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Anthony William Linnane 1930–2017

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Anthony (Tony) Linnane isolated mitochondria from bakers' yeast during his doctoral studies at the University of Sydney in the 1950s. He subsequently pioneered research into the biogenesis of mitochondria, covering enzymology, membrane biochemistry, and molecular biology and genetics, over more than two decades until the mid-1980s. These discoveries were made mostly at Monash University and earned him election as FAA (1972) and FRS (1980). Linnane thereafter broadened his research towards medical topics, especially the role of mitochondria in human ageing, together with studies on interferon and cancer-specific mucinous antigens. After retirement from Monash in 1996, Linnane worked towards ameliorating disease through bioenergetic strategies, based at the Centre for Molecular Biology and Medicine in Melbourne. He played significant roles in the Australian Biochemical Society and the International Union of Biochemistry.

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Family background

Anthony (Tony) William Linnane (Fig. 1) was born in Sydney on 17 July 1930. His father, William Patrick Linnane, born in Sydney in 1899, was a master plumber who also ran a bookmaking business.

Figure 1. Anthony W. Linnane, ~1994. Image of a framed photograph displayed in the Department of Biochemistry and Molecular Biology at Monash University (original photographer unknown; image taken by the author).

William's father Thomas had been born in County Galway, Ireland, and emigrated to Australia in the late nineteenth century. Tony's mother Irene Broadstock was born in Broken Hill in 1901. Tony was an only child and grew up in Clovelly, an eastern suburb of Sydney. William died when Tony was eleven years old. Tony did not have a close relationship with his father, who came from a strong Irish Catholic background. Tony rejected religious belief as he grew up.

After his father's death Tony was the only male in the household, living with his mother, a maiden aunt and his dominating maternal grandmother. Tony was very close to his mother and, long after Tony first came to Melbourne, she finally moved to a retirement home in Melbourne where she lived out her days until her passing in 1997.

Secondary and university education

Tony attended the Sydney Boys High School, 1942–6. His interest in science was kindled by an outstanding chemistry teacher, Leonard Basser, who had inspired many boys who went on to successful careers in science.[1] Tony completed his BSc at the University of Sydney in 1950, majoring in biochemistry and graduating with first class honours. In his honours year, he carried out research work with Eva Eden on carbon tetrachloride toxicity and its effects on creatine metabolism in rats. At university, Tony enjoyed competitive sports, including rugby and tennis.[2]

Masters and PhD research studies (1951–6)

Linnane carried out research work towards his Master of Science, and subsequently his PhD degree, under the supervision of Professor Jack Still, who was professor and head of biochemistry at the University of Sydney from 1949 to 1976. Linnane enrolled initially for an MSc degree in Still's area of interest on bacterial pigment

[1] Leonard Basser taught at Sydney Boys High School for 28 years until his retirement in 1959. He is reputed to have inspired at least eight boys who went on to become Fellows of the Royal Society, including Lord (Robert) May, a former President of the Society, and Sir John Cornforth, who shared the Nobel Prize for Chemistry in 1975.

[2] Author's note: I recall the one and only time that I played squash with Tony in the 1970s. He played his game fast and furious, always positioning himself to crowd me out so that I hardly ever had a chance to hit the ball. I had the unmistakeable impression that he only ever liked to win.

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formation, studying metabolic aspects in *Serratia marcescens*. He submitted his MSc thesis in February 1954.

For his PhD studies, Linnane took up the challenge of demonstrating that baker's yeast (*Saccharomyces cerevisiae*) contained mitochondria. This was an innovative project at the time, as it was not known if microorganisms, including bacteria or fungi, contained mitochondria. A strong hint that this yeast (already recognised as eukaryotic) contained a respiratory system had come from the pioneering genetic studies carried out in France by Boris Ephrussi and colleagues. They had already shown by 1951 that there were unusual 'petite' mutants of *S. cerevisiae* that were unable to respire and obligatorily used fermentable substrates such as glucose.[3]

To disrupt the sturdy cell wall of yeast (comprising chitins, glucans and mannoproteins), Linnane adapted physical methods for breaking open yeast cells without excessive disruption of intracellular membranes, using a shaker that abraded the yeast cells with glass beads, before isolating mitochondria by differential centrifugation.[4] His published reports demonstrated that the subcellular preparations were able to carry out respiratory processes characteristic of mitochondria, using the then available manometric and enzymological methods.[5]

Tony was initially very happy in Still's laboratory as he felt that he was being nurtured by his mentor. But a turning point came one day when Still remarked in Tony's presence that Tony would not amount to anything in science. Tony was mortified and not a little betrayed by those remarks.[6] In 1956, Tony married a local medical graduate Judith Ann Neil, who later became a psychiatrist. After Tony had completed his PhD thesis that same year, together they set off to the USA for Tony's postdoctoral experience in the laboratory of David Green in Madison, Wisconsin.[7]

Postdoctoral work at the Institute for Enzyme Research in Madison (1956–8)

When Tony arrived at the Institute for Enzyme Research at Madison (the Enzyme Institute), Green was at the height of his research endeavours. His research programs covered many core topics at the forefront of biochemistry research, including the detailed mechanism of the mitochondrial electron transport chain and oxidative phosphorylation (OXPHOS).[8] Linnane joined research projects on mitochondrial bioenergetics, concerning the component enzyme systems of the mitochondrial respiratory electron transport chain. A series of oxidation-reduction steps takes place along this chain, catalysing the oxidation of substrates such as NADH through consumption of O_2, as part of OXPHOS. As a result, mitochondria generate ATP from ADP, but the mechanism was not known at the time. The research aim was to systematically break down the mitochondria into functional components that could be eventually put back together to re-establish the full properties of intact mitochondria. In 1956, these biochemical approaches were being combined with newly developed electron microscopy techniques. Working on the so-called electron transport particles isolated from mitochondria, Linnane published five papers from his two years at the Enzyme Institute, on various aspects of the components of the mitochondrial electron transport and OXPHOS systems.[9] Such approaches in Green's laboratory (and many others in the field at the time) produced lots of enzymological and morphological information about the workings of mitochondria, but never could solve the issue of how energy from respiratory electron transport was coupled mechanistically to the formation of ATP in mitochondria.[10]

His time at the Enzyme Institute was formative for Tony. He was inspired personally by David Green whom he admired for his breadth of scientific vision and his ability to solve major research problems in biochemistry through the shrewd organisation of his research teams to address significant issues. Moreover, Linnane was thrilled to have met many internationally distinguished visitors who were, in his words, 'the mitochondrial giants of the day'.[11] He also revelled in the weekly seminars given by local and national greats of biochemistry more generally. He made many friends in the Green laboratory, many of whom he would continue to meet and interact with at conferences over the next decades. He would maintain collaborations with a few of these colleagues many years later (especially Fred Crane).[12]

[3] Ephrussi and Hottinguer (1951).

