbances, fast quenching of samples, data evaluation, de-trending, smoothing procedures and statistical evaluation must be followed (Gilbert and Ferreira, 2000). "Aliasing" avoidance requires that at least three, and preferably more, samples per cycle of the oscillation in data must be sampled. Routine calculations of specific activities of enzymes based on total protein in extracts where enzyme activities and extracted protein concentrations are oscillating out-of-phase are necessarily highly inaccurate. This is an extremely expert and valuable document.

It should be noted that despite intensive research and an enormous literature over 60 years, defining conclusions on possible functions of glycolytic oscillations are still uncertain: recently it has been proposed that they are necessary concomitants of the maintenance of low entropy and the temporally organised dynamics of water in cells (Thoke et al., 2018a,b).

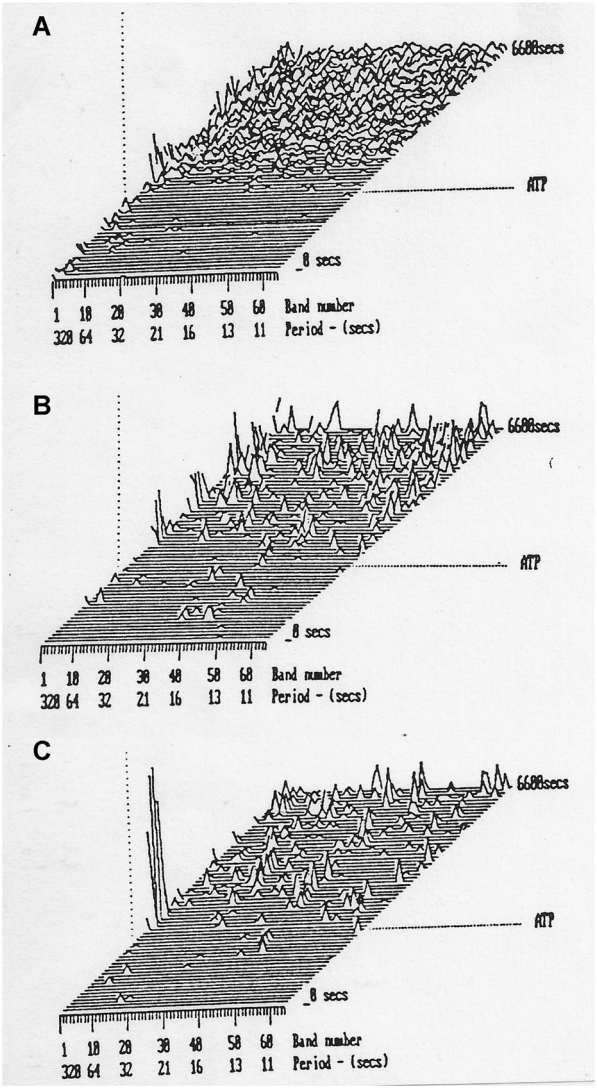

Figure 4 Overall power spectrum analyses of oscillations observed in a stirred suspension of murine erythroleukaemic cells. Changes after adding ATP in (A) cell surface activity, (B) NADH, and (C) oxidised FAD

Extremely rich oscillatory behaviour on time scales ranging from seconds to several minutes became evident in spectrofluorometric traces of the adenosine triphosphate (ATP)-enhanced autofluorescent emissions of both nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD), and also the light scattering from whole cell suspensions of murine erythroleukemia (MEL) cells (Figure 4; Visser et al., 1990, Visser, 1993). Whereas the former are ascribed to the complex networks of intracellular metabolic redox reactions, the latter are the consequences of the rapid morphological changes in cell surfaces observed microscopically ("ruffling and surface undulations").

