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MOLECULAR BIOLOGY INTELLIGENCE UNIT 9

Aubrey D.N.J. de Grey

The Mitochondrial Free Radical Theory of Aging

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PREFACE

What makes us age at the rate we do? More to the point: Why doesn't someone find out?

We now know a great deal about what human aging comprises, not only in macroscopic terms but also at the molecular level. We also have a respectable grasp of how these degenerative molecular changes interact. What is missing is a similarly detailed understanding of what — again, at the molecular level — is the process determining the *rates* of these changes. We do not even know whether one determinant of those rates is dominant or whether there are many factors with comparable influence. The layman may reasonably ask why, given such spectacular advances in so many areas of medicine, so little progress has been made on this most fundamental health hazard. For the biologist it is, frankly, somewhat embarrassing.

This book presents and discusses the hypothesis that the rate of accumulation of spontaneous mutations in our mitochondrial DNA is the principal determinant of the rate of human aging. This is a school of thought which has been developed over a period of 45 years and has incorporated ideas from a huge range of biological specialisations. It is still only a hypothesis, but recent advances in many of these contributory disciplines have strengthened it very greatly, so that it has gained the support of large numbers of professional gerontologists. Moreover, these advances have made it much more detailed and concrete, and correspondingly more readily testable; this is engendering a widespread feeling that the "wilderness years" of gerontology are drawing to a close and that progress towards a true causal understanding of aging will henceforth be rapid. What is a "true causal understanding" of a process? In my view it is best defined as a degree of knowledge that enables us to influence the process as we wish.

The book is organised broadly into four parts. The four chapters that follow the Introduction are devoted to background material, covering (respectively) mitochondria, free radicals, lipid metabolism and the major pathological features of human aging. Chapters 6 to 9 are essentially chronological, describing the development of the theory, with emphasis on recent advances in Chapters 8 and 9. Chapters 10 to 12 cover a wide range of its potential predictions; those in Chapters 10 and 11 concern compatibility with existing data, whereas Chapter 12 discusses still-available tests. Finally, Chapters 13 to 17 discuss the medium-term potential, contingent on the correctness of the hypothesis presented thus far, for the development of therapies which would greatly increase the healthy human lifespan.

I cannot overstate my profound gratitude to those who have supported my work on this book and on the research that led to it. My interest in the field of theoretical gerontology has led me to delve deeply into many disciplines, in some of which I initially lacked grounding, let alone expertise. Such wide-ranging forays into the literature are possible in the present day due to one recent advance more than any other: the availability of electronic literature databases with comprehensive scope and powerful search functionality. Despite working at an institution better endowed with library holdings of scientific periodicals than nearly any in the world, I could not possibly have made my contributions to this field without such tools; for unlimited access to them I thank my colleagues at Cambridge, who have tolerated my unsociable working hours.

I am also hugely indebted to the many colleagues who have critiqued my earlier contributions to the gerontological literature, and especially to Adam Wilkins, Jeremy Henty and John Rickard, who each read the entire manuscript and inspired numerous valuable improvements. Above all I thank Adelaide Carpenter, who not only critiqued the whole manuscript twice but also put up with me for a year when I was (to my mind, though she will have none of it) intolerably immersed in it.

Aubrey D.N.J. de Grey

DEDICATION

To my mother,
Cordelia de Grey,
artist and poet,
who set aside her own career
to devote herself to my education
and gave me my urge to understand the world.

Chapter 1

Introduction

1.1. What Is Aging?

It has been said that aging is a difficult thing to define, but in fact a relatively uncontentious definition is possible. Masoro's[1] is as good as any:

> ***deteriorative changes with time during postmaturational life that underlie an increasing vulnerability to challenges, thereby decreasing the ability of the organism to survive.***

This definition encapsulates a number of important features which the gerontologist, as well as the non-specialist, benefits from keeping in mind:

1. Aging is bad for us. One might think this is so obvious as not to be worth saying, but it goes unsaid so much that gerontologists often seem to have forgotten it.
2. Aging is not an extension of development, but a decay: thus, one can only weakly extrapolate from development when seeking its mechanisms.
3. Aging doesn't kill us: it "only" makes us steadily more killable. Thus, by this definition, strokes are not part of aging but atherosclerosis (which predisposes us to them) is.

Aging also, clearly, needs to be defined descriptively as a prerequisite to its study; here it becomes justified to be less general and to limit the description—at least of the macroscopic features—to a single species, which in this book will be humans. Chapter 5 is devoted to such a description; it is a purposely superficial one, since excellent comprehesive treatments have been published in recent years and are referenced there. Chapter 5 also probes a little way into the proximate causes of these gross symptoms of aging, preparing the ground for the proposed ultimate cause which is the book's central topic.

What about Chapters 2, 3 and 4? Perhaps regrettably, it is in the nature of biology that one must master a fearsome quantity of background information before one can really make sense of a research area. This is, arguably, truer in gerontology than in any other biological field. Hence these three chapters are devoted to providing that background information (on, respectively: mitochondria, free radicals and lipid metabolism), and aging does not feature in them at all; I must ask the reader to have patience with this and to trust that it is a prerequisite for understanding the rest of the book.

1.2. Subjectivity and Vocabulary

There is a good deal more use of the first person singular in this book than is typical in academic work. I chose to write it in this style for one central reason: it is a book about a theory. Consequently, much of the thinking that I shall describe has yet to achieve such widespread support that it can be presented as fact. If there were less material in that category, it might have been satisfactory to couch it in language which combined the impersonal with the unauthoritative, but that necessitates a good deal of circumlocution, which, above a certain density, becomes more irritating and flow-breaking than it is worth. I have therefore

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chosen the informal alternative of presenting such material explicitly as my opinions; I apologize in advance to any readers who disagree with my rationale.

I have also adopted a relatively informal style with regard to technical vocabulary. Since aging is a feature of life for everyone, I have tried to make the book accessible to a wide audience. With regard to subject matter, this has entailed the inclusion of introductory material with which professional biologists will be familiar, but by and large that appears only in the sections on the structure and function of mitochondria. In order to keep such material out of other sections, I have attempted to minimize the use of jargon that is standard to biologists but unfamiliar to others. I hope I have done so without introducing irritating verbosity.

1.3. Why Suspect Mitochondria or Free Radicals?

The history of the mitochondrial free radical theory has been a bumpy one. Harman realized in 1954[2] that toxic free radicals might be formed in the body and might cause aging; it was not until 15 years later that their toxicity was robustly supported by McCord and Fridovich's discovery[3] of an enzyme that destroys such a radical. The mitochondrial free radical theory of aging, hereafter usually abbreviated MiFRA, was truly born in 1972, when Harman suggested[4] that mitochondria had the right characteristics to be both the sources and the direct victims of toxic free radicals. Then the tide of opinion turned. The idea that mitochondrial damage could contribute to aging was powerfully challenged by Comfort, who pointed out[5] that damaged mitochondria would naturally be destroyed and replaced by the cell; it came under further pressure in the early 1990s as numerous laboratories reported that only very small quantities of mutant mtDNA were present even in very elderly individuals. Then the tide turned again: Comfort's objection was overturned with the discovery by Müller-Höcker[6,7] that mutant mitochondria not only survive but are clonally amplified when they arise, and Barja and others identified interspecies differences that compellingly correlated free radical damage to mitochondria with lifespan.[8,9] This story is told in Chapter 6.

One of the most powerful factors which have perpetually held back research into aging is that it is phenomenologically so complex. This has resulted in a paralysing imbalance between data and theory: there is so much information to absorb that no simple synthesis can possibly be made, but the frequent reaction to this is to gather yet more data in the vain hope that it will bring sudden illumination. The sad result has been that both theory and experiment in gerontology have gained a widespread reputation for, if not mediocrity, then at least paucity of top-quality work. While this reputation is to my mind undeserved by the experimentalists, it is perhaps partly deserved by the theoreticians. Chapter 7 discusses these issues in more detail.

The next two chapters cover two recent refinements of the mitochondrial free radical theory. Chapter 8 presents a mechanism to explain the age-related accumulation of mitochondrial DNA mutations; Chapter 9 a mechanism for intercellular transmission of oxidative stress which may make those mutations disproportionately harmful. Chapter 10 deals with a wide range of potential challenges to aspects of the whole theory (particularly to these recent refinements), and Chapter 11 with one more, whose refutation is complex enough to be chapter-sized.

1.4. Tests, Interventions and Consequences

With Chapter 12 the book begins to look to the future. Although each step in the mechanism set out here is already well supported by experimental evidence, there are still several clear weaknesses in this support, which translate into possible tests of components of the hypothesis.

Some may feel that all avenues for falsification of a hypothesis should be exhausted before any significant effort is invested whose payback depends on that hypothesis being correct. I do not share that view; I think that, in spite of the availability of the tests outlined in Chapter 12, it is in no way too soon to explore the possibility of plausible, feasible intervention in the aging process by subversion of one or other step in the mechanism here proposed to determine its rate. Chapter 13 is a survey of 12 formally conceivable options, in which I conclude that two are sufficiently promising to merit further attention. Chapters 14 and 15 explore those two in detail, with emphasis on the obstacles that they are already known to face and realistic approaches for overcoming those obstacles.

The last two chapters of the book will, doubtless, raise the most eyebrows—if not hackles—among my colleagues. It is very unfashionable (though perhaps not for long, given the likely impact of a recent book by the U.K.'s foremost gerontologist)[10] for professional gerontologists to assert publicly that a dramatic increase in the maximum healthy human lifespan may be only a few decades away. Indeed, and especially when addressing an audience including non-specialists, distinguished gerontologists have often gone out of their way to assert the opposite. There are two good reasons for such aggressive pessimism, when one is sure that such progress will not take place. The first is that it does nobody any good if the general public expects the impossible from its scientists. The second is that there is a great deal of hugely valuable work to be done in gerontology nevertheless, by way of making the process of aging less painful even if it cannot be slowed, and that such work might be less well supported if it became seen as unnecessarily unambitious. I mention these points at the outset, lest any colleagues think that I fail to appreciate them. Conversely, if they feel that I am unwise to voice the contrary prediction, I only ask them to hear me out.

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Chapter 2

An Introduction to Mitochondria

Mitochondria have two main characteristics which, in combination, mark them out among subcellular structures as especially plausible mediators of cellular decline. The first is their absolute indispensability to the cell: they are the intracellular machines that enable us to use oxygen, and they are also intimately involved in other aspects of cellular stability. The second is their vulnerability: they constantly inflict damage on themselves as a side-effect of aerobic respiration, and they have one small but vital component which is not reconstructible from scratch, so damage to which may be permanently harmful to the cell—their DNA. Their structure and function are both highly complex and will be covered in detail in, respectively, Sections 2.2 and 2.3; Section 2.4 focuses on the mitochondrial DNA (usually abbreviated mtDNA) itself.

This description of mitochondria begins, however, with a summary of how they are believed to have arisen during evolution. At first sight, this question may seem to be unrelated to the subject of age-related mitochondrial dysfunction and its causes and effects. In fact, however, one of the main fascinations of studying "how we got into this mess in the first place" (as evolution has often been described) is the frequency with which an understanding of it sheds light on matters of more immediate concern. The topics covered in this book are no exception.

2.1. The Evolutionary Origins of Mitochondria

The macromolecular composition of mitochondria is almost entirely proteins and lipids—the same basic components as the rest of the cell. The cell makes these on demand. But the creation of a new mitochondrion also entails the creation of a new copy of that small but absolutely vital piece of genetic material, the mtDNA. This—like the DNA in our chromosomes—cannot be made from scratch, only by duplicating an existing copy. For that reason above all, the way cells make a new mitochondrion is by replicating an existing one to make two. (It is more accurate to say that "the cell replicates the mitochondrion" than that "the mitochondrion divides," because not one of the proteins that are involved in the replication process is encoded in the mitochondrial DNA. All of them—for example, the components of the enzyme that replicate the mtDNA, which is called DNA polymerase gamma—are encoded in the nucleus.)

A logical conundrum clearly arises from the situation just described: if the only way to make a mitochondrion is by starting with a pre-existing one, then where did the first one come from? There are plenty of mitochondria in egg cells, so we have no problem transmitting them from one generation to the next; but there must have been a first mitochondrion at some time in our evolutionary history.

The fascinating answer, which is now universally agreed, was proposed in the 1920s[1]—indeed, arguably 40 years earlier[2,3]—but was almost universally rejected as altogether too preposterous, even as recently as 1966,[4] until it was revived in 1967.[5,6] Recall,

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firstly, that cells of the type we are made of are not the only ones that exist. Organisms (whether made of one or more cells) whose cell(s) have a nucleus are called eukaryotes. Bacteria are not eukaryotes; they are called prokaryotes, and their structure is much simpler. They do not have a nucleus; nor do they have mitochondria. Also, their DNA is not divided into chromosomes, as ours is; instead it is all in one molecule. Furthermore, that molecule is circular, whereas all eukaryotic chromosomes are linear. What intrigued Wallin,[1] and subsequently Margulis (née Sagan),[5,6] is that mitochondria share all these (and other) features of bacteria! (Except that Wallin didn't know about the DNA, of course.) They don't have a nucleus; they don't have their own mitochondria, and their DNA is a single circular molecule. Additional, subtler similarities have been emerging ever since. For example, superoxide dismutase (an enzyme with a pivotal role in MiFRA, which we will encounter often in this book) exists both inside mitochondria and outside, but in very different forms; the metal cofactor of the mitochondrial form is the same—manganese—as in a bacterial superoxide dismutase, whereas the non-mitochondrial one uses copper and zinc.

So, it is now firmly believed that the sequence of events which created what we now know as mitochondria was as follows (see Fig. 2.1). Early bacteria were unable to use oxygen, and in fact it was highly toxic to them. Then aerobic respiration evolved in some bacteria. Independently of this, other bacteria evolved a nucleus, linear chromosomes and the other features that made them eukaryotic cells. Then a bacterium which was capable of aerobic respiration was phagocytosed—engulfed, in plain English—by a single-celled eukaryote, and by some outlandish quirk it was not immediately destroyed. It carried on living, inside the eukaryotic cell. Furthermore, it was still able to divide, producing more bacteria, all still inside this cell.* When the cell divided, some bacteria ended up in one daughter cell and some in the other. The bacteria were extremely useful to the cells, because they sequestered any toxic oxygen that was around and made energy out of it; thus, the cells with these bacteria inside them were able to multiply faster than the ones without. (Likewise, the cells were useful vehicles for the bacteria. This kind of mutually beneficial relationship between two organisms is called symbiosis, and in this case where one of the participants is living inside the other it is called endosymbiosis. Thus, this theory is called the endosymbiotic theory.) Over time they outcompeted the cells that had no aerobic bacteria inside them, so that soon almost all eukaryotic cells had not only nuclei but also bacteria performing aerobic respiration. Since then, not much has changed.

The main thing that has changed concerns the DNA of the phagocytosed, aerobic bacteria. It is that very nearly every gene in those original bacteria has either been lost from the bacterial (now mitochondrial) DNA, due to redundancy with a gene already present in the nucleus, or else has been transferred to the nuclear DNA. This is how, as noted above, enzymes such as the mitochondrial DNA polymerase came to be encoded in the nucleus: the genes didn't evolve there, they moved. This is not a trivial event, because proteins encoded in the nucleus are constructed outside the mitochondrion and have to be transported into it to do their job, as will be discussed in Section 2.4.3 and elsewhere.

** Possibly the most implausible aspect of this engulfment—the "endosymbiotic event"—is that the bacterium was able to cope with such a sudden change in its external environment, from being in the outside world to being inside another cell. This aspect of the theory has more recently been improved by hypotheses that propose a very gradual engulfment, starting out as a normal symbiosis of two species which used each other's metabolic waste products—perhaps sulphur,[7] perhaps hydrogen[8]—as nutrients; slow changes in the geological conditions then promoted an ever-closer union of the two which eventually became an engulfment.*

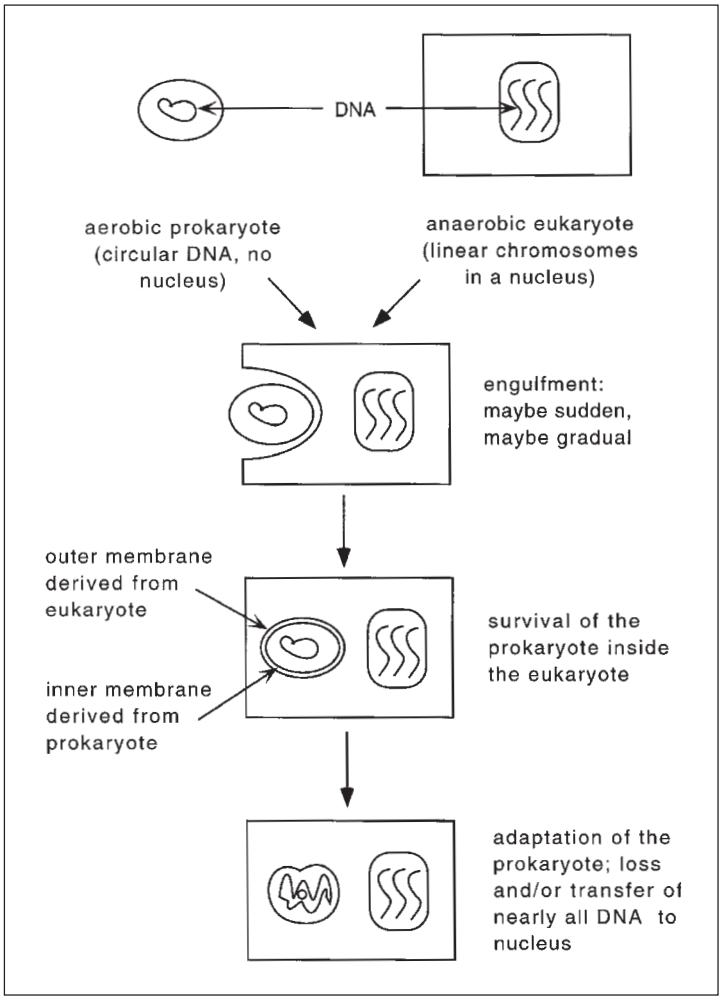

Fig. 2.1. The endosymbiotic theory of the origin of mitochondria.