[4] Later methods used in Linnane's laboratory in the late 1960s utilised a French Press to break the yeast cells that was well suited to large scale preparations of mitochondria. Later still, enzymatic digestion of the β-glucan polysaccharides of the cell wall followed by gentle osmotic lysis of the spheroplasts became widely used.

[5] Linnane and Still (1955*a*). Linnane and Still (1955*b*).

[6] In recounting this episode, Tony commented that he resolved at once that he would eventually show them what he would be capable of! After this incident he worked through his anger by walking home (a long way) in the rain, rather than taking public transport. For his part, Still had disagreements with many colleagues over the years.

[7] The door to Linnane's postdoctoral experience in Madison was opened by Still's having spent a year in Green's laboratory some years earlier, during 1948–9.

[8] Beinert and others (2004).

[9] The first such article was by Ziegler and others (1958).

[10] The search for the elusive chemical intermediate, conjectured by some biochemists in the field at the time to be a phosphorylated molecule of the putative form X∼P, was bound to be fruitless because the bioenergetically coupled systems of mitochondria (as with those of chloroplasts and bacterial cell membranes) were found some ten years later to operate by chemiosmotic processes dependent on electrical charge separation across the closed biological membranes. These discoveries of Peter Mitchell that earned him the Nobel Prize in Chemistry in 1978 certainly disconcerted many of the mitochondrial research leaders of the 1950s and 1960s as they had to give up their preconceived ideas. This is all thoughtfully described by Bill Slater in his Biographical Memoir of Mitchell after the passing of the Nobel Laureate in 1992 (Slater 1994).

[11] Linnane (2010).

[12] Fred Crane was the first to characterise Coenzyme Q_{10} (CoQ, also known as ubiquinone), a lipid soluble intermediate in the mitochondrial respiratory enzyme chain (Beinert and others (2004)). The abbreviation CoQ will be used here, encompassing variants of Coenzyme Q mentioned below, not only naturally occurring Coenzyme Q_{10}. The suffix 10 refers to the number of isoprenoid units in the hydrophobic side chain that juts out from quinone ring where the redox reactions occur. Linnane hosted visits from Crane many times in later years in Australia, especially in the 1990s when Linnane became interested in research on ageing (see Supplementary Material).

Back in Sydney as a lecturer (1959–61)

In 1959, Linnane returned to the University of Sydney to take up a lectureship in biochemistry. He turned to yeast once again to research mitochondria not only because it was a biological resource easy to obtain, but also because yeast could be manipulated physiologically as a facultative anaerobe and had a tractable genetic system. Thus, from work by Ephrussi and colleagues in France, mutants of *S. cerevisiae* were already known that mitochondrial function was compromised, namely the *petite* mutants that had been shown to lack the mitochondrial cytochromes a, a_3 and b.[13] These French studies had suggested that the genetic factor controlling the *petite* phenotype was cytoplasmically inherited controlled by a factor denoted *rho.*[14] Although such genetic and cellular properties of the *petite* mutant had been reported, definitive morphological and biochemical characterisation of these respiratory-deficient mutants were yet to be carried out.

Linnane's initial focus on return to Sydney was to characterise morphologically the mitochondria in yeast cells, in comparison to what had recently been observed in mammalian tissues. The first publication described work carried out with Linnane's first PhD student Eberhard Vitols on the overall morphology of cells of *S. cerevisiae* and their internal membranes and intracellular organelles, using an improved fixation procedure for electron microscopy.[15] Further, Vitols and Linnane were able for the first time to isolate preparations of intact yeast mitochondria that had some capacity of OXPHOS in laboratory experiments.[16] They collaborated with staff at the Electron Microscope Unit of the University of Sydney that had been established in 1958 (just a year before Tony's return to Sydney) under the directorship of D. G. Drummond.

Together with Vitols and another PhD student, Patricia Nowland, Linnane published his first definitive paper on the properties of anaerobically grown yeast cells, this time using *Torulopsis utilis*.[17] This work showed that mitochondria normally seen in aerobically grown cells are converted, under anaerobiosis, into primitive structures that neither generate measurable respiration nor contain detectable mitochondrial cytochromes (nonetheless, the primitive entities contain two typical mitochondrial dehydrogenase enzymes). This system therefore had the potential for the systematic study of how mitochondria might be formed.

Linnane made substantial progress in setting up his own laboratory, not only obtaining a grant from the National Health and Medical Research Council of Australia (NHMRC) for his research, but also gaining promotion to Senior Lecturer. Yet Sydney was not the place where Linnane could develop his laboratory, in part because of the constraining hand of Jack Still, as head of department, who preferred a department emphasising teaching rather than research. The opportunity arose for Linnane to join the recently established department of biochemistry at Monash University in Melbourne.[18]

The highly productive yeast research years at Monash (1962–80)

The early years at Monash

In 1962, Linnane was appointed as Reader in Biochemistry at Monash, where he commenced the realisation of his vision to build a multidisciplinary research endeavour in a concerted attack on a major scientific problem: the biogenesis of mitochondria. His first approach to this problem was the use of anaerobically grown cultures of *S. cerevisiae* into which oxygen was introduced to demonstrate that functional mitochondria rapidly reappeared.[19] This work was initially carried out by Pat Wallace (née Nowland), who had come from Sydney to continue her PhD studies with Linnane. The electron microscopy work by Wallace was carried out at the University of Melbourne, where Neil C. R. Merrillees had an electron microscope in the Department of Anatomy.