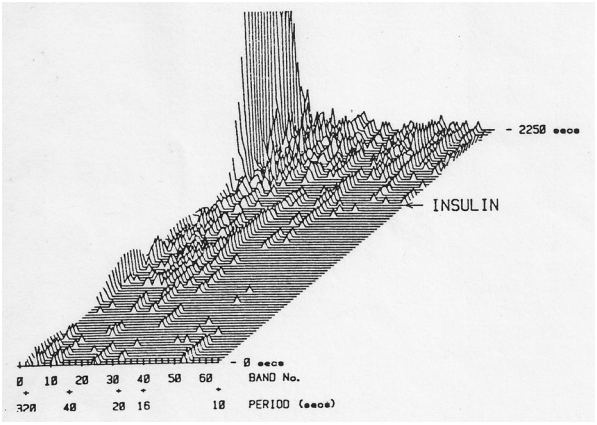

Figure 5 Power spectrum analysis of the response of the light scattering in a suspension of mouse erythroleukemia cells to an addition of insulin. Oscillations on a broad range of frequencies are enhanced by the hormone (Visser, 1993; Visser et al., 1990)

This diagram provides the successive power spectra each showing oscillations with periods of between 11 and 328 s, as obtained at 8 s intervals over a total period of 6,686 s, and showing the effect of additions of 1 μM ATP on (a) cell surface activity (light scattering due to cell surface and "ruffling" undulations, and fluorescence emission bands of: (b) NADH (450 ± 20 nm), and (c) oxidised FAD 520 ± 20 nm metabolic redox rhythms) (Visser, 1993; Visser et al., 1990).

This dynamic behaviour was stimulated by additions of insulin (Figure 5), the chemotactic peptide, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), epidermal growth factor (EGF), and transferrin (Gilbert and Visser, 1993). Whereas frequencies of redox changes were enhanced for extended times, fMLP or ATP additions had more transient effects, although just as rapidly evoked for each, and appeared to cover similar frequency ranges. After about 20–30 min the undisturbed behaviour of cells was restored. Results suggest that the observed membrane motility was autonomous.

Redox changes in NADH and in 90° light scatter in washed suspensions of intact *A. castellanii*, suspended in 130-mM NaCl, with periods of 10 and 13 s were obtained by flow cytometry at Cardiff (Visser, 1993). Confirmation for NADH autofluorescence and increased 90° light scatter was obtained using a diode array spectrophotometer in the Johannesburg lab, and a 200 s period for FAD was also noted in Enright periodograms. Similarities between amoeba and mammalian cells in these intracellular redox and cell surface characteristics indicate their evolutionary conservation over about 1.6 billion years as important and significant autonomous processes, cell–cell interactions and synchrony, and of responses to various perturbations.

Interaction between oscillators sometimes leads to further apparent complexity (e.g., bursting, intermittency and stages of transient chaos; Gilbert and Lloyd, 2000; Gilbert et al., 2000).

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In the laboratory of Hiroshi Kuriyama, Satroutdinov et al. (1992) discovered that yeast growing under defined controlled conditions at a low O_2 concentration in continuous culture self-synchronises, with intercellular signals acetaldehyde and H_2S (or related products) maintaining coordinated behaviour within the population (Murray et al., 2003; Brodsky and Lloyd, 2008); thereby a splendid system is provided for the study of the timekeeping of the ordered sequences of intracellular coherence. The Tsukuba group produced a series of papers showing that the formation of fermentation products show high amplitude oscillations (ethanol, acetaldehyde and acetate) in the growth medium, with phase differences and with a 40 min periodicity. Mitochondrial respiration, measured as dissolved O_2, provides the most easily monitored variable, and Lloyd and Murray (2000) proposed a mechanism with a redox cycle at its core. Experiments demonstrating respiratory control by additions of adenosine diphosphate (Lloyd et al., 2003), and with added inhibitors and uncouplers of energy generation indicated the pivotal role of mitochondria in the generation of the rhythm. Refinements in mass spectrometric analyses of rapidly sampled cultures further probed metabolic complexity (Murray et al., 2007, 2014), and eventually more than 1,650 oscillating chemical species were identified by using improved extraction and separation methods (Sasidharan et al., 2012). Furthermore, genome-wide transcription (Klevecz and Murray, 2001; Klevecz, 2004), as well as the dynamics of mitochondrial membrane and chromosomal changes during the cell budding cycle, are all timed on the highly pervasive 40 min cycle (Amariei et al., 2014).