2.2. Mitochondrial Location and Structure

2.2.1. Location

As explained in the previous section, mitochondria are components of cells: subcellular organelles. Different cell types contain different numbers of them; the extremes are that red blood cells (erythrocytes) contain none and egg cells (ova) contain about 100,000. Most human cells contain between a few hundred and a few thousand.

All our cells (except platelets and, again, red blood cells) have a nucleus, which is the compartment containing our chromosomes. Mitochondria are never found in the nucleus, only in the rest of the cell (the cytoplasm). The cytoplasm is also sometimes called the cytosol; strictly, the cytoplasm is everything inside the cell except the nucleus (so including

mitochondria and other organelles), whereas "cytosol" denotes the cytoplasm minus the organelles.

Mitochondria are distributed throughout the cytoplasm. Even in very long and thin cells, such as nerves, there are mitochondria all the way to the end—sometimes feet from the nucleus. They occupy a substantial fraction of the cell volume: about 1/5 in liver cells, for example.

The components of a mitochondrion which will feature in this book are shown in Figure 2.2.

2.2.2. Macroscopic Structure

"Macroscopic" in this context is relative—mitochondria are typically only a few hundred nanometres long. In this context, "macroscopic" only means "at least an order of magnitude bigger than a protein."

A mitochondrion, like all organelles, is essentially a scrap of water encased in a membrane. Unlike most organelles, though, that membrane is itself encased in another membrane. The two membranes differ in many ways. The surface area of the inner membrane is much larger than is needed to contain the material inside the mitochondrion, but is intricately folded; the folds are called cristae. The outer membrane is not folded. The outer membrane has large holes (pores, ringed by a protein called, helpfully, porin) through which small molecules—though not most proteins—can easily pass; the inner membrane, on the other hand, is impermeable to most molecules (especially charged ones). The space enclosed by the inner membrane is called the mitochondrial matrix. The space between the inner and outer mitochondrial membranes is just called the mitochondrial intermembrane space.

The membranes are both composed of the same types of molecule as all other membranes: phospholipids and cholesterol. There are some peculiarities regarding which phospholipids are present: for example, a diphospholipid called cardiolipin (see Fig. 4.1 for structure) is present in large amounts in the inner membrane, whereas it is almost absent anywhere else in the cell.[9] Conversely, cholesterol is hardly present at all in the inner mitochondrial membrane (except in the adrenal cortex, where it is metabolised to pregnenolone)[10a] but is plentiful elsewhere.[9] The two membranes are not completely separate: they come together at a few places called contact sites (whose absence from Figure 2.2 will be explained later).

The intermembrane space contains only a few proteins; some are involved in transport of proteins into mitochondria (see Section 2.4.3). Apart from those, the only one that will concern us is cytochrome c, which has a crucial role in mitochondrial function. The outer membrane contains some proteins too, apart from porin and some other protein-transport proteins, but none of them will feature here. The inner membrane and the matrix, on the other hand, each contain many molecules whose roles are relevant to the mitochondrial free radical theory of aging.

2.2.3. Composition of the Mitochondrial Matrix

The description of the mitochondrial matrix as "a scrap of water" is not in fact terribly accurate, because a fantastically high concentration of other material is suspended in it. It has been estimated that fully half the mass of the matrix is protein.[10b]

These proteins are mostly enzymes: catalysts of chemical reactions that are involved in mitochondrial function. In all, there are a few hundred different ones. The matrix also contains smaller molecules, many of which are substrates or cofactors of these enzymes (that is, participants in the reactions which they catalyse). Finally, it also contains a small

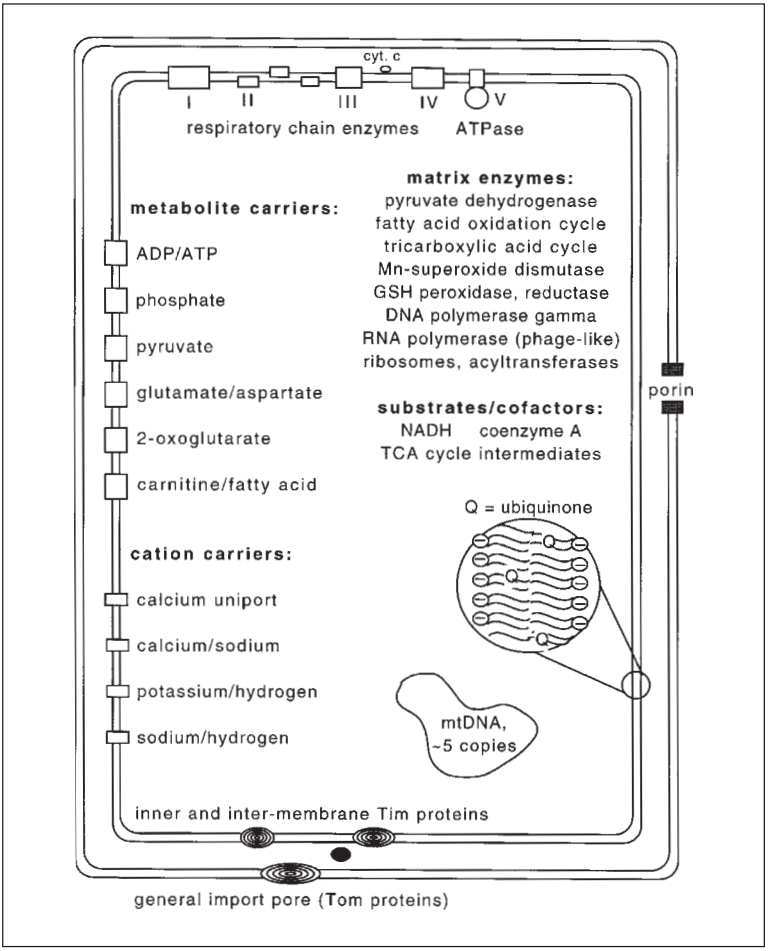

Fig. 2.2. A mitochondrion. For clarity the cristae are not shown; only items discussed in this book are noted.

amount of DNA, which will be described in detail in Section 2.4, and the machinery (including ribosomes) for decoding that DNA.

The matrix is one of the most alkaline environments in the cell. It has a pH of between 8 and 8.5, roughly half a unit higher than the cytosol.

2.2.4. Composition of the Inner Mitochondrial Membrane

I mentioned above that the inner membrane is impermeable to most molecules. However, that only means that most molecules cannot **freely** pass through it. Many molecules do indeed pass through, under the active control of proteins embedded in the membrane. These proteins are very specific—each one transfers only a particular molecule, one at a time. This rigid control exists because it is vital for mitochondrial function; the molecules that are transported in this way must be transferred when they are needed, but only in the right quantity, and without attendant, unregulated transfer of other molecules.

In addition to these "carrier" proteins, several enzymes are embedded in the inner membrane. In a sense these enzymes are the clients of the carrier proteins; their roles (discussed in Sections 2.3.3 and 2.3.4) are central to mitochondrial function, but the substrates whose reactions they catalyse must be made available on the correct side of the inner membrane.

Most of the transmembrane enzymes and carriers are actually complexes, made up of more than one protein—in one case, more than 40.[11] Each is present in a few thousand copies per mitochondrion.

2.3. Mitochondrial Function

Mitochondria enable us to use oxygen.

Virtually everything that our cells need to do to keep themselves—and hence us—alive requires energy. We use energy in macroscopic ways, of course, in movement and in keeping warm; but the microscopic processes that our cells are doing all the time, such as construction of proteins, replication of DNA and so on, also need energy. This energy comes from nutrients in what we eat; the chemical bonds in those nutrients (such as sugars and fats) are changed into other types of bond (such as in carbon dioxide and water) which have less energy. The difference between these energies is thus available to cells. Oxygen, and hence mitochondria, are intimately involved in these processes.

2.3.1. Three Ubiquitous Reservoirs

2.3.1.1. A Terminological Apology; The Proton Reservoir

Almost all the chemical reactions that will be discussed hereafter occur in water. (The rest occur within membranes.) Many of them involve acids. The characteristic that makes a molecule acidic is that, when it is dissolved in water, it tends to break apart in a particular way: one hydrogen ion (which is simply a proton) comes off. It turns out that water itself does this to a small extent—protons become detached, leaving hydroxide anions.* Conversely, the characteristic that makes a molecule alkaline is that it comes apart in water in a different way, whereby one hydroxide anion comes off.** Water, therefore, effectively does both when it comes apart. The coming-apart is very transitory: protons constantly jump between water molecules, so that individual hydroxide anions reacquire a proton almost instantly after they come into existence. The proportion of water molecules that are dissociated at any one time is about 10^{-7} in pure water at room temperature and pressure, which is why "neutral pH" is defined as pH 7. We will come back to these aspects of water in Section 11.3.1.

The tendency of water reversibly to come apart like this has a profound chemical consequence in biological systems: it means that protons are available for incorporation into a chemical reaction whenever needed, and similarly they can be discarded at will. In effect, the water that comprises 70% of our mass is a huge reservoir of protons.

Now for the terminology problem. When an acid reacts with an alkali, it forms a compound whose chemical name is constructed from those of the reagents—sodium chloride, for instance, or sodium acetate. But while such molecules are in solution, their

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** The detached protons do not, in fact, remain free in solution, but form bonds to intact water molecules making molecules of H_3O^+, which is called hydronium. We will come back to this in a discussion of water's conductivity, in Section 11.3.1, but meanwhile it need not concern us—the protons can be considered to be free in solution.*

*** Or, in some cases, whereby nothing comes off but instead a proton is removed from a nearby water molecule leaving the water as hydroxide.*

acid and alkali components are mostly not bound together. Thus, in a sense, it would be inaccurate to speak of a solution of sodium acetate; more precise would be to speak of a solution of both sodium hydroxide and acetic acid. What people have tended to do, therefore, for reasons of brevity, is to treat the term "acetic acid" as synonymous (when discussing it in the context of aqueous solution) with "acetate". This applies to all organic acids; thus the term "lactate" is identical in meaning to the term "lactic acid", and so on. In this book, I will normally use the "-ate" form in preference to the "-ic acid" form.

2.3.1.2. The Electron Reservoir

Biological reactions do not only use and/or produce free protons, however: they do the same with electrons. Indeed, the availability or disposability of electrons is vital to many of the reactions that will be discussed later on. Now, whereas protons are harmless when discarded into the medium, electrons are potentially highly damaging; in particular, they have a tendency to latch onto other molecules and make free radicals, as will be discussed in detail in Chapter 3. Evolution has discovered a molecule which helps to avoid this problem, by acting as a reversible electron carrier. This molecule is nicotinamide adenine dinucleotide, or NAD. It is capable of carrying two electrons in a very easily reversible configuration, involving the simultaneous carrying of just one proton. When not carrying the electrons it exists as a cation, NAD^+; when carrying them it is uncharged (because of the proton) so is NADH.

In fact, NAD is not the only molecule that performs this role. In a few of the reactions that we will encounter in later sections, a closely related molecule, flavin adenine dinucleotide or FAD, is used instead; it behaves in the same way except that it is always uncharged, since when it is carrying two electrons it carries two protons (and is denoted $FADH_2$). A third very similar molecule, NADP, is used instead in some circumstances, but not in any of those which will be discussed in this book.

2.3.1.3. ATP: The Energy Reservoir

Nutrients in the diet are many and varied, and processes requiring energy are just as varied. Evolution might, in principle, have developed separate systems for pairing each nutrient with each energy-requiring process, but that would have multiplied up to an enormous number of systems. Instead it has settled on a division of the problem into two. The processes that extract energy from nutrients all transfer it into the construction of just one molecule: adenosine triphosphate, or ATP. The processes that require energy, similarly, all derive it from the destruction of ATP. ATP thus constitutes an energy reservoir. It is also often described as the "legal tender" of cellular chemistry, because the merit of this system, compared to the alternative of a separate system for passing energy from each nutrient to each process, is absolutely analogous to the merit that civilisation has discovered in replacing barter with money.

The use of the words "construction" and "destruction" above is perhaps a slight exaggeration. Energy-requiring processes only break ATP into two pieces, ADP (adenosine diphosphate) and $H_2PO_4^-$ (inorganic phosphate, usually abbreviated P_i). Thus, the effective energy store in ATP is really only the energy in one chemical bond—or, more strictly, in the maintenance of a higher than equilibrium concentration of ATP relative to ADP and P_i.

2.3.2. Preliminaries to Mitochondrial Function

Mitochondria are really quite simple machines, by biological standards. They are only able to make ATP starting from a few very simple molecules. Therefore, in order that we can extract energy from the vast range of available foodstuffs, a great deal of preprocessing has to occur in order to reduce them to these few common denominators.

2.3.2.1. Precursors of ATP Synthesis: Digestion

The most obvious molecular difference between our various foodstuffs is molecular size. Some of the molecules we digest, such as starch, are enormous; others, like glucose, are relatively tiny. The first step in reducing the variety of molecules is to break down the bigger ones into three basic types of smaller molecule: amino acids, fatty acids and monosaccharides. This happens in the gut and involves neither the construction nor destruction of ATP.

2.3.2.2. ATP Synthesis, But Not Much: Glycolysis

One of the three types of product of digestion, monosaccharides, provides a source—albeit only a modest one—of ATP without the help of mitochondria, and without the help of oxygen. A sequence of ten (give or take one—see Fig. 2.3) enzyme-catalysed reactions can split the simplest monosaccharide, glucose, into two molecules of pyruvate or lactate (i.e., pyruvic or lactic acid—see Section 2.3.1.1.), and one of those reactions also makes ATP.

Glycolysis is enough to keep human cells happy for a short while, when there is a transient shortage of oxygen, but it is not adequate in the long term, not least because lactate is somewhat toxic. Some single-celled organisms can get along indefinitely with glycolysis, though; they simply excrete the final product (lactate or ethanol).

2.3.2.3. Breakdown of Amino Acids and Fatty Acids

Amino acids are not amenable to glycolysis-like reactions: without mitochondria they cannot be used as energy sources at all. Their breakdown need not, in fact, be discussed further. This is partly because they are only used for ATP production in emergency, when no other energy source is available, and partly because the way that the cell makes them available to mitochondria as an energy source is by turning them into pyruvate (exactly the same molecule that is created by glycolysis), or else into one of the molecules that mitochondria make from pyruvate in the process of breaking it down further. Therefore, all the ATP-synthesising reactions involved in amino acid breakdown will be covered in the discussion of pyruvate breakdown (below).

Fatty acids, similarly, are first broken down (by a sequence of reactions involving the incorporation of water) into several molecules of acetaldehyde, one of the products of pyruvate breakdown, so their contribution to ATP will also implicitly be covered below. In fact this happens inside mitochondria (see Section 2.3.3.1), but again it is not linked to any ATP synthesis.

2.3.2.4. Transport of Metabolites Across the Mitochondrial Inner Membrane

The subsequent processes involved in ATP synthesis, whose description will occupy the rest of Section 2.3, all happen in mitochondria. It is therefore necessary to transport pyruvate (the product of glycolysis that mitochondria use), fatty acids, protons and NADH across the mitochondrial inner membrane. Pyruvate and fatty acids are each transported by a specialised carrier protein, the fatty acid one involving the cofactor carnitine. Protons move across without any active carrier, since the membrane is somewhat permeable to water (their reservoir).[12] The two NADH molecules created by glycolysis are actually not transported through the membrane; instead, they pass their electrons to other NAD^+ molecules that are

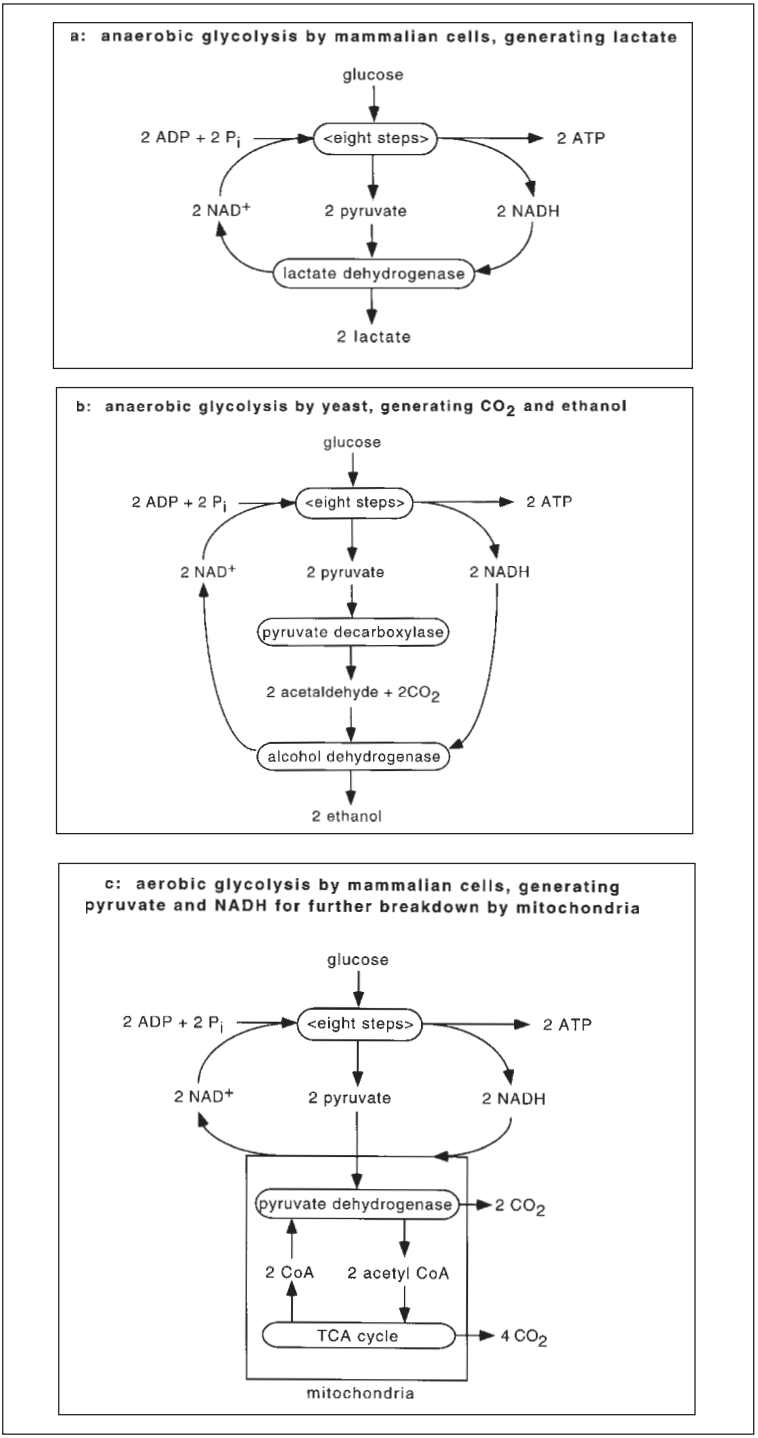

Fig. 2.3. Varieties of glycolysis

already in the matrix. This is mainly done by a group of enzymes and carriers collectively termed the malate/aspartate shuttle. There is also a backup pathway for transfer of electrons from cytosolic NADH to the mitochondrial ATP synthesis machinery. It is much simpler, involving no matrix-located components and only two enzymes. Its disadvantage is that it is less energy-efficient than the malate/aspartate shuttle. Muscles that work extremely hard for short durations (such as insect flight muscles) use it a great deal.[13] It is called the s,n-glycerophosphate shuttle.