More detailed work on anaerobically grown yeast indicated that both catabolite repression (observed with glucose, as opposed to less repressing substrates such as galactose) and the availability of unsaturated fatty acids and sterols (both unable to be biosynthesised in the absence of oxygen) contributed to the abundance and enzyme composition of the mitochondrial membranes in anaerobically grown cells.[20] David Jollow (a PhD student) carried out seminal work on defining the lipid composition of mitochondrial membranes in cells grown under various aerobic and anaerobic conditions.[21]

Linnane's successful research and participation in departmental leadership led to his promotion to Professor in 1965, at the relatively young age of thirty-five (Fig. 2). He had participated in departmental teaching programs as soon as he arrived, being one of the few academic staff members who had experience in undergraduate teaching (in his case, from the University of Sydney). During the period 1962–5 Bornstein, Linnane and others oversaw the design and construction of a new biochemistry building.[22] A wide range of technical facilities for modern biochemistry research was established

[13] Ephrussi (1953).

[14] The inheritance pattern of *rho* was distinct from that of the genes of the nucleus that were inherited in a strictly Mendelian fashion. In the mid-1950s *rho* was conjectured to be linked to DNA in mitochondria but it would be another decade before DNA in the mitochondria of yeast cells would first be definitively demonstrated (Schatz and others (1964)).

[15] Vitols and others (1961).

[16] Vitols and Linnane (1961).

[17] Linnane and others (1962). Note that *T. utilis* is also known as *Candida utilis*.

[18] Monash University took its first students in 1961, the same year that the Department of Biochemistry was established under the headship of Joseph Bornstein. The early history of the Monash Department of Biochemistry has been recounted by Nagley (1992).

[19] Wallace and Linnane (1964). Pat Wallace became the first female PhD graduate of Monash University after she submitted her thesis in 1965 (from Oxford, where she was working as a postdoctoral fellow in the laboratory of Sir Hans Krebs).

[20] Lukins and others (1966). The first author is H. B. Lukins who later made his mark with Linnane as his right-hand man on the study of mitochondrial genetics. Bruce Lukins was a graduate of the University of Sydney, who had completed his PhD on bacterial metabolism at the University of Texas at Austin, in 1961. Lukins returned to the University of Sydney as a Lecturer in 1962 before joining Monash for the rest of his career until he retired in 1994.

[21] Jollow and others (1968).

[22] The main Biochemistry building was known as 13D and fulfilled this role for more than forty years. Other buildings nearby were used for undergraduate laboratory teaching of biochemistry and molecular biology. In 2009, the vast majority of academic research staff of Biochemistry and Molecular Biology moved from 13D to new buildings (76 and 77) in the School of Biomedical Sciences. The later history is detailed by Nagley (2011).

Figure 2. Tony Linnane as Professor of Biochemistry at Monash University, ~1965 (Monash University Archives, IN27).

in the new building from 1965. For Linnane's group on the second floor there were specialised facilities for large scale microbial culture, including a large hydraulic press for use with so-called French Press cylinders to break open the yeast cells, and an adjacent electron microscope suite. Elsewhere in the department was an analytical ultracentrifuge laboratory, under the supervision of Barry Preston, that was used for DNA and RNA research in Linnane's group.

In the mid-1960s Linnane commenced his seminal studies on the mechanisms whereby mitochondria are formed in cells. The first step was the demonstration that yeast mitochondria contain a protein-synthesising system separate from that of the major population of ribosomes in the cell (known as cytoplasmic ribosomes). A team in Linnane's laboratory comprising two PhD students, Minta Huang and Dick Biggs, together with a postdoctoral fellow, Des Clark-Walker, discovered that the antibacterial antibiotic chloramphenicol inhibits the formation of mitochondria in *S. cerevisiae*.[23] The properties of the drug-treated cells were very similar to those of anaerobically grown yeast, both containing vestiges of mitochondria. A definitive publication shortly thereafter revealed great similarities between chloramphenicol-treated cells and the respiratory-deficient *petite* mutant.[24] Here, the phrase 'biogenesis of mitochondria' appeared for the first time in the title of a publication by Linnane. Over the next twenty years he published a remarkable series of 65 research articles under this generic title, plus many more without such a label, on a wide range of topics within this theme.

Clark-Walker and Linnane in 1967 interpreted their observations to suggest that mitochondria are built up from proteins contributed separately by two protein-synthesising systems: one is the 'conventional' cytoplasmic ribosomes translating nucleus-encoded mRNA, the other is the 'novel' mitochondrial ribosomal system that translates mRNA inside mitochondria, encoded by mitochondrial DNA (mtDNA). Adrian Lamb (a PhD student with Linnane) soon showed that isolated yeast mitochondria are able to incorporate radioactive amino acids into protein, in a process that is inhibited by chloramphenicol, but not by cycloheximide that inhibits the cytoplasmic ribosomes.[25] This work was consolidated over the next few years with demonstrations of mitochondrial ribosomes in yeast with sedimentation properties different from those of cytoplasmic ribosomes. The concepts were extended to mammalian systems by a team led within Linnane's group by Geoffrey Kellerman (a senior medically qualified academic biochemist who had come from Sydney).[26]

Elucidation of major principles of mitochondrial genetics and the biogenesis of mitochondria