Deterministic chaos in self-synchronised populations of yeast has since been demonstrated in three-dimensional plots of dissolved gas concentrations (O_2, CO_2 vs. H_2S). These minimally perturbed, non-invasively interrogated continuous cultures were monitored over periods of several months to provide many tens-of-thousands of points to obtain adequate data sets (Roussel and Lloyd, 2007). Deterministic chaos had previously been demonstrated in biological systems and in enzyme catalysed reactions in vitro (Olsen and Degn, 1977, 1978, 1985). Advantages may be discovered for such an "ergodic system" (one in which every point in the dynamic trajectory is visited), possibly providing opportunities for innovative performance (Lloyd and Lloyd, 1993, 1995; Lloyd, 2009; Lloyd et al., 2015). Gradual lowering of the pH of the yeast culture also results in the appearance of a strange attractor in the respiratory dynamics, one signature of chaotic behaviour (Murray and Lloyd, 2007). Kembro et al. (2018) show how mitochondrial redox-energetic behaviour lies at the edge of stable performance.

Another related characteristic of many multi-oscillatory systems (including in human physiology, Hildebrandt, 1979) is their self-similar (fractal) time structures, that is, statistical relatedness of relative period/amplitude properties and "memory" of earlier temporal relationships. Thus, spectral and relative dispersal analyses of synchronised yeast cultures oscillating with 13 h, 40 and 4 min periods (Aon et al., 2007b; Roussel and Lloyd, 2007) confirmed the predicted fractal time structure of rhythmic respiratory and enzyme activities (Brodsky, 1992; Yates, 1994; Aon et al., 2000; Yates and Yates, 2008).

Respiratory oscillations in single yeast cells have been observed in several studies using 2-photon excitation of NAD(P)H autofluorescence and inner mitochondrial membrane potential (Aon et al., 2007a), and also by microfluidic flow methods (Papagiannakis et al., 2017; Baumgartner et al., 2018), thereby avoiding possibilities of population-dependent effects. Oscillations of membrane potential in individual mitochondria from minute biopsy samples can now be measured by fluorescence or even more sensitive electronic means (Zand et al., 2017).

Zang and Wang (2019) have recently reviewed the coupling of the processes of the cell cycle to cell differentiation in lower eukaryotes as well as in tissue and organ development.

The cell division cycle in eukaryotic cells

The smooth and correctly ordered progression of growth and division is a highly ordered process of great complexity and almost always occurs faultlessly. It includes all the processes of nutrient uptake, the control of balanced ionic composite, the networks of inter-conversions of intermediary metabolism, the transcription and translation of macromolecules, the assembly of membranes and cell walls and matrices, membrane trafficking and genome replication. Networks of metabolic, genetic and developmental controls and checkpoints co-ordinate all these processes.

Between 1968 and 1982 Don pioneered the application of oscillatory dynamic theories to explain many of the experimental observations accumulating in the biological literature.

The control of rates and states characteristic of functioning metabolic pathways in vivo, had been shown to depend on allosteric feedback and feed-forward inhibition/activation by small molecule effectors of enzyme-catalysed reaction steps. However, during this era many controversies had arisen in efforts to explain control of integrated cellular processes, especially that of cell growth and division. Two conflicting schools of thought considered either that a strict sequence of reactions, once initiated, culminated at the end-point of cell division, or that the variability of the duration of the cell division cycle could better be analysed as a limit cycle oscillator.

By the 1970′s it became generally accepted that cells contain numerous negative feedback-feed-forward control

systems that are frequently invoked for their ability to maintain metabolic homoeostasis. What was still widely agreed was that similar oscillatory mechanisms could not even partially provide explanations for the varied characteristics of the cell division cycles as established for lower single cell organisms or cultured mammalian cells. The three major classes of models (transition probability, mitogen [mitotic oscillator] and dependent pathways) discussed at that time were reviewed (Lloyd et al., 1982b; Lloyd and Gilbert, 1998).

There was no reason to believe that the replicating cell is an exception to oscillatory principles, yet seemingly paradoxically it is such a highly dynamic entity in that the levels of an individual constituent may vary with time, from an undetectable amount, to such an extent as to account for as much as half the cell mass. Also, morphology and internal structure has to change so remarkably.