Also, since ATP (over and above that created by glycolysis) is needed throughout the cell and not only in mitochondria, it must be transported out of mitochondria after being made, and conversely its constituent parts, ADP and phosphate, must be imported. These processes are also done by specialised carrier proteins: ATP and ADP by one, and phosphate by another. The only other metabolites that need to cross the membrane are oxygen (which is consumed by the processes described in the rest of this section) and carbon dioxide (which is generated by those processes); they both cross it quite freely, like water, without an active carrier.

2.3.3. Mitochondrial Chemistry

2.3.3.1. Creation of Acetaldehyde, as Acetyl CoA: Oxidation

The first degradative process that occurs inside mitochondria is the conversion of pyruvate and fatty acids to acetaldehyde. These are oxidation reactions, because they involve the removal of electrons from the major reagents. This process does not make ATP, and there is no hugely compelling reason why it should not have evolved to happen in the cytosol. Ostensibly that would have been simpler, because then only one molecule destined for destruction—acetaldehyde—would need to be imported into mitochondria, as against two different, larger ones (pyruvate and fatty acids). The likely reason is that, whereas pyruvate and fatty acids diffuse freely in the cytosol, acetaldehyde is always attached to a carrier molecule, coenzyme A (CoA), which is quite large (see Fig. 2.4) and is not transported through the mitochondrial membrane. Both fatty acid oxidation and pyruvate oxidation end by attaching a molecule of acetaldehyde to CoA, forming acetyl CoA.[14,15] The attachment involves the liberation of two hydrogen atoms, whose constituent protons and electrons go to the usual reservoirs.

2.3.3.2. More ATP, but on Borrowed Oxygen: Oxidation (Again)

Acetaldehyde contains two carbon atoms, one oxygen and four hydrogens, and in mitochondria it is converted into two molecules of carbon dioxide. Clearly this needs a supply of three atoms of oxygen for each molecule of acetaldehyde. But this is not where the oxygen we breathe is used: that comes later. Instead, the oxygen is recruited by breaking down three molecules of water.

The other imbalance between acetaldehyde and carbon dioxide is the hydrogens. The conversion of one molecule of acetaldehyde and three of water to two of carbon dioxide releases ten of them. Two of these have already been accounted for by the attachment of acetaldehyde to CoA; the other eight are released now. All ten go to the reservoirs—ten protons into the aqueous medium, and ten electrons into five two-electron carriers, which is this case are four NADH and one $FADH_2$.

The breakdown of acetaldehyde is done by a complex series of reactions (see Fig. 2.5) that was worked out in the 1930s. The key breakthrough, identification of the cyclic nature of this series, was achieved in 1937 by Hans Krebs,[16,17] which is why it is often called the Krebs cycle. Krebs preferred to call it the tricarboxylic acid (TCA) cycle, so I will stick to that term.

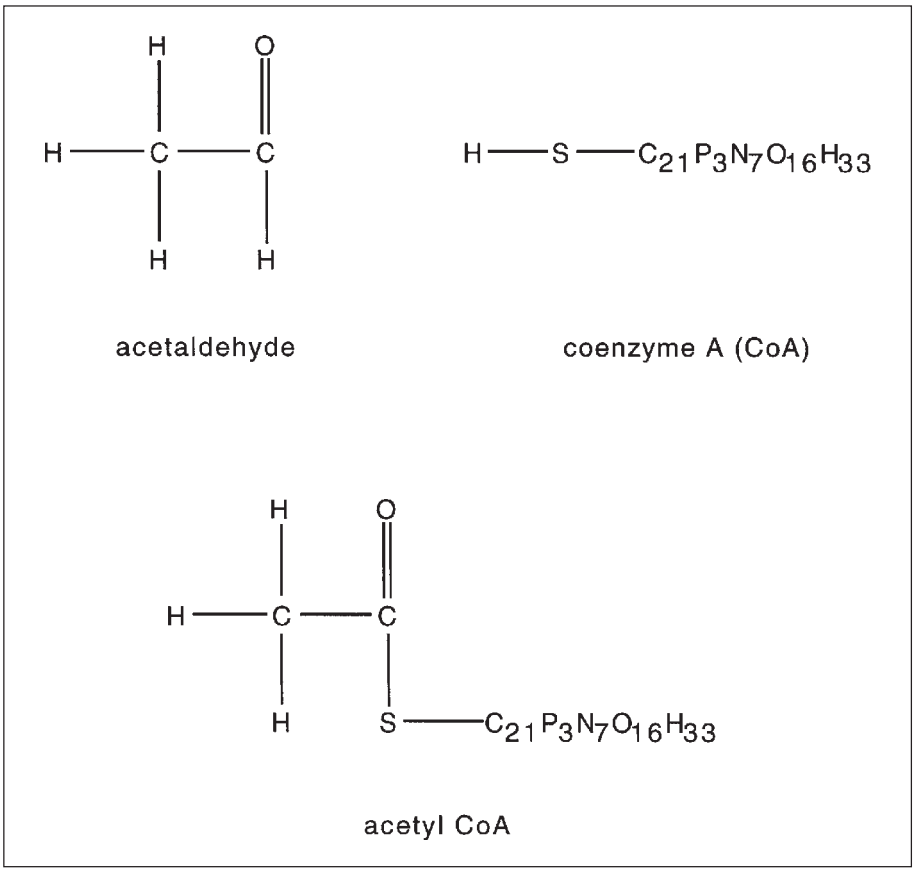

Fig. 2.4. Acetaldehyde, CoA and acetyl CoA.

I say it was worked out in the 1930s, but in fact one very important step was absent until 1953. Until then, it was thought that the TCA cycle created no ATP. But in fact, one of the steps in Krebs's original formulation—the conversion of α-oxoglutarate to succinate—occurs in two steps, with coenzyme A as a cofactor, and the latter of these steps includes the creation of one molecule of guanine triphosphate (GTP) from GDP and phosphate.[18] This reaction is followed by a transphosphorylation reaction, mediated by the enzyme nucleoside diphosphate kinase, in which the GTP is turned back into GDP and the released phosphate is transferred to ADP making ATP. Many bacteria skip this last complication and phosphorylate ADP directly in the succinate synthesis step.[19]

2.3.3.3. Repaying the Oxygen Debt: Respiration

At this point, the destruction of one molecule of glucose has consumed six molecules of water and yielded six molecules of carbon dioxide. Additionally, it has stored up 24 hydrogen atoms—12 from the original glucose and 12 from the water—whose protons are free in the aqueous medium, while their electrons are carried by ten molecules of NADH and two of $FADH_2$. There is a limit to our supply of NAD^+ and FAD, and also to the number

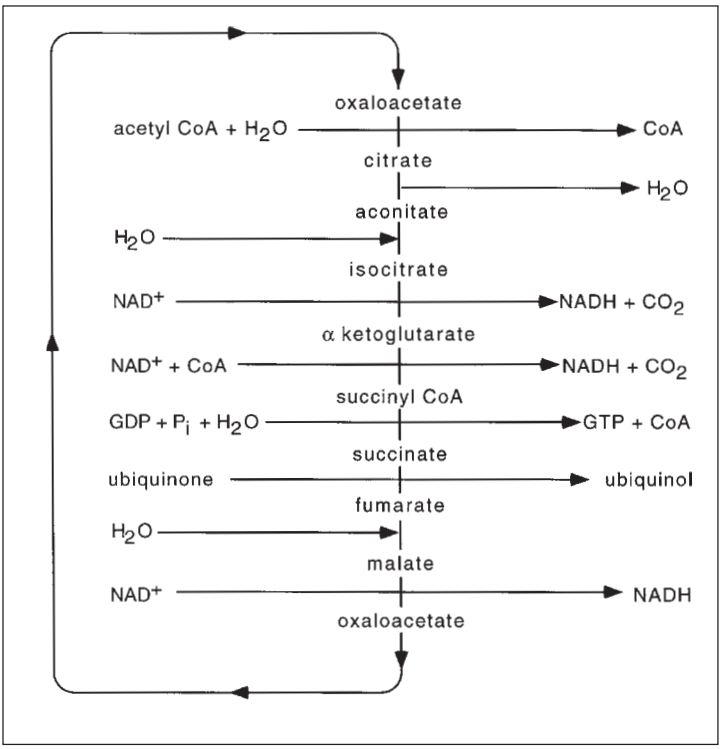

Fig. 2.5. The tricarboxylic acid, or citric acid, or Krebs cycle.

of protons that can be discarded into the matrix without changing its chemical properties, so this is only a sustainable process if the electrons and protons can be off-loaded. This is done by combining them with oxygen: and this time, the oxygen used is the straightforward, molecular oxygen (O_2) that we breathe, not borrowed from some other molecule. Each molecule of O_2 is combined with four electrons and four protons (that is with four hydrogen atoms) to make two molecules of water. Thus, six molecules of O_2 are used to get rid of the hydrogen that came from oxidizing one molecule of glucose.

This process may sound quite simple, but the machinery that achieves it is extremely elaborate, involving dozens of proteins formed into four large structures called Complexes I, II, III, and IV. (These structures also have long enzymatic names, respectively, NADH dehydrogenase, succinate dehydrogenase, ubiquinol-cytochrome c oxidoreductase and cytochrome c oxidase.) It also involves two other small molecules: coenzyme Q (abbreviated "CoQ"), which is not a protein but a quinone (see Fig. 2.6) and which transports electrons from Complexes I and II to Complex III, and cytochrome c, which is a small protein that transports electrons from Complex III to IV. Complex IV is the place where the electrons are combined with oxygen. This sequential arrangement of components gives rise to the terms "respiratory chain" or "electron transport chain" to describe the whole system starting at Complexes I and II and ending at Complex IV (see Fig. 2.7).

How does an electron get passed along the respiratory chain? For the most part, it is carried by atoms of metals that are able easily to vary the number of electrons they carry (their valency). Such metals (iron, for example) are called transition metals. They will feature

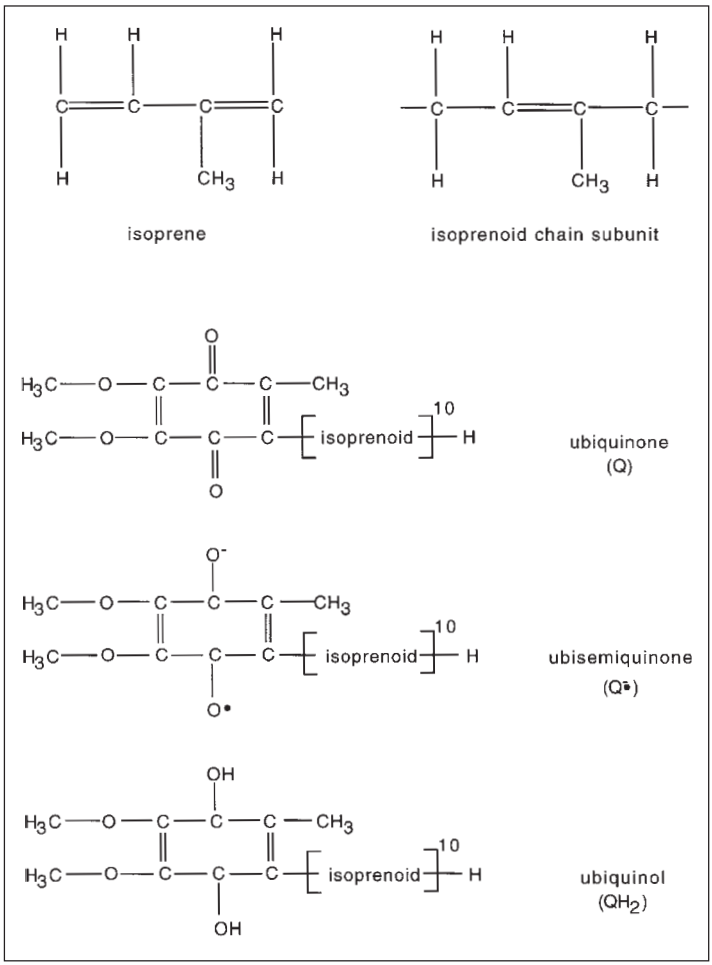

Fig. 2.6. Coenzyme Q, in its three forms.

frequently in this book because in certain situations they can contribute greatly to the toxicity of free radicals. But in the respiratory chain, these metal atoms are tightly bound with the enzyme complexes and cytochrome c, and are not toxic.

The non-protein member of the respiratory chain, coenzyme Q, does not harbor a transition metal atom to carry the electrons: it carries them itself, like NAD and FAD. It has one very striking difference from NAD and FAD, though: as shown in Figure 2.6, it exists not in two alternative states but in three. The first and third states, ubiquinone and ubiquinol, differ in content by two electrons just like the two states of NAD and FAD. In fact, these two are the only ones which exist free in the membrane (moving between the enzyme complexes). The intermediate form, ubisemiquinone, exists only fleetingly while CoQ is interacting with Complexes I or III, and is fairly tightly bound to them during this time. However, its existence is (for the purposes of this book) the weak link in the respiratory chain, because it can spontaneously revert to ubiquinone, and the electron it releases in so doing can, in due course, do immense harm. We will cover the details of this toxicity in Chapter 3.

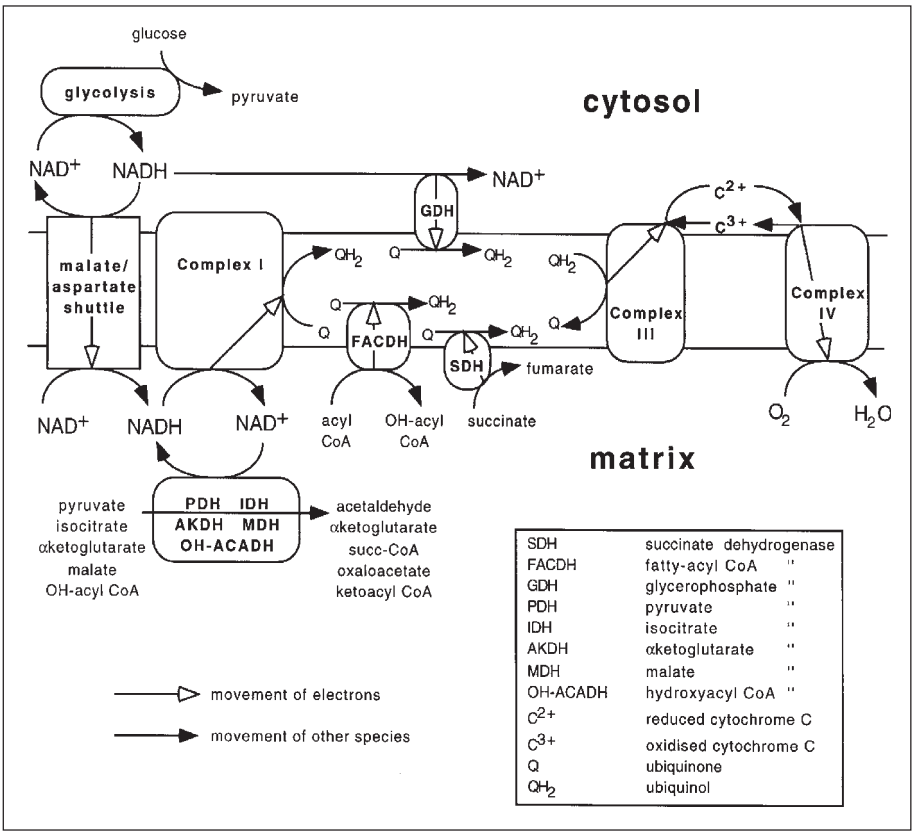

Fig. 2.7. The respiratory chain.