The study of antibiotic resistant mutants of *S. cerevisiae* provided the breakthrough for Linnane's laboratory to make definitive contributions to the molecular biology of mitochondria. The entrée to this work was facilitated by the visit of David Wilkie (a British yeast geneticist) to Monash in the mid-1960s. Wilkie had an interest in phenomena of drug resistance in *S. cerevisiae* and during his stay at Monash a series of mutants resistant to chloramphenicol, erythromycin or tetracycline were characterised for the degree of tolerance to each antibiotic and also for cross-resistance to these and other anti-bacterial antibiotics.[27] The key erythromycin-resistant strain was that whose mitochondrial protein synthesising system showed insensitivity to this drug compared with that of wild-type yeast.[28] Subsequent genetic analysis of that mutant revealed the genetic determinant for erythromycin resistance to be inherited cytoplasmically, in an extrachromosomal manner.[29] Elliot Gingold (a PhD student at Monash) clarified the relationship between the erythromycin resistance determinant (ER^R) and the genetic factor *rho* controlling the respiration-deficient *petite* phenotype (defined at the time as rho^+ in respiratory competent cells and rho^- in *petite* mutants). Gingold showed by a clever genetic test that the spontaneously arising *petite* derivatives from a parent rho^+ ER^R strain often but not always retained the erythromycin resistance determinant.[30] However, treatment of these parent cells with strong inducers of *petite* mutants, euflavine or ethidium bromide, led to almost complete loss of the ER^R determinant from the rho^- cells

[23] Huang and others (1966).
[24] Clark-Walker and Linnane (1967).
[25] Lamb and others (1968).
[26] Towers and others (1972). Kellerman was involved in medical teaching at Monash and later became Dean of Medicine at the University of Newcastle in NSW.
[27] Wilkie and others (1967).
[28] Linnane and others (1968*a*).
[29] Linnane and others (1968*b*).
[30] Gingold and others (1969).

generated by that drug. The notation ER^0 was first used to describe the loss of the erythromycin resistance determinant, as opposed to ER^S and ER^R for sensitivity or resistance, respectively.[31] The concept of differential loss of mitochondrial genetic information in *petite* mutants, presumed to arise from deletions within the mitochondrial genome, was a fundamental contribution to the field of yeast mitochondrial genetics.

Linnane and colleagues made a further contribution that was seminal to the development of the mitochondrial genetics field, namely the concept of heteroplasmy whereby two (or more) different mitochondrial genomes can co-exist in the cytoplasm of the same cell.[32] Gary Saunders, a PhD student, analysed the mitochondrial genetic content of the progeny diploids formed by mating two rho^+ haploid yeast cells, one ER^S and the other ER^R. This entailed micromanipulation of the buds arising from the zygotes (the first cells arising after mating) and testing the colonies that were formed for their ability to grow in the presence of erythromycin. The patterns of mixed ER^S and ER^R genomes in the individual cells were inferred from the proportions of drug-sensitive and drug-resistant colonies. The variations in such patterns from zygote to zygote (and from successively generated buds) were interpreted to arise through the random reassortment amongst a population of multiple mitochondrial genomes in a single cell. Heteroplasmy is a fundamental idea that has stood the test of time and applies to most eukaryotic species including humans.[33]

Linnane's work up to the end of 1969 had made considerable advances on two other fronts. First was the detailed characterisation of the mitochondrial structures in anaerobically grown cells, taken to much finer levels of morphological detail compared with earlier work due to improved electron microscopy techniques.[34] Second was a thorough description of the role of mitochondrial protein synthesis in quantitative terms in normal and *petite* mutant cells under a variety of growth conditions.[35] The findings collectively cemented the notions elaborated some years earlier by Linnane that mitochondria are formed through the concerted biosynthetic programs of the nucleus-coded general genetic system of the cell plus a specialised system inside mitochondria relying on the mitochondrial genome and its own dedicated protein synthesis system (much of which is generated by the main cellular nucleus-coded system).[36]

One of the major outcomes of all the above contributions of Linnane to mitochondrial research, as represented by the substantial number and impressive content of his publications in the international literature, was his election as Fellow of the Australian Academy of Science in 1972.

The molecular definition of the yeast mitochondrial genome and mapping of the genes

The introduction of research directly on mtDNA and mitochondrial RNA in the Linnane group commenced after the arrival of Phillip Nagley, who joined the research team as a PhD student in 1969.[37] He discovered that *petite* mutant cells devoid of detectable mtDNA were generated after prolonged ethidium bromide treatment of the parent rho^+ cells.[38] The term rho^0 was introduced by Nagley and Linnane for the mutant cells lacking mtDNA.[39] By contrast, rho^- cells contain mtDNA, representing incomplete segments of mtDNA but at the same total abundance as in the parent rho^+ cells. This led to the important concept that rho^- cells provided a biological system for the purification or cloning of segments of mtDNA that became amplified in inverse proportion to the amount of mitochondrial genetic information retained in each such *petite* strain.[40]

Two further developments enabled the Linnane laboratory to take the leading position in the international race to map the overall yeast mitochondrial genome. First, was the development of methods to enable the proportion of the rho^+ mitochondrial genome retained in the mtDNA of any given rho^- strain to be accurately determined. A quantitative DNA-DNA hybridisation method was developed by a postdoctoral fellow K. S. Sriprakash, working with Nagley.[41] The procedure also enabled the extent of sequence overlap between the residual mtDNA genomes of any

[31] Saunders and others (1971).

[32] Saunders and others (1971).

[33] This feature underlies the genetic and physiological properties of many human mitochondrial diseases, whose clarification first took place in the early 1980s.

[34] Watson and others (1971).

[35] Kellerman and others (1971).

[36] These innovative concepts turned out to be applicable to mitochondria in all eukaryotes during the mid to late 1960s and also to apply to chloroplasts in the plant kingdom. It was, therefore, very timely that in December 1969 a major international conference entitled 'Autonomy and Biogenesis of Mitochondria and Chloroplasts' was arranged through the energetic organisational skills of Linnane and N. K. (Keith) Boardman (Division of Plant Industry, CSIRO, Canberra). This conference was held at the Australian Academy of Science in Canberra and was sponsored by that Academy together with the US National Academy of Sciences and the International Union of Biochemistry (IUB). The conference proceedings (Boardman and others 1971) became a seminal volume in the field of organelle biogenesis.