By 1990, the problem of understanding cell division had become simplified through discussions about clocks and dominoes (Murray and Kirschner, 1989), and by the turn of the century, decisions on periodic dependence, oscillatory or "hourglass" mechanisms (Gilbert and Lloyd, 2000; Rensing et al., 2001). An earlier physiologically plausible dynamic model (Lloyd et al., 1992) that exhibits the possibility of deterministic chaos in the cell division cycle thereby remains a possibility.

Gilbert (1968) had always maintained that apparent inconsistency between theory and observation is easily resolvable if (a) the events of the cell cycle reflect the oscillatory generation of most of the regulatory processes and (b) proliferation control is exerted via transitions between periodic and aperiodic (or damped periodic) states as the result of changes in the values of the parameters determining the behaviour of the entire system. This concept can be briefly discussed in relation to: the wide variety of agents that can affect replication (Lloyd et al., 1982b); the existence of distinct non-proliferative states; the continuous control of proliferation rate; variations in the sensitivity toward cell cycle inhibitory agents; senescence; the "loss" of control of cell division in cancer (Gilbert and MacKinnon, 1992; MacKinnon and Gilbert, 1992). The importance of these hypotheses now finds intensive research applications in anti-cancer drug discovery (e.g., of specific protein kinase inhibitors, Bai et al., 2017).

Contemporary ideas (Lloyd et al., 2018; Murray et al., 2019) overwhelmingly envisage the cell division cycle as an emergent and phenomenally complex process (Klevecz et al., 2008; Chin et al., 2014), whereby a heterarchy of networked controls operate simultaneously and continually from molecular levels upwards, and downwards from the levels of whole cell, tissue, organ and whole organism physiology. Predominantly oscillatory at every stage and level, it employs clock-like synchrony or "timekeepers" as well as spikes, intermittency, simple limit cycle mechanisms, delays, futile cycles, other multi-oscillatory modes and non-linear constructs (quasi-periodic and chaotic).

Cellular dynamics

Don moved to South Africa in 1972, began research at the National Institute for Virology Sandringham, and with the financial support of the National Cancer Association of South Africa continued to write a series of contributions on the temporal order of cells. The processes to be elucidated include, not only the time-dependent structure and function of cells during their continuing state changes, but their even longer term dynamic processes of reorganisation and disorganisation. A life-long interest in the kinetics and control of the processes of normal cellular growth and division of cells, was Don's main preoccupation. This led him to proceed to model the changes evident on the attainment of normal tissue formation during development by cellular differentiation, and of the dynamics of abnormal changes during oncogenesis. Also considered were the natural processes leading to the declining capacities of cells and organisms during the ageing processes. Therefore, advances in understanding presaged in his 1968 paper were further elaborated in a seminal series of sole-author papers published until that dated Gilbert (1995b). The key concept underlying the dynamics of change in these phenomena was that all had in common features of living systems, that is, cellular biochemical periodicities, the oscillatory or rhythmic alterations in concentrations and/or activities of their individual chemical constituents. These continual changes require the operation of a network of controlling interactions, and normality is ensured by autonomic action, restraint or acceleration (Gilbert, 1971, 1984b). Disturbance of this state by oncogenic agents or spontaneous malfunction is a reflection and outcome of irregularity of control resulting from temporal perturbation. The process of fertilisation of the ovum represents an initiation of a complex sequence. Ageing and the gradual changes during senility is a consequence of loss of periodicity and dampening of essential life processes (Gilbert, 1995a,b). Don's deep understanding of enzymology helped him to progress to basic considerations of these key unsolved biological questions and to place them into the context of recently developed mathematical treatments of dynamic systems analyses as employed in biomathematics (Gilbert and Joosting, 1994), widely used by electrical and mechanical control engineers, and applicable to biology to great effect (Gilbert and Ferreira, 2000). Bacterial rhythms (Malarczyk and Pazdzioch-Czochra, 2000) are often not as well characterised as those in eukaryotes: one difficulty lies in their high sensitivity to perturbation.

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Disturbing influences (light, temperature and chemicals) detected by arrays of membrane receptors evoke easily-switched on/off signalling pathways (damped oscillators) to activate responsive reaction chains.

Underlying biological functions in normal and pathological states can now be assessed from time series analyses of stationary and non-stationary biological variables (Guzman et al., 2017; Kurz et al., 2017). Methods used include power spectra, relative dispersional, wavelet and metabolomics-fluxomics determinations (Cortassa et al., 2012).