Like the earlier steps in the destruction of pyruvate, all these components are located in mitochondria. In this case they are all embedded in the mitochondrial inner membrane, except for cytochrome c which moves within the aqueous space between the mitochondrion's inner and outer membranes (the intermembrane space). Complex II is a slightly special case: it is both a part of the TCA cycle and a part of the respiratory chain. This is because, unlike NAD, FAD does not exist free in solution. Instead, Complex II contains a "trapped" molecule of FAD; in its TCA cycle role it causes the transfer of electrons from succinate to that FAD making $FADH_2$, and in its respiratory chain role it causes the transfer of those electrons from the $FADH_2$ to coenzyme Q, regenerating FAD. The s,n-glycerophosphate dehydrogenase also uses a trapped FAD, but with that exception all the other electrons that enter the respiratory chain from the processes discussed so far—glycolysis, the pyruvate dehydrogenase cycle and the TCA cycle—are delivered by NADH, which donates them to Complex I. NADH molecules are not trapped but diffuse freely in the mitochondrial matrix, so there is no similar physical association of Complex I with NADH-producing components of these cycles.

A final note about fatty acids is appropriate here. Their breakdown to acetaldehyde also releases hydrogen atoms; in this case, the electrons are donated in equal quantity to NAD^+

and FAD. The resulting NADH goes straight to Complex I, but the $FADH_2$, just like that in Complex II, is trapped within the relevant enzyme, fatty acyl CoA dehydrogenase. Again like Complex II, this enzyme resides in the inner membrane and also catalyses the transfer of the electrons from $FADH_2$ to coenzyme Q and on to Complex III.

The depiction of the respiratory chain in Figure 2.7 implicitly includes the fatty acyl CoA and s,n-glycerophosphate dehydrogenases and the malate/aspartate shuttle as members. This is not in fact customary: more usual is to define the respiratory chain as composed only of Complexes I to IV. I think this is illogically TCA-ocentric, however, and prefer the expanded definition implied in Figure 2.7.

2.3.4. Mitochondrial Physics: Oxidative Phosphorylation

The alert reader may have noted the conspicuous absence of ATP from Section 2.3.3.3. It will resurface here, along with an explanation of why the conversion of oxygen to water involves such suspiciously intricate machinery.

2.3.4.1. The Curious Isolation of Complex V

Everything discussed is Section 2.3.3 was known by 1953. It was also known that cells made hugely more ATP than could be accounted for by the contributions of glycolysis and the TCA cycle; moreover, the enzyme which made the "missing" ATP was characterised not long afterwards.[20] Like the components of the respiratory chain, it is a highly sophisticated structure in the mitochondrial inner membrane; naturally it is termed Complex V (or, alternatively, ATP synthase or ATPase).

There was a problem, however. It was quite clear that the respiratory chain had to be, somehow, driving ATP synthesis by Complex V. It was releasing the required amount of energy—it had to be, because it was mediating the reaction of hydrogen with oxygen to make water. This clear linkage gave rise to the term oxidative phosphorylation, or OXPHOS, to describe mitochondrial function—the linkage of oxidative processes (described above) with the phosphorylation of (addition of phosphate to) ADP making ATP. The difficulty was that there was no apparent physical or chemical linkage (coupling, as it is usually termed) between the respiratory chain and Complex V. Somehow there had to be a transfer of energy between the two, but exactly how was a mystery that resisted elucidation for many years more. This transporter of energy was so central to the description of other biochemical processes that biochemists resorted to a special notation for talking about it: it was denoted "~". I think this was a good choice, evoking very clearly the despair felt by those struggling to identify it.

2.3.4.2. The ATP Goldmine: Chemiosmosis

This last major part of the puzzle of mitochondrial function fell into place, with rather a fight, starting in 1961 with the publication by Peter Mitchell of the chemiosmotic hypothesis.[21] Mitchell's pivotal breakthrough was to identify an assumption that everyone was making but which was unfounded. The assumption was that, in cells, the only way to get energy from one molecule to another was in chemical reactions. Granted, that was how it had always been found to be done; but just because a process was happening in cells, that did not prohibit the process from using the other types of energy that are familiar in physics. Mitchell realised that electrochemical potential energy was a perfectly realistic candidate in this case. The enzymes between which energy was being transferred all reside in the inner membrane of mitochondria, and that membrane is, by and large, impermeable to ions. Therefore, it was conceivable that the respiratory chain was generating a disparity in the concentrations of one or another ion on either side of this membrane. Basic thermodynamics tells us that that disparity takes energy to create, and that energy is released as and when it is

dissipated. So, if the respiratory chain were generating such a disparity, then the ATP synthase could be drawing on it for energy to make ATP, and there would be absolutely no need for the chemical link that had so long been sought. In Mitchell's own words: "The elusive character of the 'energy-rich' intermediates of the orthodox chemical coupling hypothesis would be explained by the fact that these intermediates do not exist."[21]

Most specialists found Mitchell's hypothesis unattractive at first, but experimental data in support of it eventually emerged from other labs[22] and it is now universally accepted.* The ion in question is simply the proton: Complexes I, III and IV (but not the FAD-linked enzymes) all extract energy (by "normal," chemical bond-making and -breaking processes) from the electrons they are shuttling, and use this energy to translocate protons from inside the inner membrane to outside to create the required disparity. (The movement of water across the membrane, noted in Section 2.3.2.4, does not affect this.) These protons return into the mitochondrion through Complex V, and the potential energy that they release in doing so is converted into the creation of the bond between ADP and phosphate that makes ATP (see Fig. 2.8). This situation can equally be described in terms of force rather than energy; Mitchell named the force generated by the respiratory chain, which drives the ATP synthase, the proton-motive force.

Exactly how the respiratory chain proteins move the protons, and how the ATP synthase constructs ATP, are still matters of intense research. For example, Mitchell proposed in 1975 a mechanism of action for the simplest proton-pumping respiratory chain enzyme, Complex III,[23] and his hypothesis—the Q cycle—is still broadly accepted, but there are some details of it that may need to be revised,[24] and new hypotheses are still emerging to fill in its details at the level of enzymatic binding of substrates.[25-27] Recent progress has been most dramatic with regard to ATP synthase, which is now known[28,29] to work as a turbine, with the centre of the complex rotating within the rest of it.

2.3.5. A Note About Other Energy-Transducing Biological Systems

The release of energy from sugars, by combining them with oxygen, is—to a first approximation—the reverse of photosynthesis, the process by which plants (using organelles also derived by endosymbiosis, chloroplasts) construct sugars and oxygen from carbon dioxide and water, using sunlight as their energy source. It is thus no surprise, given the ubiquitous reversibility of enzymes, that the machinery of chloroplast photosynthesis is very similar to that of respiration. Likewise, bacteria have energy-transducing systems which exhibit strong similarities to those of mitochondria. Comparisons between mitochondria and these other systems (chloroplasts, in the case of plants) might in principle be useful, as are, for example, comparisons between mitochondria of different animal species (which will be the topic of Section 6.5.3). But not in practice. I have found that the differences between mitochondria and their non-animal counterparts are in fact more confounding than helpful, so I will not introduce them.

2.4. Mitochondrial DNA, or mtDNA

2.4.1. The Discovery of Mitochondrial DNA

In about 1961, as Mitchell was elucidating the fundamentals of mitochondrial function, a surprising discovery was made regarding mitochondrial structure. I say "about," because this discovery was one of the messy ones which it is not really fair to attribute to a particular

** This universal acceptance applies to the "core" chemiosmotic hypothesis set out in 1961, but there is still deep disagreement regarding refinements of it which were first made explicit in 1966. We will explore this topic in Section 11.3.*

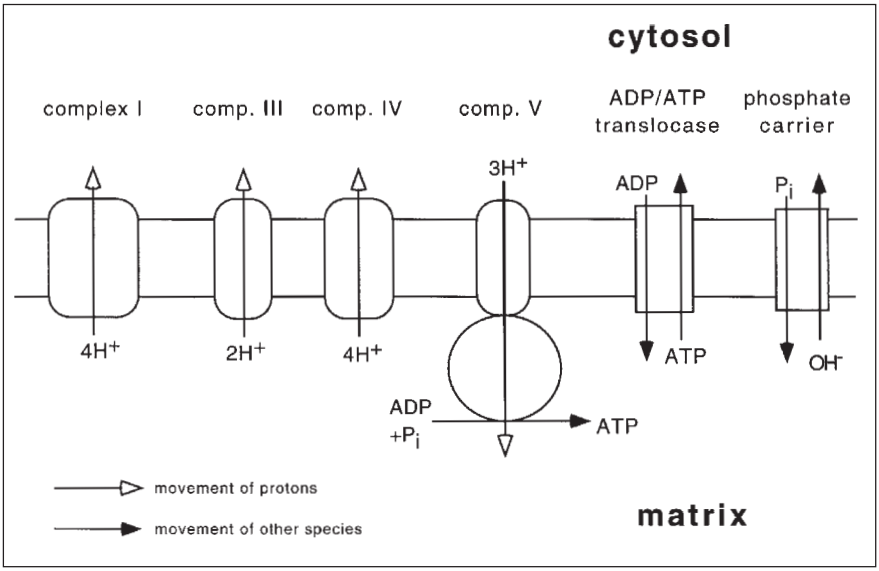

Fig. 2.8. The chemiosmotic proton circuit.

laboratory at a particular time. The discovery was that mitochondria contained their own DNA, and the best that can be said with fairness is that it spanned a period between 1959, when Chèvremont and colleagues first reported evidence for it,[30] and 1963, when Nass and Nass published a clear demonstration that a frequently isolated component of mitochondria was indeed DNA.[31] No one had any reason to suppose that mitochondria needed DNA, so there was initially a tendency to ascribe its presence in mitochondrial preparations to nuclear contamination. Indeed, mitochondria are the only components of animal cells' cytoplasm that have any of their own DNA;* all the rest of our genetic material is stored in our chromosomes, in the nucleus.

This reticence was somewhat compensated in the following years. There was still a lingering suspicion that the mitochondrial DNA might be of nuclear origin after all, but this was swept away by the discovery that it is a circular molecule, in contrast to the linear character of all eukaryotic chromosomes. This discovery was made in 1966, and published with alacrity, by three labs independently.[32-34]

Each mitochondrion has several copies of the mtDNA. The exact number varies between species; in humans it is typically about five.

2.4.2. Inheritance of mtDNA

Erythrocytes are the only mammalian cells with no mitochondria, but there is another cell type which has very few: sperm. Mitochondria are passed on from one generation to the next in egg cells, but almost none are contributed by sperm.** This is largely because sperm

** There is still some debate on this point with regard to centrioles, but the view that they have their own DNA is not currently favoured.*

*** It is widely stated that no mtDNA whatever is paternally inherited, but this is incorrect: see Ref. 35 for an eloquent dissection of this "meme."*

are so small, containing under 100 mitochondria (located in the tail); also, there may be a system whereby any paternal mtDNA that gets into the fertilized egg is then actively destroyed.[36] This situation is of course in complete contrast to the way that the rest of our DNA is inherited, namely that we receive one copy of each chromosome from each parent (with the exception of the sex chromosomes). The almost solely maternal inheritance of mitochondrial DNA has proved very useful in many areas of science: it has helped us to discover the patterns of migration of early man,[37] and also to pin down the genetic cause of certain degenerative diseases, which were found to be maternally inherited and then to be associated with particular mitochondrial DNA mutations (see Section 6.6.5).[38,39]

2.4.3. The Nuclear-Coded Parts of Mitochondria; Protein Import

Quite soon after mtDNA and its maternal inheritance were discovered, genetic studies began to show that some of the enzymes known to be present in mitochondria exhibited normal Mendelian inheritance, which meant that their genes must be carried in the nucleus, not in the mtDNA. In 1981, when the human mtDNA was sequenced,[40] it was established that just 13 proteins are encoded in it—a small fraction of those known to be mitochondrially located. The hundreds of other proteins that are found in mitochondria are all encoded in nuclear, chromosomal DNA, and are constructed in the cytoplasm just like all other chromosomally encoded proteins. They are then imported into mitochondria. The import mechanism is highly sophisticated, and is still the subject of intense study. It carries molecules across the energy-transducing inner membrane, so it must be as fastidious as all the other carrier systems described earlier (see Section 2.3.2.4) in not allowing free transit ("leakage") of protons or other ions; but it must do so while translocating a molecule far larger than is carried by those other systems. Also, since proteins are too big to fit through the pores of the outer membrane, they must be dragged through both membranes. This has traditionally been supposed to occur at specialised membrane locations, the "contact sites" mentioned earlier, but there is now good evidence that the protein translocation systems of the outer and inner membranes are separate except when engaged in translocation:[41] the contact sites are indeed the sites of import, but they are created by the import process pulling the membranes together. (This is why they were not shown in Figure 2.2.) The proteins involved are all found to be located strictly in one or the other membrane (or in the intermembrane space), and are in fact now named accordingly—"Tom" and "Tim" (followed by a number indicating the particular protein's molecular weight) for "Transporter of the outer (inner) mitochondrial membrane."* The channel formed by the Tom proteins is also often termed the general import pore.

One of the first things to be discovered about the import process was the characteristic by which a protein destined for the mitochondrion is recognised as such. Nearly all of those initially studied have a "signal" sequence of a few dozen amino acids, at the beginning of the protein, which has no role in the protein's ultimate function—in fact, it is chopped off once the protein is safely inside the mitochondrion.[42] But prior to import, it acts to tell the cell's transport machinery to take it to a mitochondrion. Experiments have shown that typical cytosolic proteins (ones that are not normally imported into mitochondria) will be imported into mitochondria if such a signal is attached to them.[43]

** This nomenclature, due to Nikolaus Pfanner, is so attractive that it has been copied by botanists to describe the corresponding proteins of chloroplasts, which are called Tocs and Tics. Some primitive organisms have organelles with three or four membranes; I await the corresponding nomenclature with interest.*

The journey of nuclear-coded proteins whose eventual destination is not the matrix but the inner membrane (or, in a few cases such as cytochrome c, the intermembrane space) is rather more heterogeneous. Some of them have no signal sequence and pass only through the outer membrane.[44] Others have two signal sequences of the sort described above: one which causes them to be imported completely into the matrix, and another (which is invisible to the relevant machinery until the first has been cleaved) that directs it part of the way out again.[45] Yet others also have two, but the second acts as a barrier to import, so that the mature protein stays in the intermembrane space even though the primary signal sequence penetrates into the matrix (where it is chopped off).[46] A few inner membrane proteins, particularly the carriers of anions such as ATP and phosphate, appear to find their way to mitochondria despite having no presequence, but they have been presumed to use the same pathway, because they were found to have internal sequences which are characteristic of standard presequences, so may fold into a hairpin-like shape that makes the internal sequence look like a presequence.[47] Quite recently, this has been elucidated in more detail:[48,49] these anion carrier proteins do indeed use broadly the same "Tom" machinery to get across the outer membrane, but they use a different "Tim" machinery, which embeds them in the inner membrane from the outside, rather than diverting them into the matrix. The varieties of mitochondrial protein import are depicted in Figure 2.9.

2.4.4. Curiosities of mtDNA

The mtDNA of many species has been studied and sequenced. In animals it is always tiny: in humans, for example, it comprises only 16,569 base pairs,[40] of which virtually every one is necessary for its function. In most plants and fungi, however, it is much larger, containing large introns and other "junk DNA."[50] This indicates that there has been considerable evolutionary pressure for conciseness of mtDNA in animals, but sometimes less elsewhere. We do not know the basis for this difference. In fact it may be somewhat less dramatic than it seems, because the compactness of animal mtDNA is permitted mainly by a single trick—the use of transfer RNA sequences as sites for chopping-up of a primary transcript of almost the whole genome into separate RNAs for each of the proteins—which relieves it of the need for any of the regulatory, untranslated sequence present in fungal and plant mtDNA.[51,52]

One rather surprising thing about the mtDNA is that it exists at all, since ostensibly it is a major and unnecessary inconvenience. There is no formal need for it, because (as just noted) cells have a system for getting proteins into mitochondria even when they are encoded on nuclear genes: they are constructed in the usual way by ribosomes in the cytoplasm, and then they are transported to a mitochondrion and hauled through its membranes. Reasons why it has not been evolved away are explored in Section 10.2.

But even more surprising was the discovery[53,54] that mitochondrial DNA—again, in animals but not plants—has a different genetic code than the nuclear DNA. The process of turning a sequence of nucleotides into a sequence of amino acids involves the recognition of triplets of nucleotides as coding for a particular amino acid, or else for "this is the end of the protein." Since there are four different nucleotides, there are $4^3 = 64$ possible triplets of nucleotides—"codons"—and, since there are 20 amino acids, 21 things to encode. The mapping between them is called the genetic code. The nuclear genome of virtually every free-living organism uses exactly the same code (though there are a few exceptions).[55] This is no great surprise, since any mutation causing a change to the code would necessarily change the amino acid sequences encoded by huge numbers of genes; a few might not matter, but there would inevitably be many for which the change was a lethal mutation.