[37] Phillip Nagley was a graduate of the University of Sydney and had carried out research there on bacterial DNA replication with Robert Gerard (Gerry) Wake for his MSc degree during 1968, where he had gained experience with the analytical ultracentrifuge applied to quantification of DNA species of differing buoyant density in gradients of heavy salts such as caesium chloride. In the first year of his PhD studies in mid-1969 he participated in an international training course in Italy on RNA-DNA hybridisation (an emerging technique in the analysis of gene structure and gene expression) under the auspices of the International Cell Research Organisation.

[38] Nagley and Linnane (1970). Later that year, Goldring and others (1970) published their study showing that ethidium bromide treatment of *S. cerevisiae* specifically prevents the replication of yeast mtDNA, eventually resulting in cells lacking mtDNA. The first author was Elizabeth Goldring (née Dennis) who had completed her PhD at Sydney with Gerry Wake. Liz Dennis eventually went on to a distinguished career in plant molecular biology at CSIRO in Canberra.

[39] Nagley and Linnane (1972). The term rho^0 was applied many years later by King and Attardi (1989) to cultured mammalian cells lacking mtDNA that had been generated by prolonged ethidium bromide treatment of the human cell line 143B TK^-. These rho^0 cells could be repopulated by a complementation process using exogenous mitochondria, generating cybrids that opened up cell-based studies of mitochondrial disease (where the donor mitochondria were from patient-derived cells containing mutant mtDNA).

[40] Reviewed by Nagley and others (1977).

[41] Sriprakash and others (1976).

two *rho*$^{-}$ mutants to be measured. Second, was the identification of several more mitochondrial genetic markers in *S. cerevisiae*. These included antibiotic resistance markers that encompassed a further series affecting mitochondrial ribosomes including spiramycin, chloramphenicol and paromomycin (in addition to the erythromycin long-studied by Linnane) and those affecting other enzymes complexes of mitochondria including antimycin A (Complex III) and oligomycin (ATP synthase).[42] The retention or loss of each of these genetic markers amongst collections of *rho*$^{-}$ mutants could be determined. The integration of these two factors led quickly to the unambiguous physical mapping of these markers onto a circular mitochondrial genome. It was thereby possible to locate the retained segment of mtDNA in each *rho*$^{-}$ strain in relation to the particular antibiotic resistance markers that it had retained or lost.[43]

A further major addition to mitochondrial genetics in the mid-1970s, just as the development of physical mapping techniques came to fruition, was the identification of *mit*$^{-}$ mutants, defined by Slonimski and Tzagoloff.[44] These are defective in a single mitochondrial enzyme complex and contain a point mutation or small deletion in mtDNA (but not the major deletions characteristic of *rho*$^{-}$ mutants). A driving force for the isolation of such *mit*$^{-}$ mutants in the Linnane group was a visiting Polish scientist, Joanna Rytka, who devised a clever mutagenesis, selection and clonal purification strategy for novel *mit*$^{-}$ isolates. By genetically testing these *mit*$^{-}$ strains in crosses to the panel of mapped *rho*$^{-}$ petites, the map position of the new *mit*$^{-}$ locus could be readily determined. Further, by biochemical analysis of the *mit*$^{-}$ mutant cells grown in bulk (cytochrome content and enzyme activities), it was possible to determine which of the respiratory complexes was primarily affected by the *mit*$^{-}$ mutation.[45] These additional genetic markers, together with further molecular markers (namely, the two mitochondrial ribosomal RNA (rRNA) genes, studied by RNA-DNA hybridisation techniques with mtDNA from the panel of mapped *rho*$^{-}$ petites) were incorporated into the physical map of mtDNA. The first comprehensive map of the mitochondrial genome of yeast was thereby generated.[46] These insights into the physical placement of genes in the circular yeast mitochondrial genome were all the more remarkable because the map derived at Monash was congruent with the fragmentary maps that were being built up by other laboratories in the USA and Europe, based on the application of restriction enzyme analysis to the mtDNA of smaller series of *rho*$^{-}$ collections carrying defined genetic markers.[47]

Biochemical studies from 1970 focussed on the membranes of mitochondria, particularly using techniques to alter the lipid composition of the inner mitochondrial membrane. This was achieved primarily by use of a fatty acid desaturase mutant of *S. cerevisiae* (*ole1*) and manipulation of the concentrations of unsaturated fatty acids provided in the culture media. A powerful tool to study membrane-associated enzyme systems in mitochondria containing various levels of unsaturated fatty acids was introduced by means of Arrhenius plots that involves measurements of the change of activity of a particular function with temperature. The approach was first applied in collaboration with John K. Raison (CSIRO Plant Physiology Unit, in Sydney) on mitochondrial protein synthesis in rat liver, in work carried out by Neale Towers (a PhD student) working with Kellerman.[48] This work showed elegantly that mitochondrial ribosomes are membrane-associated. This approach was soon applied to yeast mitochondrial protein synthesis by Sangkot Marzuki (PhD student), who used chemostat cultures of the *ole1* mutant to generate bulk quantities of cells containing particular levels of unsaturated fatty acids.[49] The biochemical analyses of this work were overseen in the laboratory by James (Jim) Haslam, a lecturer from the UK. These studies not only confirmed the functional association of yeast mitochondrial ribosomes with the mitochondrial membranes, but also showed that a minimal threshold of 28% of unsaturated fatty acids was required for the mitochondrial protein synthesis system to function at all. This type of work was extended to encompass DNA synthesis in isolated mitochondria by John Mattick (PhD student) and Ruth Hall (postdoctoral fellow) that was also shown to be a membrane-associated function. Terry Spithill (PhD student) carried out further work on characterising the mitochondrial ribosomes in wild-type and various mutant yeast strains.

In recognition of Linnane's pioneering achievements on mitochondrial biogenesis, primarily in yeast, in 1980 he was elected a Fellow of the Royal Society, London. Tony was then at the peak of his scientific career (Fig. 3).