For other recent reviews, see Brodsky (2014); Poon (2016); Ahn et al. (2017); Palmisano et al. (2017); Papagiannakis et al. (2017); Murray et al. (2019); Bokes and King (2019).

Temporal variations and controls in protein expression and enzyme activity

During his time in Johannesburg (Figures 6A and 6B), first at the National Institute for Virology and subsequently in the Departments of Biochemistry and Medical Biochemistry at the University of the Witwatersrand, Don developed further his ideas concerning the nature of the cell cycle and the control of cell proliferation, and provided experimental data in support of the concepts (Gilbert, 1973, 1977, 1978a,b,c, 1980, 1981, 1982a,b, 1984a,b, 1988, 1995a,b).

The evidence that the reversible phosphorylation of proteins influences virtually all aspects of metabolism was overwhelming but at that time little attention had been paid to the dynamic aspects of these processes. In his theoretical studies (Gilbert, 1974a,b), Don had predicted the involvement of dynamic control of phosphorylation in the regulation of cell differentiation and cancer (Gilbert, 1971) and his work in South Africa focussed on the oscillatory nature of such reactions. Again, there was an element of adverse criticism and disbelief toward his ideas and findings, but nevertheless he continued undeterred. I (KH) have to admit that I was also a sceptic when first introduced to these concepts, but having been persuaded by Don to participate in such research and to examine the methods used very critically, I had to accept that time-dependent fluctuations in content and activity of cellular proteins and enzymes really did occur. In collaborative studies, we and our colleagues—we called ourselves "The Dynamic Research Group"—provided a wealth of indisputable evidence for the existence of cellular oscillations in enzyme protein expression and activities and for modification of the waveforms by differentiating agents, hormones and growth factors.

It is pertinent to mention that Gilbert and Tsilimigras (1981) and Ferreira et al. (1996a,b) described the existence of oscillations in the activities and isoenzyme patterns of certain glycolytic enzymes in various mammalian cell lines demonstrating that this is a universal phenomenon, at least in cultured cells, and therefore of universal importance. The fluctuations in the amount of protein extractable from the cells were not primarily responsible for the enzyme rhythms, although protein oscillations may have contributed to the enzyme waveforms and become coordinated with them. A controversial observation for lactate dehydrogenase was that both the activity and the amount of active isoenzyme oscillated in cell- and particle-free preparations; it was suggested that this may relate to covalent modification of the enzyme protein by phosphorylation.

The special phosphorylated compound, ATP, a component of widespread significance, necessary for many reactions of interest, is produced by glycolysis and mitochondrial action. Analysis of ATP oscillations in several cell lines (Figure 7), gave support to the argument that the ATP level in a cell reflects the summation of several

Figure 6 (A and B) Don in Bryanston Tea Gardens near Johannesburg, South Africa; and with Kay in CapeTown

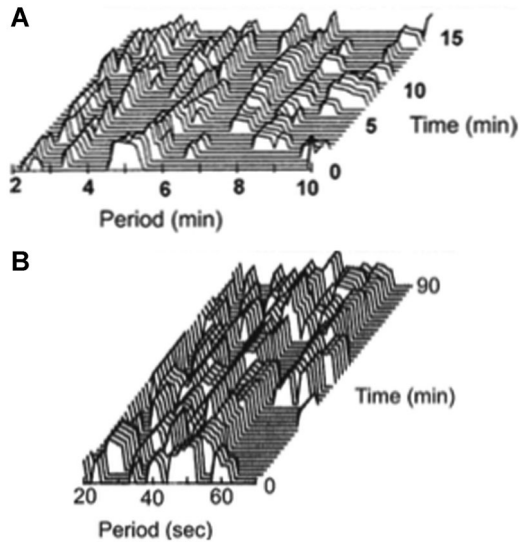

Figure 7 The total cellular pool of ATP produced and consumed by so many processes reflects the summation of many rhythms and forms parts of distinct control systems that can oscillate relatively independently. Enright periodograms; time series analyses of fluctuations in two mouse cell lines: (A) L cells, and (B) MEL cells. (Gilbert and Hammond, 2008). ATP, adenosine triphosphate; L cells, intestinal entero-endocrine cells; MEL, mouse erythroleukemia cells