Conversely, it is less amazing that animals' mitochondria can be more flexible about their code, since there are only 13 potential "victims." Indeed, the mitochondrial genetic

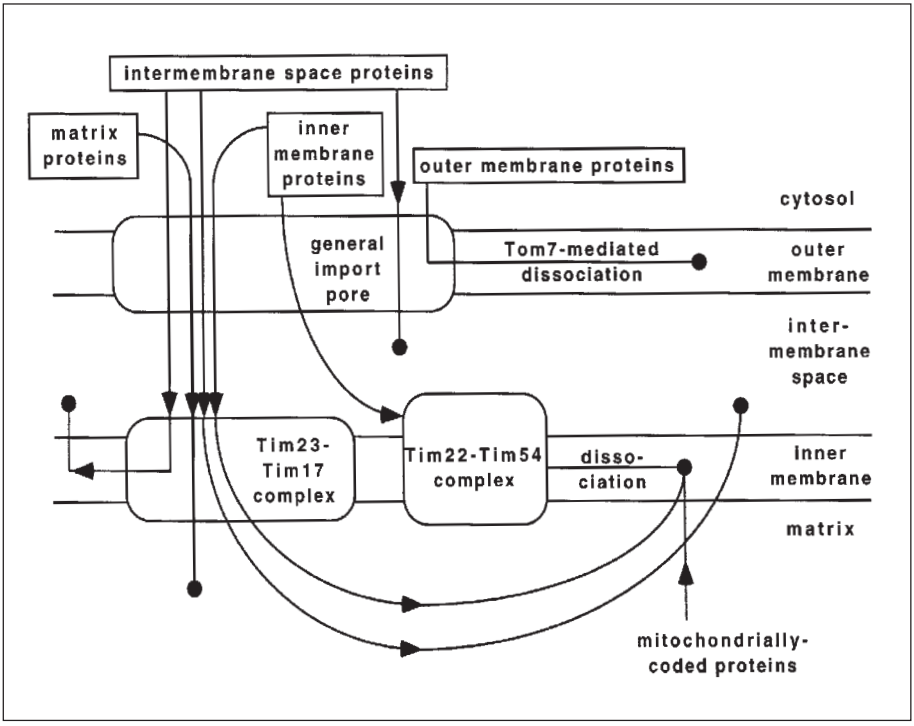

Fig. 2.9. Types of protein import into mitochondria.

code is not simply different from the nuclear code, but also differs from one species to another. Evolution seems to have allowed it to drift.

All animals whose mitochondrial DNA has been sequenced encode exactly the same 13 genes on it, except that nematodes[56] and some mussels[57] encode only 12 of them. (The last, ATPase subunit 8, may be encoded on a nuclear gene, or it may have been completely lost; the latter option is plausible because the encoded protein is not a structural component of the complex but is merely involved in its assembly.) Nearly all plants so far examined* also have broadly the same set, plus usually a selection of extra ones.

Some (though not all)[58] yeasts' mitochondria only encode about half of the proteins encoded in animals' mtDNA, but in this case there is not the uncertainty that attends nematodes' ATPase 8: all the missing ones are subunits of Complex I, and these yeasts simply do not have a Complex I at all, so the genes for its subunits are absent from either genome. These yeasts do have an enzyme that takes electrons from matrix NADH and passes them to ubiquinone, but it is nothing like normal Complex I in structure; in particular it does not pump protons.[59] The evolutionary causes and effects of various organisms' mitochondrial genetic complements will be discussed in Section 10.2.

** We will come back to the exceptions in Chapter 15, since they may be extremely useful in the development of one particular life-extension therapy.*

2.4.5. The Vulnerability of mtDNA and Mitochondrial Function

The 13 proteins encoded in the mtDNA are absolutely essential for OXPHOS to occur. It is therefore just as important for an organism to maintain its mtDNA intact (in all cells, not simply the germ line) as to maintain its nuclear, chromosomal DNA. However, for reasons connected with mitochondria's evolutionary origin (which was described in Section 2.1), cells fail to maintain their mtDNA to the nuclear standard. The mtDNA is much more prone to suffer spontaneous changes to its sequence, which result in the production of incorrect or truncated proteins—or, in some cases, failure to produce any protein at all.

This vulnerability takes several forms. Firstly, the mtDNA is much more exposed than the nuclear DNA to free radicals that can induce mutations. This is because mitochondria are the main cellular site of production of free radicals; this will be explained in Section 3.3. There are no mitochondria in the nucleus, so the nuclear DNA is exposed to a much lower concentration of free radicals.

A second source of mtDNA vulnerability is that it is "naked." Nuclear DNA is always wrapped around special proteins called histones, which keep it somewhat protected from mutagenic attack. mtDNA does not have these.[60]

Another reason is that, once damage is done to it, mtDNA is less well repaired than nuclear DNA. Certain classes of damage to DNA are rapidly reversed when they occur in the nucleus, but do not get mended in mitochondria. For some years it was believed that mtDNA was not repaired at all, but this was a piece of bad luck: the first researchers to address this question[61] had happened to analyse a class of damage called a pyrimidine dimer,* which indeed is not repaired at all in mtDNA, and most investigators incorrectly presumed that this was true of other types of damage too.

Yet another problem for mtDNA is that it contains a high level of short sequences which appear twice, some way apart. This is dangerous, because it is possible for the circular mtDNA molecule to wrap around into a figure of eight, with the two identical sequences lying next to each other. When that happens, there is occasionally a "crossover"—the strands come apart and join up the other way, just as chromosomes do in meiosis. For a mtDNA molecule, however, this is fatal, because the single circle of DNA becomes two circles, each with only a subset of the necessary genetic material (see Fig. 2.11). Worse yet, one of those two circles will be without the D loop, a small stretch of DNA which contains no genes but which is essential for initiating replication of the molecule. Thus, the circle without the D loop will never be replicated and will eventually be lost, so that the final result is a deletion of a large chunk of the mtDNA. Indeed, these are easily detected in humans in vivo. In theory, the longer the stretch of duplicated sequence the greater will be the tendency for this to occur. In practice, one particular deletion is much more prevalent than any other[62a]—so

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** The four nucleotides that endow DNA with its informational content are classified into two sets of two, on account of their atomic structure. Cytosine and thymine are pyrimidines, with a single-ring structure; adenine and guanine are purines, which have a double-ring structure. Thus, the complementary bases on opposite strands of the double helix are always one pyrimidine and one purine. The other discriminant, which causes (e.g.) adenine always to be paired with thymine rather than with cytosine, is that adenine and thymine are prone to form two hydrogen bonds whereas guanine and cytosine form three (see Fig. 2.10). A pyrimidine dimer is the result of a covalent bond being formed between adjacent pyrimidines on the same strand.*

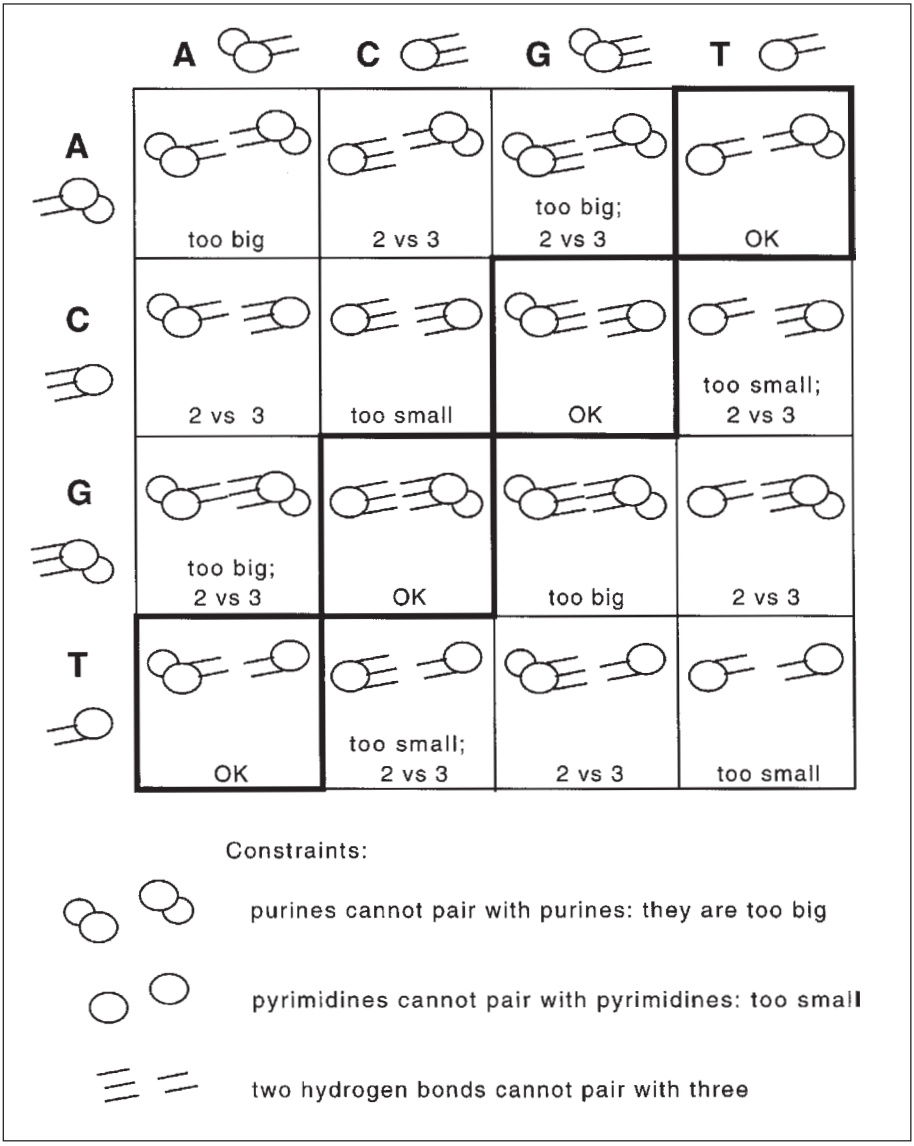

Fig. 2.10. Why the DNA bases pair only with their complements.

much so that it is usually called the "common deletion"—and, indeed, it is precisely flanked by the longest relevant* direct repeat in the human mtDNA, 13 base pairs.[40]

** Actually there are three other 13-bp direct repeats and even one 15-bp one. These are not observed to give rise to deletions. But this is no surprise, because two of these four have one of the repeats actually within the D loop region (so neither of the resultant circles would have a complete D loop), and the other two would put the D loop on one circle and the light strand origin (which is also needed for replication) on the other. Thus, each of these only create molecules that cannot be replicated.*[62b]

Table 2.1. The human mitochondrial genome, using the standard numbering conventions

Position	Gene	Component of
577-647	phenylalanine tRNA	
648-1601	ribosomal RNA, subunit 12S	ribosome
1602-1670	valine tRNA	
1671-3229	ribosomal RNA, subunit 16S	ribosome
3230-3304	leucine tRNA (UUA or UUG)	
3307-4262	NADH dehydrogenase, subunit 1	Complex I
4263-4331	isoleucine tRNA	
4329-4400	glutamine tRNA	
4402-4469	methionine tRNA	
4470-5511	NADH dehyrogenase, subunit 2	Complex I
5512-5576	tryptophan tRNA	
5587-5655	alanine tRNA	
5657-5729	asparagine tRNA	
5761-5826	cysteine tRNA	
5826-5891	tyrosine tRNA	
5904-7444	cytochrome c oxidase, subunit 1	Complex IV
7445-7516	serine tRNA (UCN)	
7518-7585	aspartate tRNA	
7586-8262	cytochrome c oxidase, subunit 2	Complex IV
8295-8364	lysine tRNA	
8366-8572	ATP synthase, subunit 8	Complex V (non-structural)

Table 2.1. The human mitochondrial genome, using the standard numbering conventions, cont.

Position	Gene	Component of
8527-9207	ATP synthase, subunit 6	Complex V
9207-9990	cytochrome c oxidase, subunit 3	Complex IV
9991-10058	glycine tRNA	
10059-10404	NADH dehydrogenase, subunit 3	Complex I
10405-10469	arginine tRNA	
10470-10766	NADH dehydrogenase, subunit 4L	Complex I
10760-12137	NADH dehydrogenase, subunit 4	Complex I
12138-12206	histidine tRNA	
12207-12265	serine tRNA (AGC or AGU)	
12266-12336	leucine tRNA (CUN)	
12337-14148	NADH dehydrogenase, subunit 5	Complex I
14673-14149	NADH dehydrogenase, subunit 6	Complex I
14674-14742	glutamate tRNA	
14747-15887	cytochrome b	Complex III
15888-15953	threonine tRNA	
15955-16023	proline tRNA	

There have also been reports that mitochondrial DNA is more inaccurately replicated than nuclear DNA,[63] though this has since been disputed.[64] However, in non-dividing cells (a category which includes nerves and muscle fibres, among others) there is quite definitely a greater risk of replication error of mtDNA than nuclear DNA, for a much more direct reason: their nuclear DNA is not being replicated at all, so its risk of replication error is necessarily zero. Their mitochondrial DNA, on the other hand, is still being replicated (as will be discussed in the next section), so its risk of replication error, however slight, is greater. In fact, there is circumstantial evidence that replication error is the main source of mtDNA mutations in somatic cells. This is that only a small percentage of mtDNA point mutations seen in vivo, whether in evolution[65] or in cultured cells,[66] are transversions—changes of a purine to a pyrimidine or vice versa. All the rest are transitions, which change a purine to

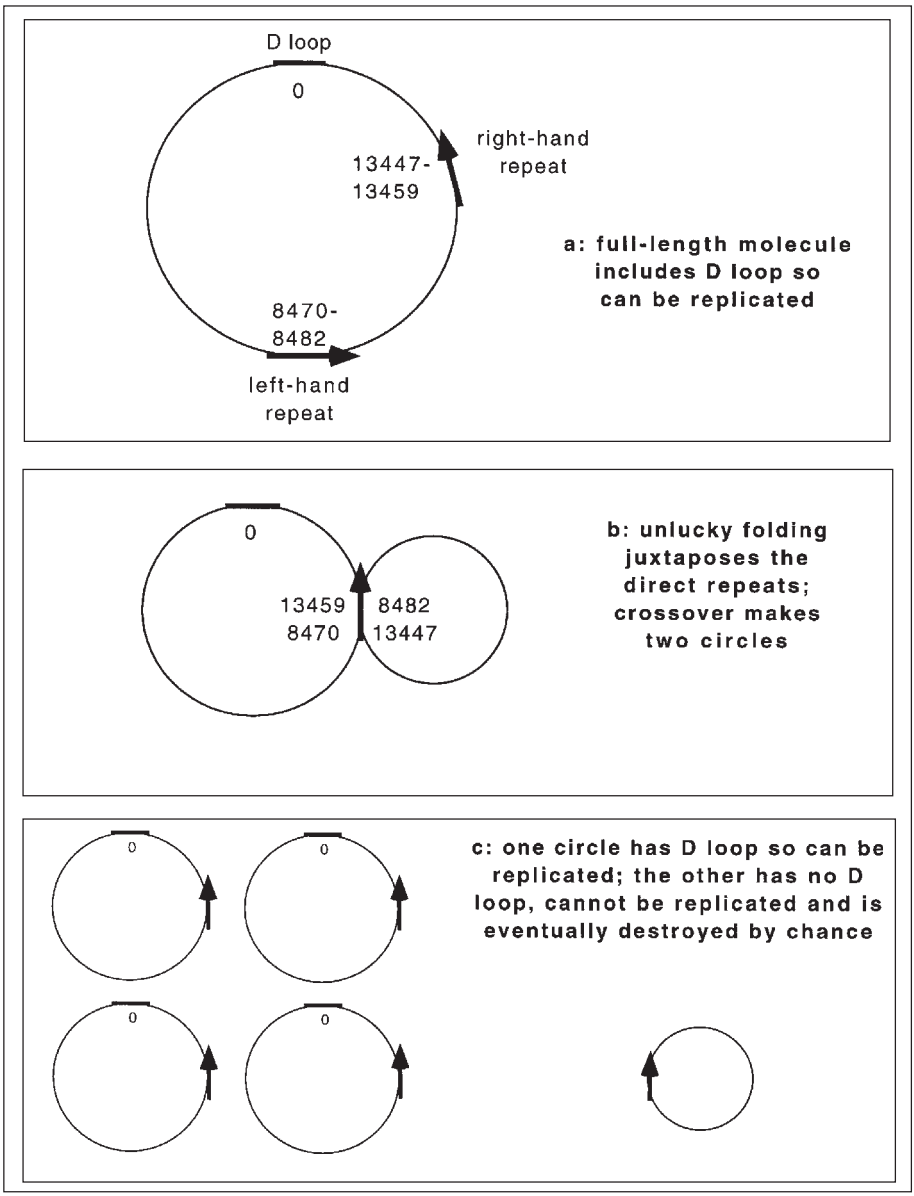

Fig. 2.11. Formation of a mtDNA deletion by illegitimate recombination.

the other purine or a pyrimidine to the other pyrimidine. Replication error is known to be strongly biased towards transitions,[67] basically because purines are a very different shape from pyrimidines so the replication enzymes are less likely to mistake one for the other;* many mutagens, by contrast, are known to be biased towards transversions, because damage

**For a more specific reason see Section 5.2.*

to certain nucleotides makes (for example) guanine "look like" thymine.[68] The predominance of replication error as a source of mutations may also explain why the mitochondrial tRNA genes are particularly prone to pick up spontaneous mutations: relative to nuclear DNA, an unusually long stretch of the mtDNA is single-stranded during replication, and while a tRNA gene is in that state it may have a tendency to fold up into the configuration it adopts as RNA, which would impede DNA synthesis of the other strand and increase the chance of errors.[69]

2.4.6. Mitochondrial Turnover

Around the same time that mtDNA was identified, a related discovery was made. Each time a cell divides, its mitochondria must also divide—not necessarily at once, but eventually—or else the daughter cells will have progressively fewer mitochondria, which will eventually deprive them of energy. But in 1961 it was shown that mitochondria divide more often than that. As noted in the previous section, many cells in the human body are permanently non-dividing, or postmitotic: they can do their job in the body, but they can never divide again. Nerves and muscle fibres are prominent examples. Numerous other cell types, such as glia and fibroblasts, are in almost the same class: they can divide, but in practice they virtually never do so except in response to tissue damage (such as a wound). Paradoxically, even in non-dividing cells (postmitotic or not), there is a turnover of the mtDNA: the average mtDNA molecule lives less than a month.[70] Now, all the proteins in a mitochondrion could—in theory—be recycled individually, rather like parts of a car, without actually having the mitochondrion divide. But if the mtDNA is being recycled, the mitochondria themselves must be dividing. This means that mitochondria must also be getting destroyed in postmitotic cells, since otherwise the cells would fill up with mitochondria. This logic was soon confirmed by direct electron-microscope observation:[71,72] they are engulfed by lysosomes, which are the cell's generic garbage collector.