From a personal point of view, his family had grown by the arrival of two children, Ruth in 1967 and Nicholas (Nick) in 1972. In 1978 he and Judith separated. After the divorce, Tony married again in 1980, to Daryl Ann Woods. The marriage with Daryl that lasted

[42] Many (but not all) of these were first identified in the Linnane laboratory, as reviewed by Nagley and others (1977). The antimycin A marker was first identified through another drug, mikamycin. Many PhD students (including Kaye Trembath, Peter Molloy and Rodney Devenish) working with Lukins, made significant contributions to these genetic analyses.

[43] Sriprakash and others (1976).

[44] Slonimski and Tzagoloff (1976).

[45] The contributions of the Linnane laboratory are summarised in two papers in the proceedings of a conference held in Munich, Germany, in August 1976, entitled 'Genetics and Biogenesis of Chloroplasts and Mitochondria' (Cobon and others (1976); Rytka and others (1976)). This volume represents the ongoing legacy of the conference held Canberra in 1969, as the field had truly accelerated over the successive six and half years. There were many laboratories internationally contributing to this highly competitive field, including the 'veterans' of 1969 and many newcomers.

[46] Linnane and others (1976).

[47] The relevant contributions are published in the proceedings of a conference held near Bari, Italy in May 1976, entitled 'The Genetic Function of Mitochondrial DNA' (Saccone and Kroon (1976)). The correlation of these maps available at the time was achieved at that conference in a very direct manner. Nagley managed to get various contributors to draw their maps onto transparency sheets for visualisation using the overhead projector at the discussion following presentations at the relevant session. Remarkably, all the other maps overlaid beautifully onto the Monash map! This conference was one of a series that for many years was organised by colleagues at the University of Bari to cover many topics in mitochondrial function and biogenesis.

[48] Towers and others (1973).

[49] Marzuki and others (1975). Marzuki was an Indonesian medical graduate (1968) who had carried out work for his MSc (1971) at Mahidol University in Bangkok, working on bacterial cell physiology and biochemistry. He had met Adrian Lamb (former PhD student of Linnane) who was then working at Mahidol. Lamb recommended him to join Linnane's laboratory at Monash, where he arrived in 1972.

Figure 3. Tony Linnane in his office at Monash in 1980 (Monash University Archives, IN450).

until his death 37 years later had implications for Tony's professional life in relation to his establishment of the Centre for Molecular Biology and Medicine (CMBM), as set out in the Supplementary Material.

From yeast mitochondria to human ageing (1981–2008)

Adenosine triphosphate synthase

The yeast research now concentrated on mitochondrial ATP synthase, continuing work commenced in the late 1970s. Adenosine triphosphate synthase is responsible for ATP formation during OXPHOS, by pumping of protons through the membrane embedded F_O sector of the complex.[50] Two of the membrane-associated proteins of the complex (as known when this work started), were coded by mtDNA, namely subunits 9 and 6, encoded by the *oli1* and *oli2* genes respectively.[51] The remaining ten or more subunits are encoded by nuclear genes, the proteins being imported into mitochondria where they assemble into the complex. Linnane and colleagues (Fig. 4) focused on the genetics and assembly of the enzyme complex.[52] The genetical studies were overseen by Lukins and the work on biochemistry and assembly of the complex was led by Marzuki. The DNA sequencing analyses that took place under Nagley's supervision enabled detailed specification of the sites of mutation conferring resistance to oligomycin and other drugs, as well as those causing *mit*$^{-}$ mutations blocking assembly or function of the complex. In 1982 a third gene in yeast mtDNA encoding a subunit of ATP synthase was discovered, namely the *aap1* gene encoding subunit 8.[53] This subunit is thought to be an ancillary protein spanning the inner mitochondrial membrane, alongside the other two subunits 9 and 6 directly involved in the proton channel.[54]

Mitochondria and ageing

Linnane became interested in the role of mitochondria in human ageing, through contact with Edward Byrne, who was the Director of Neurology at St Vincent's Hospital in Melbourne, with a research interest in mitochondrial myopathies.[55] Marzuki, who had a medical background, had initiated these interactions as his own interest in mitochondrial diseases developed in the mid-1980s. Linnane had another colleague in Japan, Takayuki Ozawa (University of Nagoya), with whom he had interacted at mitochondrial conferences. In the late 1980s, Linnane and Ozawa began to have discussions on the generality of the mutations in human mtDNA that had been described to occur in various mitochondrial diseases. Such prior research had led to the recognition that in many mitochondrial myopathies, the mtDNA in muscles of the patients carried a

[50] John E. Walker and Paul D. Boyer shared the Nobel Prize in 1997 for their elucidation of the detailed molecular structure and mechanism of the soluble F_1 component of the complex that catalyses the synthesis of ATP from ADP plus inorganic phosphate (or the reverse ATPase reaction, namely ATP hydrolysis). However, the detailed molecular structure and proton-pumping mechanism of the membrane-embedded F_O sector remains the subject of active research to the present time. F_O is so-called because the engagement of this sector with F_1 confers sensitivity of the enzymic activity of the complex to the drug oligomycin that blocks the proton channel.

[51] Murphy and others (1978). Roberts and others (1979).

[52] Of the three senior colleagues of Linnane at Monash who were actively involved in yeast work in the mid-1980s (Fig. 4), Lukins remained active in yeast mitochondrial and nuclear genetics until he retired in 1994. He had developed a special interest in nuclear genes that are required for expression of mitochondrial genes or assembly of mitochondrial enzyme complexes containing products of genes in mtDNA. Marzuki shifted his interest to human mitochondrial disease and ageing in the mid-1980s; he also did some work on interferon with Linnane (see Supplementary Material). Marzuki left Monash in 1992 to help rebuild the Eijkman Institute in Jakarta, Indonesia (holding the position of Director of the Eijkman Institute for Molecular Biology from 1992 until 2014). Marzuki built up Indonesian research in the areas of human genome diversity and infectious diseases, and he served as President of the Indonesian Academy of Sciences. Nagley continued working at Monash with Rodney J. Devenish on various aspects of the genetics and molecular biology of the ATP synthase until 2003. Nagley's interests also included mtDNA mutations in human ageing (collaborating with Linnane until 1996), after which he diverged into studies on mammalian cell death and the cellular response to stress, especially in neuronal systems. He retired from Monash University in 2012 as Emeritus Professor. Remarkably, each of Linnane, Marzuki and Nagley became Members of the Order of Australia (AM).