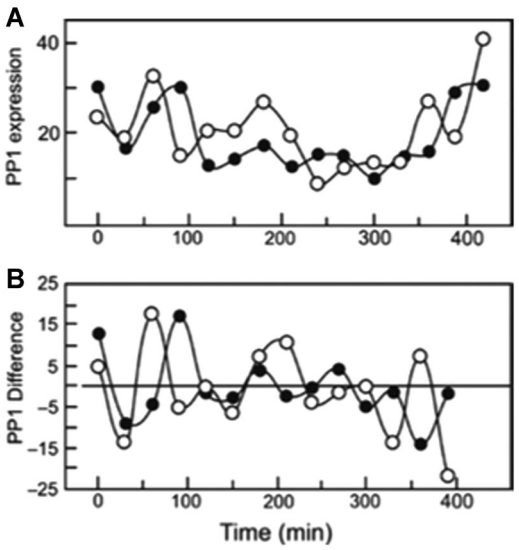

Figure 8 Variations in the expression of PP1 can be seen in proliferating (•) and HMBA-treated, differentiating (°) MEL cells (A). The difference curves (B) provide further illustration of the dynamic nature of the system (Gilbert and Hammond, 2008). HMBA, hexamethylene bisacetamide; MEL, mouse erythroleukemia cells; PP1, protein phosphatase 1

rhythms, consistent with the view that cells are multi-oscillators (Gilbert, 1974b).

The highly dynamic nature of protein phosphorylation was initially illustrated by Don and his co-workers in a study of changes in the phosphorylation potentials of certain specific proteins (Ferreira et al., 1994a,b), using MEL cells, which can be induced to differentiate and lose their malignant characteristics. The complex, periodic nature of the variations observed may have been the result of alterations in the activities of protein kinases and phosphoprotein phosphatases and the extents and multiplicity of phosphorylation, reflecting the involvement of several different enzymes affecting different residues. Cyclic behaviour of kinases and phosphatases was demonstrated in a series of further studies.

Suitable phosphorylated protein substrates were not obtainable at first. However, spectrophotometric assays for measuring the hydrolysis of phosphotyrosine, phosphoserine, and phosphothreonine were developed by the group (Hammond et al., 1985; Hammond et al., 1989a,b; Ferreira et al., 1996c). The activities of all three phosphatases in MEL cells showed cyclic variations; on treatment of the cells with inducers of differentiation or with insulin, differences in amplitude, frequency and phasing of the rhythms were observed.

When specific antibodies to key serine/threonine phosphatases became available, studies of the serine/threonine phosphatases, protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), and of protein tyrosine phosphatase 1B (PTP1B) enzyme protein expression during proliferation and differentiation of MEL cells (Figure 8), and HL60 cells, showed marked temporal variations in the intensities of immunoreactive bands. The magnitude of the fluctuations for PP1 was as much as 7–8-fold and for PP2A and PTP1B up to 10–12-fold (Hammond et al., 1998; Bhoola and Hammond, 2000; Bodalina et al., 2005).

In MEL cells, for all three proteins there were differences between the patterns of expression in proliferating and hexamethylene bisacetamide (HMBA)-induced differentiating cells; in some cases there were changes in amplitude and phasing of the rhythms, while in others there was a dampening or an overall increase in expression as compared with controls. Differentiation of HL60 cells along the granulocytic and monocytic pathways, induced by all-trans retinoic acid (ATRA) or phorbol myristate acetate (PMA), respectively, was associated with variations in the patterns of expression in all cases; the findings indicated that the dynamic control mechanisms operating for the two pathways were different. The wide variety of modulatory effects following treatment of cells with various differentiating agents reflected their dynamic nature and the complex time-dependent interactions between the individual regulatory processes within the cell, between the metabolic control systems of different cells and within the environment.