Incidentally, this explains why cells need not bother to repair pyrimidine dimers in mtDNA. The enzyme responsible for mitochondrial DNA replication is not able to copy a pyrimidine dimer—in fact, it cannot even jump across and resume replication on the other side of one.[61] So mtDNA molecules that have suffered that particular type of damage are never replicated, and are eventually destroyed, by random chance, along with their host mitochondrion. This acts as a perfectly good mechanism to stop them accumulating, so no active repair process is necessary.

There is another feature of mitochondria in non-dividing cells which will be central to the theory outlined later on, and which is therefore worth mentioning here. Tissues composed of non-dividing cells, especially ones which use a lot of energy (such as the heart), are found to accumulate much higher levels of mutant mitochondrial DNA during aging than dividing cells.[73] We will come back to this feature of mtDNA decline in Section 6.6.

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Chapter 3

An Introduction to Free Radicals

3.1. Another Terminological Apology: "Pairing" of Electrons

It is most unfortunate that the term "free radical" has become so firmly entrenched in the vocabulary of gerontology, especially in the popular press. The problem is that the meaning which is generally attached to it has several major differences from its strict chemical definition.

Free radicals are a class of molecule with a very simple definition. The nature of atomic structure and of the covalent chemical bond, the features that give an atom its valency, are underpinned by the rule that electrons occupy orbitals of atoms, such that an orbital can contain zero, one or two electrons, and that electrons carry less energy when they are one of a pair in an orbital than when they are unpaired. A molecule is a free radical if and only if it possesses any unpaired electrons. That's all. Some are composed of only two atoms; some are huge, being made by removing one electron from, for example, a protein or a chromosome.

If one were to try to infer what a free radical is solely from reading the gerontological literature, however, one would form the impression that a free radical is a small, highly reactive, highly toxic, oxygen-centred molecule which can be rendered less toxic by the addition or removal of one electron. This is a clear case of a little knowledge being a dangerous thing; it is near enough to the real definition to seem trustworthy, but it can easily cause misunderstanding of important details.

So, what are the specific differences between these two definitions? Superficially, "unpaired" might be thought to connote that free radicals have an odd number of electrons, whereas all other molecules have an even number. Unfortunately, "pairedness" is defined in terms of the physical interactions of a molecule's electrons, and those interactions are much more complicated than that. For a start, the vagaries of atomic structure in fact allow a molecule to have two unpaired electrons at once. Such molecules are called diradicals. In practice, however, the only diradical which will concern us is molecular oxygen (O_2), and its electrons are arranged in such a way that, for purposes of reactivity, its unpaired electrons behave rather as if they are paired after all.*

Another class of chemical which sometimes breaks the correlation between pairedness and evenness is atoms. Atoms that contain an odd number of electrons are not called free radicals, and indeed those that exist in cells do not quite behave in the same way as bona fide free radicals do. Some of the most biologically important examples are atoms of transition metals, which can carry varying numbers of electrons; iron and copper, in particular, will feature extensively in this book. But curiously, these atoms are both more "free-radical-like" when carrying an **even** number of electrons!

** The electrons of O_2 can be arranged differently, so that it does behave like a radical: it is then termed singlet oxygen. This variant of O_2 is written $^1O_2$ and plays a role in light-induced skin damage;[1a] see also Table 3.3 and Section 11.2.3.*

The Mitochondrial Free Radical Theory of Aging, by Aubrey D.N.J. de Grey.

Finally, some free radicals have decidedly un-free-radical-like chemistry. (That is to say, to the chemist they are perfectly free-radical-like, but they diverge sharply from the popular impression.) Nitric oxide (NO), for example, is a free radical, but its only likely toxicity arises from possible reactions with other free radicals;[1b] in my view it probably plays an insignificant role in free radical toxicity, whereas it certainly has many vital beneficial functions. This is the last you will hear of NO in this book.

There is a term commonly in use in the literature which may appear to remedy this terminological deficiency, but in fact it is very little better. The term reactive oxygen species, or ROS, is used to cover free radicals that behave as radicals are popularly imagined to, but not ones that are beneficial. But the utility of this term is completely spoilt by its being defined also to include certain non-radicals, particularly hydrogen peroxide, which are highly prone to react with radicals and reduced metal ions. This, together with the non-inclusion of atoms or molecules which behave similarly but are not oxygen-centred, destroys any chance that there might have been to use "ROS" to describe the relevant chemistry in an orderly manner that might be easy to master.

In view of this dismal state of affairs I have decided to coin a new term, defined as covering those molecules or atoms which behave, in biological systems, in a manner that accords with the popular impression of what a free radical does. Such atoms or molecules all possess a "lonely" electron—one which either (a) is prone to detach from its host atom or molecule and move to another (is reductively reactive), or (b) is prone to pull another electron (which may not itself have been lonely) away from some other atom or molecule (is oxidatively reactive). This includes many molecules that would be called free radicals by the strict definition, but not diradicals like molecular oxygen; it also includes transition metal ions that are in a reductively reactive state where one electron is prone to escape. So these atoms or molecules will hereafter be termed "lonely electron carriers" or LECs.

The explanatory value of this term will, I hope, become clear in the next three sections. Meanwhile, Table 3.1 summarises the terminological alternatives.

3.2. What Do LECs Do?

LECs react—with each other and also with non-LECs. You may have wondered what was so special about having a lonely electron that merited being given a name; this is the answer. It is vital for life that cells keep tight control over what reactions do and do not occur, because if unwanted reactions occur then the chemicals that we are made of—proteins, lipids, nucleic acids—are damaged or destroyed. LECs, if and when they arise in cells or in the extracellular medium, are particularly prone to initiate unwanted reactions, and cells employ fantastically sophisticated means to keep them under control. This control is not always negative—in certain environments, LECs are produced on purpose and are useful—but MiFRA concerns the more typical situations in which they are toxic to us.

3.3. Where Do LECs Come From In Vivo?

A class of reaction that LECs are very prone to undergo, and which will be discussed extensively in this book, starts with one LEC and one non-LEC and transfers one electron between them, so that the LEC becomes a non-LEC but the non-LEC becomes a LEC. (The electron may move from the LEC to the non-LEC, or from the non-LEC to the LEC: this is determined mainly by which LEC is involved, and to a lesser extent by which non-LEC.) This is therefore a chain reaction, "passing the parcel." But of course one can't get this chain reaction until one has a LEC in the first place.

Unfortunately there is a way that LECs can, and often do, arise de novo inside cells. In principle, one could create two LECs from none, by taking a molecule all of whose electrons

Table 3.1. "Free radical," "reactive oxygen species," and "lonely electron carrier"

Term	Basis of definition	Do all degradative reactions relevant to MiFRA involve at least one?	Does it provide a simple rule governing all reactions relevant to MiFRA?
free radical	molecular structure; not atoms	no, e.g. reaction 8 in Table 3.3	no
reactive oxygen species	reactivity; importance of oxygen	no, e.g. reaction 17 in Table 3.3	no
lonely electron carrier	single-electron reactivity	yes	LEC parity (see Section 3.5)

are happily paired and splitting it in two, partitioning the electrons so that there is a lonely one on each side. Equally, the starting point could be two or more atoms or molecules none of which had any lonely electrons. In general, processes of either of these types do not occur to a significant degree in the body, but there are exceptions.

By far the most prevalent exception is OXPHOS itself. The oxygen that is consumed by OXPHOS is turned into water; each oxygen molecule thus becomes two molecules of water by the addition of four protons and four electrons. As was explained in Sections 2.3.2 and 2.3.3, the protons and the electrons reach their target by completely different routes. The electrons, in particular, are painstakingly carried, one by one, along a chain of molecules, three of which remove a little of the electron's energy and use it to pump protons out of the mitochondrion (see Section 2.3.3). The fact that it is one by one, rather than two by two, is the problem: the atoms and molecules that do the carrying are turned into LECs for part of the time. If they can pass their lonely electron on to the next carrier, no harm is done; but this is somewhat error-prone (particularly, it seems, in the case of the only non-metal LEC in the chain, ubisemiquinone), and a few percent of the electrons are "fumbled" at some stage in the chain. These electrons are annexed by free non-LECs (that is, ones which are not in the chain) to make free, potentially toxic LECs.

3.4. The LECs and Related Molecules Relevant to MiFRA

It happens that the electrons fumbled by the respiratory chain are almost all annexed by just one non-LEC: molecular oxygen, O_2. The resulting molecule is called superoxide, and its chemical formula is $O_2^{\bullet -}$. The "$^{-}$" indicates that it has one more electron than proton, making it an anion; the "$^{\bullet}$" indicates that it is a free radical. Since I am going to talk about LECs in preference to free radicals, I will attach "$^{\bullet}$" to all LECs and nothing else; this is a little cheeky, but I think no worse—and better motivated—than the existing convention of not attaching "$^{\bullet}$" (or even "$^{\bullet\bullet}$") to O_2.

A type of LEC which will not be considered explicitly here is those formed by modification of proteins or nucleic acids. Such LECs are undoubtedly toxic, but MiFRA has nothing to say about them over and above what it says about another class, those formed by modification of lipids. By contrast modified lipids have a specific role in MiFRA which modified proteins or nucleic acids do not play.

The LECs that are discussed throughout the rest of this book, and especially in the rest of this chapter, are listed in Table 3.2. Also listed are the molecules which, though not LECs, are important in MiFRA because they react with LECs and/or are formed by reactions involving LECs.

3.5. LEC-Related Reactions Relevant to MiFRA

The reactions that will concern us in the rest of this book are described in Table 3.3; several of the most important destructive ones are also depicted in Figure 3.1. The reactions fall into the following categories:

a. donation of an electron by a LEC to a non-LEC — reduction
b. removal of an electron by a LEC from a non-LEC — oxidation
c. donation of an electron by a LEC to another LEC — usually disproportionation
d. fusion of a non-LEC with a LEC forming a LEC — condensation
e. fusion of a LEC with another LEC forming a non-LEC — termination
f. fission of a LEC forming a LEC and a non-LEC — dissociation
g. reactions not involving LECs but of relevance nevertheless

Because some of the reactions involve joining or splitting of lipids to form other lipids, the equations that involve more than one lipid use subscripts a, b etc. to distinguish them.

Tables 3.2 and 3.3 may seem rather intimidating, but there is no need to absorb them; they are supplied here mainly for ease of reference through the rest of the book. At this point, the most important thing to appreciate about these reactions is the reason why I defined "LEC" as I did: as noted in Table 3.1, reactions that happen in biological systems preserve "LEC parity." That is, if you start with an odd number of LECs you end with an odd number, and if you start with an even number of LECs you end with an even number. This is not so for free radicals, nor for reactive oxygen species.

It is also worth stressing at this point that not all LECs behave in the same way. For example, the reaction between two ascorbate radicals and that between two lipid radicals are completely different: the former involves the incorporation of two protons, whereas the latter uses no protons but instead makes a bond. This is a crucial difference, because the bond is permanent, whereas the oxidised ascorbate (dehydroascorbate) is still an independent molecule which can be restored to its reduced form. This property of ascorbate, together with its extreme readiness to undergo disproportionation, is the main reason why it is so valuable to us.

A third crucial feature which should be observed in this list is that some pairs of reactions cause the change of one participant to a different form and then back again. For example, superoxide turns ferric iron into ferrous, and hydrogen peroxide turns it back to ferric. Putting those two reactions in sequence:

$$O_2{\bullet}^- + Fe^{3+} \qquad O_2 + Fe^{2+}\bullet$$
$$H_2O_2 + Fe^{2+}\bullet + H^+ \qquad HO\bullet + H_2O + Fe^{3+}$$

$$O_2{\bullet}^- + H_2O_2 + H^+ \qquad HO\bullet + H_2O + O_2$$

gives us a chemical change which, if thought of as a single reaction, doesn't involve iron at all.

But that cumulative reaction does not appear in Table 3.3, and the reason it doesn't appear is that it doesn't happen. Iron (or copper, or some other ion that behaves the same way) needs to be present so that the intermediate state is available. Iron therefore acts as a catalyst, causing a normally impossible reaction to be achieved by a detour that needs less activation energy. This is the way that all catalysts work, and why a reaction needs so much

Table 3.2. Molecules of relevance to MiFRA

LECs that are free radicals	$O_2\bullet^-$	Superoxide
	HO•	Hydroxyl radical
	$HO_2\bullet$	Perhydroxyl radical
	L•	Lipid radical
	LOO•	Lipid peroxyl radical
	LO•	Lipid alkoxyl radical
	$Q\bullet^-$	Ubisemiquinone
	TocO•	Tocopheryl radical
	$Asc\bullet^-$	Ascorbate radical
	GS•	Glutathiyl radical
LECs that are not free radicals	$Fe^{2+}\bullet$	Ferrous iron ion
	$Cu^+\bullet$	Cuprous copper ion
	cyt-$c^{2+}\bullet$	Reduced (ferrous) cytochrome c
Non-LECs: simple hydrogen/oxygen derivatives	H^+	Proton, or hydrogen cation
	OH^-	Hydroxide anion
	O_2	Molecular oxygen
	H_2O	Water
	H_2O_2	Hydrogen peroxide
Non-LECs: respiratory chain components	Q	Ubiquinone
	QH_2	Ubiquinol
	cyt-c^{3+}	Oxidized (ferric) cytochrome c
Non-LECs: lipids and peroxidation derivatives	L, LH, LH_2	Lipid
	LOOH	Lipid hydroperoxide
	LO	Lipid aldehyde
Non-LECs: antioxidants (non-enzymatic) and derivatives	TocOH	Tocopherol (or Vitamin E)
	$AscH_2$	Ascorbate (or ascorbic acid or Vitamin C)
	Asc	Dehydroascorbate
	GSH	Glutathione (reduced)
	GSSG	Glutathione disulphide
Non-LECs: metal ions	Fe^{3+}	Ferric iron ion
	Cu^{2+}	Cupric iron ion
Non-LECs: reduction/oxidation reaction substrates	NADH	Nicotinamide adenine dinucleotide (reduced)
	NAD^+	Nicotinamide adenine dinucleotide (oxidized)
	$FADH_2$	Flavin adenine dinucleotide (reduced)
	FAD	Flavin adenine dinucleotide (oxidized)
Non-LECs: antioxidant enzymes	SOD	Superoxide dismutase
	CAT	Catalase
	GPx	Glutathione peroxidase
	GR	Glutathione reductase

Table 3.3. Reactions involving or protecting against LECs

no.	type	reagent(s)	product(s)	notes
1	a	$Q\bullet^- + O_2$	$Q + O_2\bullet^-$	
2	d	$O_2\bullet^- + H^+$	$HO_2\bullet$	mainly at pH < 4.7[2]
3	f	$HO_2\bullet$	$O_2\bullet^- + H^+$	mainly at pH < 4.7[2]
4	c	$O_2\bullet^- + O_2\bullet^- + 2H^+$	$O_2 + H_2O_2$	catalyzed by SOD
5	c	$HO_2\bullet + O_2\bullet^- + H^+$	$^1O_2 + H_2O_2$	not catalyzed
6	c	$HO_2\bullet + HO_2\bullet$	$^1O_2 + H_2O_2$	not catalyzed
7	a	$O_2\bullet^- + Fe^{3+}$	$O_2 + Fe^{2+}\bullet$	Cu^{2+} can replace Fe^{3+}
8	a	$H_2O_2 + Fe^{2+}\bullet + H^+$	$HO\bullet + H_2O + Fe^{3+}$	$Cu^+\bullet$,$Q\bullet^-$ can replace Fe^{2+}
9	g	$H_2O_2 + H_2O_2$	$2H_2O + O_2$	catalyzed by CAT
10	g	$H_2O_2 + 2GSH$	$2H_2O + GSSG$	catalyzed by GPx
11	g	$GSSG + NADPH + H^+$	$2GSH + NADP^+$	catalyzed by GR
12	b	$GSH + L\bullet$	$GS\bullet + LH$	
13	e	$GS\bullet + GS\bullet$	$GSSG$	
14	b	$HO\bullet + LH$	$H_2O + L\bullet$	
15	b	$HO_2\bullet + LH$	$H_2O_2 + L\bullet$	
16	b	$L_a\bullet + L_bH$	$L_aH + L_b\bullet$	
17	d	$L\bullet + O_2$	$LOO\bullet$	
18	b	$L_aOO\bullet + L_bH$	$L_aOOH + L_b\bullet$	
19	a	$LOOH + Fe^{2+}\bullet + H^+$	$LO\bullet + H_2O + Fe^{3+}$	$Cu^+\bullet$,$Q\bullet^-$ can replace Fe^{2+}
20	f	$L_aO\bullet$	$L_d\bullet + L_cO$	
21	e	$L_a\bullet + L_b\bullet$	L_c	cross-linking

Table 3.3. Reactions involving or protecting against LECs, cont.