[53] Macreadie and others (1983).

[54] Nagley (1988). Note that it was only in the late 1970s that Linnane did not include his name on all papers published from the mitochondria group. His mentor David Green had the generous practice of allowing senior postdoctoral fellows to publish important papers even without his name (Beinert and others (2004)) but Linnane felt that in Australia, to identify and protect the 'brand', his name had to be on almost everything published from his laboratory. Nagley, in establishing his own laboratory whilst maintaining strong collaboration with that of Linnane, broke that mould. But most others associated with Linnane at Monash were not able to do that.

[55] Byrne was a medical graduate of the University of Tasmania, who had carried out postgraduate medical studies in the mid-1980s with John Morgan-Hughes, a UK pioneer of research into mitochondrial disease at the Institute of Neurology in Queens Square, London. Byrne's research in Melbourne took place at St Vincent's Hospital where he was the founding Director of the Melbourne Neuromuscular Research Unit. He became Director of the Centre for Neuroscience at the University of Melbourne and later became Dean of Medicine at Monash University (2003–7). After a relatively brief period in the UK, Byrne returned to Monash as President and Vice-Chancellor (2009–14). He then became Principal and President of King's College, London.

Figure 4. Tony Linnane with senior colleagues at Monash University, Clayton, in 1986. *From left*: Sangkot Marzuki, Phillip Nagley, Tony Linnane, Bruce Lukins (Monash University Archives, IN467).

pronounced frequency of molecules carrying a deletion of 4977 base pairs (known as $mtDNA^{4977}$).[56] Familiar with the genetic properties of *petite* mutant yeast cells containing mtDNA carrying deletions and their properties including heteroplasmy, Linnane interpreted in 'yeast terms' the emerging literature on the properties of mtDNA mutations in humans, especially those of the $mtDNA^{4977}$ genotype. He extended these ideas to the ageing process, in terms of the progressive shifts that can occur over time in the proportion of mutant mtDNA relative to normal mtDNA. Together with Marzuki (at Monash), and Masashi Tanaka (a postdoctoral researcher with Ozawa at Nagoya), the seminal proposals of the role of mtDNA mutations in ageing and degenerative diseases were published by Linnane and Ozawa in the *Lancet* in 1989.[57] This turned out to be a landmark publication in the field of mitochondrial involvement in ageing and became the most highly cited of Linnane's publications over his entire career.[58] The proposals made in this article were supported by an accompanying article in the *Lancet* from Byrne and Marzuki reporting an age-related decline in mitochondrial function in human muscle biopsy samples.[59]

Linnane and his Monash colleagues described the variety of mtDNA mutations in various human tissues during the ageing in a long series of publications. The first reported in 1990 that the $mtDNA^{4977}$ genotype was detected by the polymerase chain reaction (PCR) in relatively large amounts (up to 1% of total mtDNA) in all adult tissues examined (including skeletal muscle, cardiac muscle and brain).[60] In contrast, $mtDNA^{4977}$ was found in much less abundance in corresponding tissues from infants, indicating that the accumulation of $mtDNA^{4977}$ is not necessarily associated

[56] The deletion in $mtDNA^{4977}$ occurs between nucleotide positions 8470 and 13459 of the human mitochondrial genome and is flanked by a 13 bp direct repeat.

[57] Linnane and others (1989). These insights were the basis of a long-running grant from the Japanese Ministry of Education, Science and Culture (*Monbu-shō*), under which during the 1990s colleagues from Monash travelled to Nagoya, and those from from Nagoya to Monash, for the purposes of discussions enabling the refinement of concepts and furthering developments in the field of mitochondrial involvement in ageing and disease.

[58] This article by Linnane and others (1989) has been cited more than 900 times, nearly three times more frequently than his next most cited article (Wolvetang and others (1994)).

[59] Trounce and others (1989).

[60] Linnane and others (1990). The human tissues used by Linnane and colleagues at Monash were obtained from autopsy samples at the Victorian Institute of Forensic Medicine in South Melbourne, through the good offices of Professor Stephen Cordner. Strict adherence to Human Ethics protocols was required for access to, and use of, these tissues in this research.

with particular mitochondrial diseases but occurs in all individuals with increasing frequency during normal ageing. Further work went on to characterise different types of age-associated mtDNA deletions. One highly cited paper from Linnane's group (led by a postdoctoral researcher from China, Chunfang Zhang, working with Nagley) reported multiple types of mtDNA deletions in individual tissues of a single human subject.[61] Another avenue of research in Linnane's laboratory emphasised the accumulation of point mutations in mtDNA during ageing.[62]

It was, however, more difficult to demonstrate that the mtDNA mutations accumulating with age causally diminished mitochondrial function. Linnane pursued this proposition by examining accumulation of mtDNA mutations with age using novel PCR methods (termed 'extra-long PCR', to capture the range of mtDNA deletions displayed by agarose gel electrophoresis) and correlating the results with the observed depletion of cytochrome oxidase activity in skeletal muscle, where a mosaic of normal and functionally depleted fibres could be seen to accumulate with age.[63] Further work, carried out by Sergey Kovalenko (a Russian postdoctoral researcher with Linnane, who had previously worked with Tanaka and Ozawa in Japan) enabled the development of PCR amplification procedures to quantify $mtDNA^{4977}$ in individual muscle fibres.[64] This method was extended to utilise the 'extra-long PCR' in these experiments; the accumulation of mtDNA mutations (termed mtDNA rearrangements) with loss of mitochondrial function in skeletal muscle fibres (measured by cytochrome oxidase staining) was thereby correlated.[65] Linnane was not, of course, alone in this field and many other authors had contributed to the development of the ideas that linked accumulated damage to mtDNA through oxidative stress mechanisms and other forms of mutational damage to the loss of bioenergetic functions and tissue damage that could also lead to cell death.