Studies of the dynamics relating to protein concentrations and the activities of protein tyrosine phosphatase (PTP) and protein tyrosine kinase (PTK) provided even more evidence of

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oscillatory rhythms (Calvert-Evers and Hammond, 2000, 2002, 2003). Time-dependent variations were observed for the enzyme activities of PTP and PTK and for the amount of total protein extracted from both proliferating and differentiating HL60 cells. Treatment of the cells with ATRA significantly altered the patterns of the waveforms. Once more a wide variety of modulatory effects could be seen, including dampening or suppression of activity, partial phase shift, phase shift and aperiodic random effect. However, no obvious relationship could be distinguished between the PTP or PTK enzyme activities and the protein concentration for either proliferating or differentiating cells; the poor correlation suggested relative independence of these sets of oscillators. Since no particular relationships could be detected for these periodicities, it was possible that the frequencies of the rhythms were different, or that multiple interacting rhythms were involved. Further analysis of the data showed that period and amplitude of PTP and PTK enzyme activities varied with time and there were definite changes in these characteristics during induced differentiation. A very noticeable decrease in amplitude was seen when there was dampening or suppression of activity during differentiation. This observation suggested an extra dimension of metabolic control through differential modulation of rhythmic characteristics such as amplitude.

Protein kinase C (PKC) plays an essential role in regulating signal transducing networks including certain mitogenic pathways that control cell proliferation and differentiation. Results had suggested a role for PKC in the differentiation of MEL cells and certain isoforms were implicated (Sprott et al., 1991). Subsequently, the dimension of time was introduced in a study of the expression of the PKC isoforms α, ε, and ζ, representing the novel, classical and atypical groups, respectively. Cyclic behaviour was apparent for expression of all three isoform proteins and on induction of differentiation, using HMBA, changes were apparent (Hammond et al., 2000a). This study also presented the first report of cyclic changes in the expression of messenger RNA (mRNA). The mRNA for PKCα and PKCε was analysed. There were definite differences between the patterns in proliferating and differentiating cells; the effects differed in different experiments, reflecting the complexity of the system, but in general there was a change in amplitude and phasing rather than frequency. Modulation of the dynamics of the signals delivered by the different PK isoforms could be one of the molecular mechanisms involved in MEL cell differentiation. The rhythms of the different isoforms may vary in concert with each other as well as with those of signal transducing cascades such as Ras/Raf in order to produce a coordinated response to extracellular effectors.

The ras genes are among the most frequently activated oncogenes in cancer. The Ras proteins interact with a wide spectrum of effector molecules, including Raf which is modulated by PKA and PKC. This interaction initiates phosphorylation cascades known to regulate cell proliferation and differentiation and to play a role in cell-cycle control and apoptosis. Time studies of the expression of Ras and Raf proteins and the mRNA specific to the H-ras and N-ras oncogenes showed cyclic behaviour; modulation of the patterns on induced cellular differentiation, suggested a temporal mechanism of regulation of this process through the Ras/Raf pathway (Hammond et al., 2000b,c).

Another protein of interest to us was the p53 tumour suppressor protein. This is a nuclear phosphoprotein, which when activated rapidly inhibits cell growth by arresting proliferation or inducing cell death, minimising the processes leading to malignant transformation of cells. Dynamic variations in the expression of this protein were detected in both untreated and HMBA-treated MEL cells (Bodalina et al., 2007). In all cases, as with other systems studied, the effects were complex with variations in amplitude frequency and phasing of the rhythms; during differentiation the patterns were modified in rhythmic fashion with respect to period and amplitude.

These studies of temporal control of phosphorylation and dephosphorylation and of signal transducing molecules in model cell systems have provided considerable insight into biological functions, lending support to the view that modulation of the dynamics is an important mechanism involved in cell proliferation and differentiation and the development and reversal of cancer. Don's theoretical and practical contributions in this field cannot be overestimated. During this period he was a guide and inspiration to many colleagues and research students.

After leaving the University of the Witwatersrand and returning to England, Don continued to provide support to his colleagues and friends working in Johannesburg. He began to write a book "Are you dead or Alive?" This was to be a synthesis of his work and ideas but sadly it was not completed. Don was a dedicated and passionate pioneer who throughout his career held true to his beliefs and convictions.

"The living cell is most surely dynamic,

It's behaviour so very erratic.

Why is it so true that all but a few

Insist on treating it static?"

D. A. Gilbert

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Conflicts of interest

No conflicts of interest.

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