22	b	TocOH + L•	TocO• + LH
23	b	TocOH + LOO•	TocO• + LOOH
24	b	$AscH_2$ + TocO•	$Asc^{\bullet -}$ + TocOH + H^+
25	c	$Asc^{\bullet -}$ + $Asc^{\bullet -}$ + $2H^+$	$AscH_2$ + Asc
26	g	Asc + NADH + H^+	$AscH_2$ + NAD^+

less of them than of the reagents that are used up. Most enzymes use metal atoms to achieve their catalytic function: superoxide dismutase, for example, contains atoms of either manganese, copper or zinc (different ones depending on the enzyme's location).

3.6. Fenton Chemistry

Reactions 8 and 19 in Table 3.3 are distinguished by having a name: Fenton reactions. They are particularly important for two reasons: one chemical, one conceptual. The chemical reason is that they are the reactions which generate two major players in MiFRA: the hydroxyl radical, HO•, which is far and away the most reactive substance that will concern us in this book, and the alkoxyl radical, which is also highly toxic due to its involvement in branching of chain reactions (see Section 3.7). The conceptual reason is that they are the main reactions that switch the reactivity of their LEC participants from reductive to oxidative: the reactive precursor, ferrous iron, is an eager electron donor, whereas the reactive product, hydroxyl or alkoxyl, is an eager electron acceptor. Fenton reactions have attracted more attention from gerontologists over the years than they may biologically merit: it is very unclear that the levels of hydrogen peroxide or lipid peroxides anywhere in the body are sufficient to make Fenton reactions a practical problem. If they did not need to alternate with other reactions (see Section 3.5), they would be much more problematic: the attention they have attracted in gerontology is possibly because some observers have overlooked this feature of the chemistry. The only other reaction in Table 3.3 which is a switch from reductive to oxidative reactivity is reaction 2, the **protonation** (condensation with a proton) of superoxide to form perhydroxyl radical; this reaction, by contrast, may matter in vivo far **more** than has traditionally been supposed. We will return to the role of perhydroxyl radical in detail in Section 11.2.

3.7. Chains and Branches

Several of the reactions listed in the previous section fit together in patterns that are much more elaborate than pairs, and it is their role in these patterns which underlies their relevance to MiFRA.

A cumulative reaction that is capable of causing very rapid damage to membranes is the lipid peroxidation chain reaction. It is composed of two of the reactions listed in Table 3.3, which can alternate indefinitely in the presence of a supply of oxygen:

$L_a\bullet + O_2$ $\quad\quad$ $L_aOO\bullet$
$L_aOO\bullet + L_bH$ $\quad\quad$ $L_aOOH + L_b\bullet$

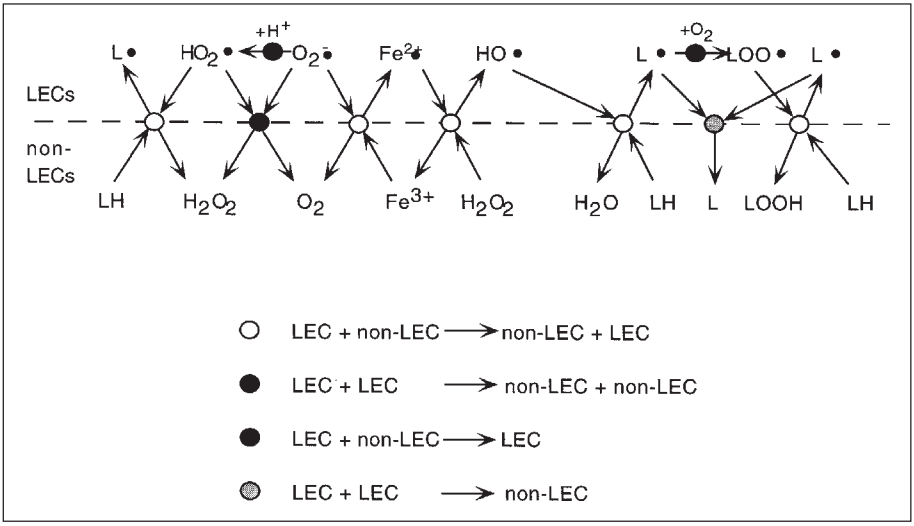

Fig. 3.1. The major destructive reactions relevant to MiFRA, showing their LEC parity.

By this means, arbitrary amounts of oxygen can become incorporated into a material composed of lipids—such as a membrane—starting from just one lipid radical. This is deleterious for two reasons. Firstly, the resulting lipid hydroperoxides (LOOH) are less hydrophobic than the original lipids, so that the membrane will be more permeable to protons and other ions and thus less able to sustain OXPHOS.[3,4] Secondly, lipid hydroperoxides are somewhat unstable, and are prone to undergo many kinds of molecular rearrangement, forming cyclic endoperoxides and other baroque species[5] (see Section 3.8).

But a much worse situation comes about when the medium contains a source of superoxide and also a trace of a transition metal such as iron. The lipid hydroperoxides formed above have ceased to be LECs: they are unwelcome in the membrane, but only mildly so as compared to LECs themselves. But the superoxide is a LEC, and with the help of the metal atom it can pass its toxic parcel to form a brand new lipid radical, not to mention an aldehyde which (like a hydroperoxide) is prone to subsequent rearrangements.

$O_2{\bullet}^- + Fe^{3+}$	$O_2 + Fe^{2+}{\bullet}$	
$L_aOOH + Fe^{2+}{\bullet} + H^+$	$L_aO{\bullet} + H_2O + Fe^{3+}$	a Fenton reaction
$L_aO{\bullet}$	$L_b{\bullet} + L_cO$	

This is known as "branching" of lipid peroxidation chain reactions, because if we put these three reactions in sequence after the two above we start with one lipid radical and end with two:

$L_a{\bullet} + O_2$	$L_aOO{\bullet}$	
$L_aOO{\bullet} + L_bH$	$L_aOOH + L_b{\bullet}$	
$O_2{\bullet}^- + Fe^{3+}$	$O_2 + Fe^{2+}{\bullet}$	
$L_aOOH + Fe^{2+}{\bullet} + H^+$	$L_aO{\bullet} + H_2O + Fe^{3+}$	a Fenton reaction
$L_aO{\bullet}$	$L_d{\bullet} + L_cO$	
$L_a{\bullet} + O_2{\bullet}^- + L_bH + H^+$	$L_d{\bullet} + L_a{\bullet} + L_cO + H_2O$	

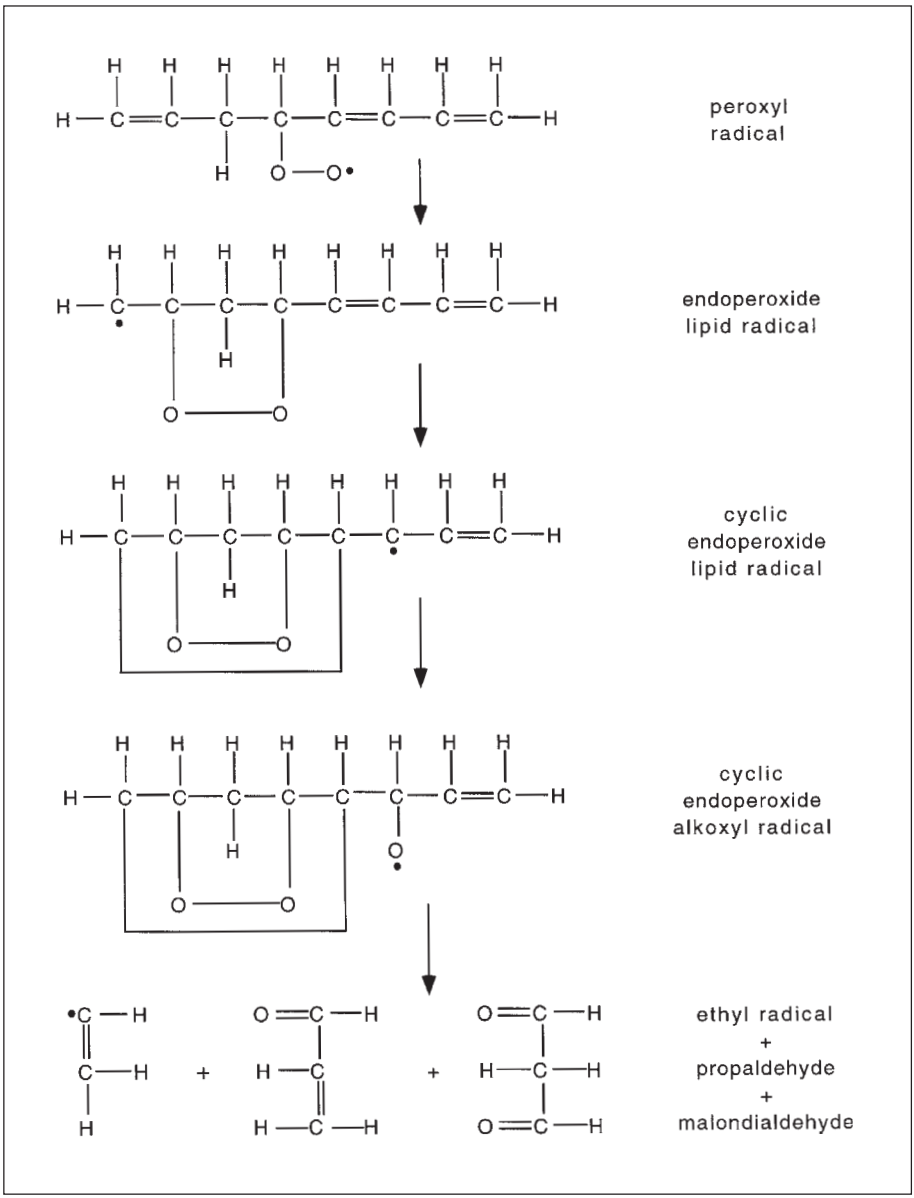

Fig. 3.2. Formation and rearrangement of endoperoxides and cyclic radicals.

Obviously the more branching there is, the more peroxidation there can be in total (given a supply of superoxide), so this is a particularly dangerous sequence. The main physiological role of Vitamin E (tocopherol) is thought to be its ability to terminate a chain reaction by reaction 22 in Table 3.3, following which it is restored by vitamin C (ascorbate) to a state where it can do it again (reaction 24).

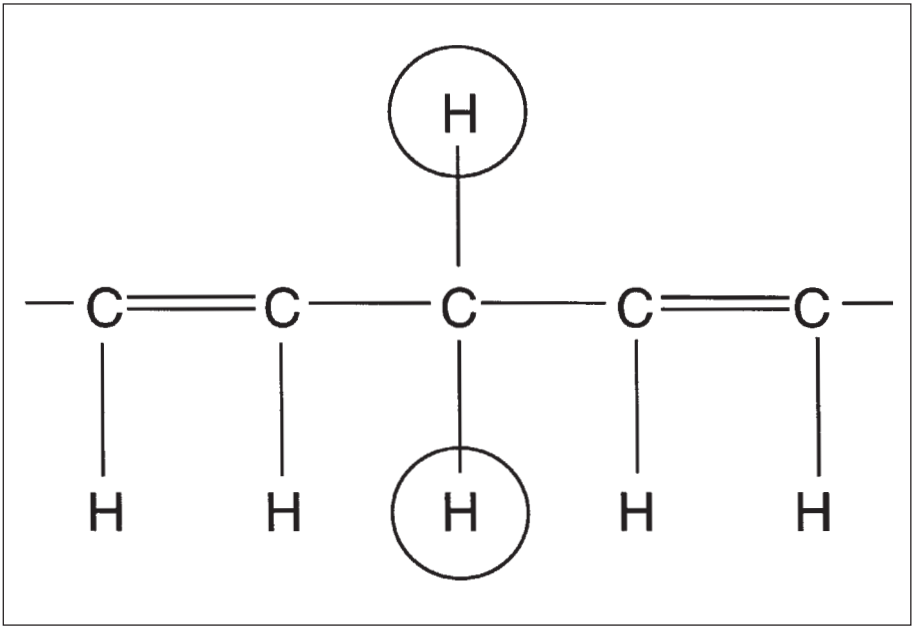

Fig. 3.3. A highly oxidizable, but necessary, juxtaposition of C=C double bonds.

3.8. Downstream Products—A Virtually Infinite Variety

One class of reaction involving LECs was not included in Table 3.3, because in order to describe it one must describe the structure of the participating lipids in detail. Reaction 18, whereby a lipid peroxyl radical becomes a hydroperoxide, can sometimes occur intramolecularly: the unpaired electron forms a bond with a carbon atom of the same carbon chain, making a ring. The molecule is still a radical: a lipid radical that is also an endoperoxide (Fig. 3.2). Molecules with an endoperoxide ring are particularly unstable with regard to their molecular structure: the oxygens can move around the molecule, creating even more exotic multi-ringed molecules (Fig. 3.2).[6] Eventually these complex molecules are prone to break apart, and one of the major products (both in vitro and in vivo) is malondialdehyde (Fig. 3.2), which can be used in biological systems as a measure of the rate of lipid peroxidation. The other molecules most often used as measures of lipid peroxidation are ethane and pentane, which are two of many unreactive molecules formed by reactions 20 and 16 but are the easiest to assay because they are gaseous.[7]

3.9. Fatty Acid Double Bonds: Fluidity but Oxidizability

In the above description of lipid peroxidation I have treated all lipids as equal, but they are very far from that. Lipids are a large family of compounds, and some are much more oxidizable than others. Unfortunately for us, the characteristic that makes lipids particularly oxidizable is one that we cannot do without, because membranes need lipids of that type in order to maintain physical integrity in vivo. This characteristic is the possession of carbon chains with C=C double bonds spaced at a particular distance, as shown in Figure 3.3. The circled hydrogen atoms, attached to the carbon in the middle, are called doubly allylic, or bisallylic: they are particularly susceptible to removal by oxidizing LECs.[8]

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Chapter 4

An Introduction to Lipid Metabolism

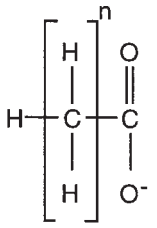
When the mitochondrial free radical theory was first conceived, researchers presumed that the process of steady mitochondrial decline was happening independently, and roughly equally, in all cells of a given type. It is now quite certain, however, that the process is very uneven indeed: some cells undergo complete OXPHOS collapse, whereas others apparently undergo none. Moreover, the cells that undergo OXPHOS collapse are extremely few in number. The evidence for the above will be discussed in later chapters. But it has a crucial corollary: if MiFRA is "correct"—that is, if the mechanism that MiFRA proposes is really the main driving force in aging—then these few anaerobic cells must somehow be doing harm to their mitochondrially healthy colleagues, or else there would be no macroscopic (that is, tissue-wide) consequences of it. The mechanism for this that I believe to be the most plausible, and which will be presented in Chapter 9, is a side-effect of the body's systems for moving lipids—fats and related molecules—between tissues. An introduction to these systems is therefore necessary.

4.1. The Major Fat-Related Molecules in Cells: Structural Roles

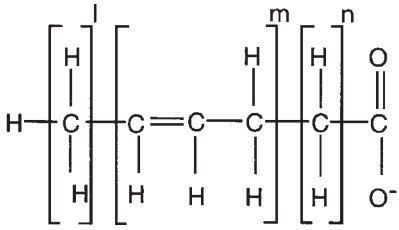
We saw in Section 2.3.2.3 that fats in the diet can be broken down and fed, two carbon atoms at a time, into the same metabolic pathway that derives energy from breaking down sugars. But, unlike sugars, fats and related molecules also have a fundamental structural role in cells. They are the building blocks of membranes—cell membranes, mitochondrial membranes, and many others. All these molecules—the fats and the related molecules—are collectively termed lipids.

Most of the structural molecules in membranes are in fact not exactly fat molecules: they are modified in a generic way. A fat molecule is a triglyceride*:* a molecule of glycerol with a many-carbon organic acid attached to each of its three carbons. When a fat molecule is broken down, the acids are stripped off and fed into the tricarboxylic acid cycle as described in Section 2.3.2.3. When a fat molecule is to be used in a membrane, however, only one of the three chains is removed; also, rather than being replaced by a hydrogen atom, it is replaced by a group centred around a phosphorus atom. The resulting molecule is called a phospholipid. Two phospholipids can thus differ in the nature of the phosphorus-centered group (the head group) and/or in the two fatty-acid side chains that are retained (see Fig. 4.1). Neither makes an enormous difference to the chemistry of the molecule, but differences in the head groups have more effect on that than differences in the side chains, so the nomenclature of phospholipids is based on the head groups. (The head groups of membrane phospholipids also have different effects on the surrounding water: we will return to this in Sections 11.2 and 11.3.) What the side chains do affect is the **physical** properties of a membrane made of phospholipids: if one of the chains is polyunsaturated (contains two or more C=C double bonds) then the molecule takes up more room, so a membrane with large amounts of that type of phospholipid is more fluid than otherwise, which is a property

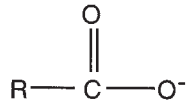
The Mitochondrial Free Radical Theory of Aging, by Aubrey D.N.J. de Grey.