Information on mitochondrial involvement in cell death emerged during the 1990s from studies on the mechanism of apoptosis (programmed cell death, whereby the cell activates internal pathways to suicide), which implicated mitochondria in hitherto unanticipated ways.[66] In 1994, it was directly demonstrated by Linnane's group that respiratory chain inhibitors could induce apoptosis (cell death) in cultured human cells.[67] Although the detailed mechanism of this was not clear at that time, this observation directed attention to the possibly lethal consequences to cells of mitochondrial functional deficits caused by mtDNA mutations as part of the ageing process.

In order to express these new ideas neatly, Linnane introduced the theme 'the universality of bioenergetic disease' to encompass his newly emergent research vision.[68] He was also much taken with the idea of ameliorating the bioenergetic defects in cells that evidently arose during ageing. Linnane considered that one needed to understand in more detail the biochemical and physiological roles of molecules with redox activity, such as CoQ and, from those understandings, to derive rational approaches to pharmacological therapies. But he disavowed the prevailing ideas of his international colleagues that CoQ acted strictly as an anti-oxidant.[69] As summarised in the Supplementary Material, Linnane was never able to realise his objectives to develop fully a bioenergetically based strategy for ameliorating aspects of ageing.

Summation of research achievements

Linnane achieved a career-long *h*-index of 51 (Web of Science), with more than 10,770 citations at the time of writing this article (December 2018). His longitudinal citation record shows two distinct waves of citations. The first and more pronounced wave is from 1956 to 1982, peaking at ~400 citations/year in 1973. This period encompasses his major works on the biogenesis of mitochondria. For these achievements, he was elected as both Fellow of the Australian Academy of Science (FAA) in 1972 and Fellow of the Royal Society London (FRS) in 1980. The trough between the waves in his longitudinal citation record during the period 1983–90 shows ~100 citations/year for each of those years, reflecting the continuing residue of citations of his yeast work as well as those of his emerging interferon research (see Supplementary Material). The second prominent wave of citations commences in 1991 and peaks in 1997 at 300 citations/year. This reflects Linnane's significant contributions to the field of biomolecular gerontology, particularly his work on accumulation of mtDNA mutations and the universality of bioenergetic disease. Indeed, in Linnane's top ten most cited papers, five are from his yeast mitochondrial work in the 1960s and five are from his mammalian mitochondrial work from 1989 onwards.

Linnane was admitted as a Member of the Order of Australia (AM) in 1995 for 'service to medicine particularly in the fields of biochemistry and molecular biology'. He also received the Centenary Medal in 2001 for 'service to Australian society and science in molecular biology'. He was elected as Fellow of the Australian Academy of Technology and Engineering (FTSE) in 1999.

The period of instability and conflict in the Department of Biochemistry at Monash during 1993–5 that led to departure of Linnane from Monash and relocation of CMBM has been described elsewhere.[70] Linnane left Monash in 1996 with the title Emeritus Professor. His official photograph (Fig. 1) was displayed in the main departmental meeting room with those of other heads of

[61] Zhang and others (1992). The subject had no mitochondrial disease. DNA sequencing analysis of PCR products showed that short direct repeat sequences (5 to 13 bp) flanked all deletion breakpoints; in most cases one copy of the repeat was deleted. This suggested the mechanism of formation of deletions was an error in mtDNA replication (such as template switching across the homologous sequences) but this has been very difficult to prove.

[62] Zhang and others (1993).

[63] Kovalenko and others (1997*b*).

[64] Kovalenko and others (1997*a*).

[65] Kopsidas and others (1998).

[66] The status of this field in the late 2000s has been reviewed by Smith and others (2008).

[67] Wolvetang and others (1994).

[68] Linnane (1992).

[69] Linnane (2010).

[70] Nagley (2011).

department, in recognition of his contributions to the department over more than three decades.

Linnane's contributions to societies and international organisations are outlined in the Supplementary Material. These include his participation in the Australian Academy of Science and his contributions to the Australian Biochemical Society (ABS), the Federation of Asian and Oceanian Biochemists (FAOB) and the International Union of Biochemistry (IUB).

Personal reflections

In the half century that I have known Tony Linnane, as student, academic colleague and friend, I have come to know well his strengths and other features of his complex personality. He was a highly accomplished insightful scientist in his productive years and was able to build up outstanding teams under his direction at various times in his career. He encouraged and supported many of his colleagues and subordinates in matters concerning career development, fellowships, academic appointment or promotion. When such backing was not reciprocated (as was the case with some of those whom he had encouraged), he felt disappointed and betrayed.[71]

Tony had a charming and gracious side that served him well in making contacts at all levels of professional and personal life. In the most recent decades (Fig. 5) he enjoyed many happy times with his family, often travelling overseas with his second wife Daryl and his stepchildren (Daniel Woods, born 1969, and Kristen Woods, born 1970), together with his son Nick. Tony took great delight in explaining the historical aspects of places they visited together or in selecting the most appropriate wine to drink at many a fine restaurant around the world. As the personal beneficiary of such cultural and historical mentoring on several occasions during attendance at conferences Europe or the USA, I can well appreciate the scientist and the often larger than life personality that was Tony Linnane.

Figure 5. Tony Linnane, in 2017 (image courtesy of Daniel Woods).

Conflicts of interest

The author declares no conflicts of interest.

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[71] Tony's feeling that he was not appreciated suitably by his peers fuelled his intense desire for recognition, especially later in life. Tony had been much influenced by the way in which the bioenergetics field was revolutionised in the 1960s by an 'outsider', Peter Mitchell, who had pursued his ideas in the face of entrenched opposition from a mainstream scientific community that had collectively missed a major feature of the OXPHOS system they had been studying for many years (this led to Mitchell eventually receiving a Nobel Prize in 1978; see above). In later life, Tony thought of himself as such an outsider in the ageing field and, in his view, the misplaced ideas of his international colleagues would not eventually lead to clinical successes; he thought that such successes could only arise according to his alternative standpoint.

[72] Nagley (2018). © International Union of Biochemistry and Molecular Biology.

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