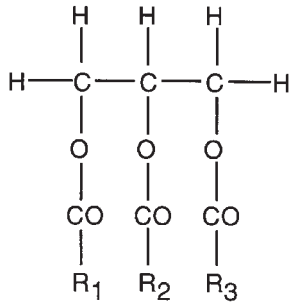
saturated fatty acid
n=15: palmitate
n=17: stearate



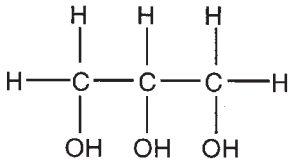
unsaturated fatty acid
l=7, m=1, n=6: oleate
l=4, m=2, n=6: linoleate
l=1, m=3, n=6: linolenate
l=4, m=4, n=2: arachidonate



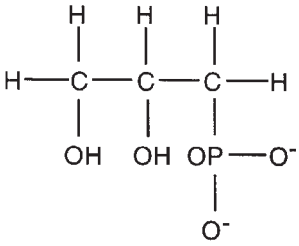
generalized fatty acid
(R = any carbon chain)



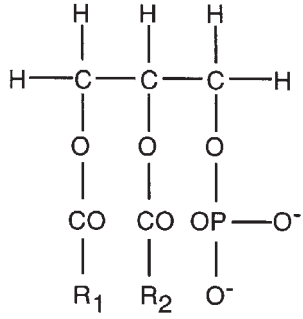
triglyceride
(neutral)



glycerol

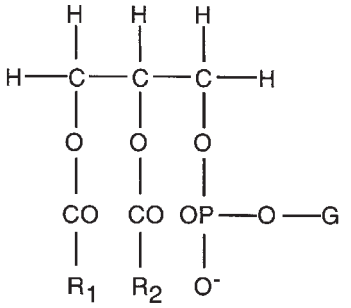


glycerol 3-phosphate



phosphatidate
(divalent anion)

Fig. 4.1. Some types of lipid (and their components) prevalent in biological systems.



generalized phospholipid, abbreviated PL^--G
R1, R2 = fatty acid side chains
G = head group (esterifies phosphatidate)
overall charge = (charge on G) - 1

$HO\text{-}CH_2\text{-}CHNH_3^+\text{-}COO^-$

serine (no net charge)

$PL^-\text{-}CH_2\text{-}CHNH_3^+\text{-}COO^-$

phosphatidylserine$^-$

$HO\text{-}CH_2\text{-}CH_2\text{-}NH_3^+$

ethanolamine$^+$

$PL^-\text{-}CH_2\text{-}CH_2\text{-}NH_3^+$

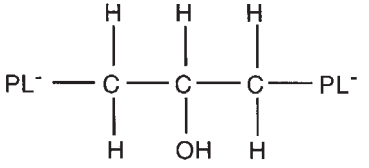
phosphatidylethanolamine
(no net charge)

$HO\text{-}CH_2\text{-}CH_2\text{-}N^+(CH_3)_3$

choline$^+$

$PL^-\text{-}CH_2\text{-}CH_2\text{-}N^+(CH_3)_3$

phosphatidylcholine
(no net charge)



cardiolipin^{2-}
(diphosphatidylglycerol)

Fig.4.1., cont. See opposite page.

that membranes need in order not to rupture. There is a trade-off here with regard to oxidisability, as was explained in Section 3.9.

Mitochondria also have a lot of a diphospholipid called cardiolipin (so named because it was first discovered in the heart). Cardiolipin is formed by joining two copies of the simplest phospholipid, phosphatidate, at their phosphates; its molecular structure is shown in Figure 4.1. Cardiolipin is the only anionic lipid present in significant amounts in the inner mitochondrial membrane, and its concentration decreases with age;[1] this change has been shown to cause reduced performance of certain membrane proteins.[1,2] A possible mechanism for this effect is that loss of cardiolipin causes an increase in the pH of the water right next to the membrane (see Section 11.2.2), which may markedly affect mitochondrial function (see Section 11.3.7).

All membranes also have a component which is rather more distantly related to fats: it is classed as a lipid, but unlike phospholipids it is not derived from fats but instead is built from scratch in the liver, as well as being extracted from food by epithelial cells in the gut. It is cholesterol. Cholesterol (see Fig. 4.2 for structure) is a steroid, a molecule only about half the length of the average phospholipid, and its presence in membranes increases their fluidity, which is necessary to keep them intact. Cells in culture that have been genetically modified to lack cholesterol are very prone to suffer membrane rupture,[3] which is of course instantly fatal to the cell; the same effect has been shown in red blood cells.[4]

4.2. Synthesis and Transport of Fatty Acids and Cholesterol

Most of our cells, despite needing cholesterol so vitally, do not make it themselves—at least, not in the quantity they need.[5] They are also rather ineffective at destroying it (or packaging it away) when they transiently need less of it, though they do package it to some extent. They can afford this because a few types of cell, particularly ones in the liver and in the gut, have a very high capacity for cholesterol synthesis and degradation (for liver) or absorption and release (for gut), and can therefore buffer the less capable cells elsewhere. But in order to achieve this buffering, the liver and gut cells must somehow exchange cholesterol with all other cells. They do this via the blood stream. Cholesterol is secreted by the liver and gut and imported by other cells in a particle called a low-density lipoprotein, or LDL;* and it is transported the other way in a similar (but easily distinguishable biochemically) particle called a high-density lipoprotein (HDL).[6] Both of these transport processes are highly regulated by the cells that are doing the import and export. The particles themselves are also highly structured, being organised around a protein scaffolding (just one big polypeptide in the case of LDL; many more for HDL) and being wrapped in a coat of phospholipid, which is necessary in order to allow the particle to move freely in the blood, since it makes the particle more water-soluble.[7]

A similar situation exists for fatty acids. Most cells are capable of building the phospholipids (and related molecules) that they need, but not from absolute scratch: they need the component fatty acids to be supplied in the blood. Fatty acids are present in LDL, as explained above, but the amount of LDL imported (or HDL exported) is fixed by the cell's cholesterol requirements and is thus not (necessarily) adequate for the cell's fatty acid requirements. Most fatty acid is acquired by a different mechanism: it circulates in the blood bound to albumin, which mediates its uptake by cells.[8] In contrast to cholesterol, however, there is no "reverse transport" system to rid the cell of excess fatty acid: this is not necessary,

* *Strictly, what the liver and gut secrete into the blood is not LDL but precursors of it, called chylomicrons (from the gut) and VLDL, very low density lipoprotein (from the liver). These are converted to LDL by an enzyme called lipoprotein lipase.*[7]

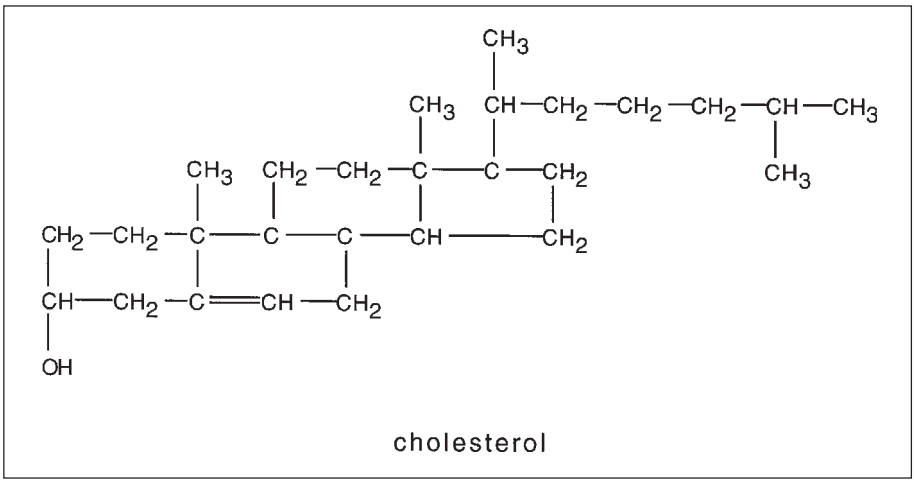


Fig. 4.2. Cholesterol.

because it can be used as fuel for the TCA cycle (which is always needed), so homeostasis is achieved by regulating the uptake and storage of the TCA cycle's other main fuel, glucose.

4.3. Good and Bad LDL

The blood stream is used as a transporter not only of cholesterol but of all nutrients, so it is not an especially predictable environment. LDL and HDL particles can, in particular, suffer chemical changes during their journey which can, potentially, render them toxic to the cells that are their intended destination. The main such modification is oxidation. In the case of HDL, this is not much of a problem, because the destination cells (in the liver) are purpose-built to deal with all manner of toxins and are not likely to suffer unduly. The situation with LDL is much more serious. We know that LDL particles can undergo modifications that make them toxic to most cells, not only because of in vitro experiments[9] but also because of the extreme care that the body takes to avoid this damage. Firstly, the intracellular machinery that imports LDL specifically rejects (fails to bind) LDL that has been significantly oxidized.[10] Secondly, a certain type of white blood cell called a monocyte has the potential to turn into a vacuum cleaner for oxidized LDL, by attaching itself to the artery wall and expressing a different type of surface protein that imports oxidized LDL even more assiduously than unoxidized.[11] (We will consider a highly deleterious side-effect of this process—atherosclerosis—in Section 5.1.) Thirdly, the blood contains high levels of various chemicals that limit the rate at which LDL oxidation occurs in the first place; these will also be discussed in detail later.

There is, however, a major paradox concerning the presence of oxidized LDL in the blood stream. It was shown in 1984[12] that the oxidation of LDL by cultured cells is totally abolished by the addition to the culture medium of antioxidants such as vitamin E, and moreover that the concentration one needed to add was a good deal less than is present in the blood stream. The same is true of the arterial intima.[13] Thus, ostensibly, LDL oxidation should not be happening in the body at all! The only way out of this is to presume the existence of oxidation "hot spots" in the extracellular medium, in which the concentration of toxins (such as LECs) is much higher than average and/or that of antioxidants is much lower. No such non-uniformity has been identified experimentally, but a possible one will be described in Chapter 9; see also Section 10.13.

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Chapter 5

A Descriptive Introduction to Human Aging

It would be rather strange to present a theory of aging without saying (in more than just that one word) what the theory sets out to explain. Furthermore, a descriptive analysis of the deleterious changes that occur in our bodies late in life offers some very instructive insights into what features are likely to be present in any plausible biochemical theory of how they occur. (The eminent evolutionary gerontologist Michael Rose recently expressed this well:[1] "Discussing aging as a purely biochemical phenomenon would be like discussing *War and Peace* purely as a geography lesson.") In the next few sections I shall not, in fact, keep strictly to description: I shall also include a summary of the proximate, local causes of each symptom. This allows me, in the closing section of this chapter, to draw together the major features of these early processes and explore their commonalities, which give hints to the even earlier processes that are the main topic of this book. However, this is only one short chapter and is correspondingly superficial; the reader who wishes a more solid grounding in the physiology of aging is urged to consult some of the excellent texts (in terms of both content and readability) that have appeared in the past decade.[2-5]

5.1. Atherosclerosis

The accumulation of fatty deposits in the cardiovascular system is the symptom of aging most likely to cause death or serious disability. It is the main cause of heart attacks and strokes. The detailed mechanism of how atherosclerosis comes about—atherogenesis—has taken a long time to become clear, but the following chain of events is now widely agreed.

The inside of our entire cardiovascular system is coated with a single-cell layer of specialised cells called endothelial cells. These cells have a protective function. One aspect of that function is the removal from the blood of oxidized fats. They do not do this directly, but rather by changing their surface in such a way that another type of cell, which is normally free in the blood, attaches to them. This is a type of white blood cell called a monocyte, and once fixed to the artery wall it changes its character sufficiently that it is given a different name. It is called a macrophage. Macrophages have a multi-faceted defensive role, but the one that concerns us here is that they absorb LDL particles which have undergone peroxidation.* This is in contrast to most cells, which import LDL particles (mainly for

** Macrophages in fact have a higher affinity for oxidized LDL than unoxidized, but not all that much higher.[6] It is interesting to speculate why macrophages do not achieve much greater resilience to high plasma LDL, by the simple expedient of importing only oxidized LDL, rather than taking everything. The simplest hypothesis is that the problem of atherosclerosis is new, resulting only from modern man's tendency towards a diet much higher in fat than has been typical through our evolutionary history. There is only slight evolutionary pressure for macrophage specificity for oxidized LDL even now, since atherosclerosis only strikes in middle to old age, after the main reproductive period; hitherto there was no such pressure whatever.[7]*

The Mitochondrial Free Radical Theory of Aging, by Aubrey D.N.J. de Grey.

cholesterol supply) that are unoxidized, but which are not receptive to them once they have become significantly oxidized.[8] Thus this is a defence mechanism that prevents the build-up of oxidized LDL in the blood, as noted in Section 4.3. Initially, and ideally, macrophages ingest oxidized LDL and rapidly break it down.

However, especially in people with a high-fat diet, this mechanism is saturable. Macrophages eventually become unable to cope with the rate of incoming LDL and cease to function properly. Macrophages that have reached this point are called foam cells. Once foam cells begin to accumulate in a region of arterial wall, things go from bad to worse: a fibrous cap forms over the foam cells, probably in an attempt to contain it and stop it from injuring the endothelial cells nearby.[9] A feature of this stage that is often overlooked, though it has been repeatedly confirmed,[10,11] is that the growth of this lesion is then further accelerated by hyperplasia: advanced lesions are found to be clonal, indicating that mutations in cell-cycle control genes (probably caused, in large part, by the high levels of oxidized, reactive lipids within the lesion) have caused it to become precancerous. Eventually the lesion becomes so big that it impedes blood flow, and in the end it begins to fragment, releasing material into the blood stream. They may become stuck elsewhere, actually arresting the flow of blood to, for example, a region of the heart or brain.

Thus, the central cause of heart attacks and strokes is oxidation of LDL in the blood. Moreover, a low-cholesterol diet does not have a greatly inhibitory effect on the rate of progress of atherosclerosis, because plasma LDL levels are not much altered;[12a] presumably the liver makes up the deficit from the gut. This is no surprise, because ultimately we need to supply our cells with cholesterol, a vital component of membranes which most cells do not make in sufficient quantity for their needs (see Section 4.2). Only the liver and gut can supply it fast enough; it is thus necessary to have it (as LDL) in the blood in order to make it available elsewhere. In line with this, the genetic and environmental risk factors which cause a wide variation in different people's susceptibility to atherosclerosis are largely related to the later, hyperplastic stages of lesion formation:[12b] foam cells are apparent at about the same (early) age in all individuals.[12c]

If we try to move one more step back in the chain of events, however, we hit a problem. Only if and when some plasma LDL becomes oxidized are monocytes induced to attach to the artery wall. The source of this oxidation, and in particular the reason why it becomes more severe with age, is unknown;[7] indeed, the fact that it happens at all is highly paradoxical (see Section 4.3). Somehow or other, though, it must be mediated by the preponderance of LECs, since LECs are the chemicals which perform oxidation in vivo.

5.2. Cancer

Cancers—malignant tumors—form from the unregulated division of cells that are only supposed to divide rarely. They do the most harm when they start to fragment—metastasize—and malignant cells are carried away in the blood, eventually attaching and forming new tumors all around the body. (The ability to metastasise is what distinguishes a cancer from a benign tumor.) They occur as a result of spontaneous mutations in multiple genes, particularly cell-cycle regulatory genes, of a single cell. Mutations are happening all the time in all our cells, and if both copies of a gene in a given cell are mutated the result can be the failure of specific functions in that cell; but such "double hits" are extremely rare, so almost all cells remain capable of all their vital functions throughout life. Furthermore, the few cells that do lose their function by this means are not a serious problem, because other cells can stand in. With cancer, however, the body does not have the latter safeguard: just a single cell going cancerous is enough to give rise to a potentially fatal tumor. Therefore, cancer is probably by far the most serious pathological consequence of spontaneous nuclear mutations.

Our DNA is actually suffering spontaneous damage at a much greater rate than is indicated by mutations, but the vast majority of this damage is repaired quickly and correctly. Damage of this sort might fairly be called "proto-mutations." The class of DNA damage which happens more often than any other is the simple falling-off of a base: purines (adenine and guanine: see Fig. 2.10) fall off much more often than pyrimidines, and their loss is called depurination. This is easy to correct accurately, because the other strand of the DNA has the complementary base. Another is the loss of an NH_2 (amine) group in favour of a hydroxyl group (OH): this is called deamination.* Again, the deamination of any nucleotide of DNA always produces a molecule that is not a normal constituent of DNA, so it is detectable and reversible.

There are two crucial determinants, therefore, of the rate at which mature, irreparable mutations occur. They are the rate at which proto-mutations occur and the quality of those proto-mutations' repair—that is, the proportion of proto-mutations that are repaired correctly. A major determinant of the quality of repair is the speed of repair. If a mutation happens and nothing is done about it for a while, another one may happen close by. The double mutation may be harder to repair correctly than two single ones each repaired at once. A more frequent situation is when there is only one proto-mutation but it is replicated before being repaired. For example: if a cytosine is deaminated, it becomes uracil, which is not normally part of DNA (only RNA), but some cytosines in DNA are deliberately methylated (a hydrogen atom is replaced by a CH_3 group) as a regulatory mechanism, and deamination of a methylated cytosine (methylcytosine) makes thymine. This therefore makes a mispair—a thymine paired to a guanine.[13] There is a specific enzyme that detects thymines paired to guanines and turns them back to cytosine, so in general there is no problem. But if a cell replicates its DNA while there are deaminated methylcytosines in it, this mispairing will be lost: the thymine will be seen as legitimate by the DNA polymerase and the new strand will be constructed with an adenine. The mutation thus becomes permanent.

It is interesting to note that the rates of these two processes (damage and repair) are likely to be sensitive to a factor that also regulates the rate of atherogenesis: namely, the preponderance of LECs. In this case, the place where the LECs matter is in the nucleus. LECs are mutagenic, since they react with nucleic acids just as readily as with lipids (see Section 3.4), so their levels will influence the rate of occurrence of proto-mutations. The rate of repair is also likely to suffer from increasing LEC levels, since the proteins which perform that repair are themselves vulnerable to damage—and hence inactivation, or (worse) erroneous function—by LECs.

We also appear to possess an anti-cancer mechanism which works in a different way, and which may also be impaired by LECs. Cells that have suffered some, but not all, of the mutations necessary to cause a tumor seem to be identified and induced to die. This process is up-regulated in rodents undergoing caloric restriction, a regime which substantially increases their lifespan (see Section 6.5.4), and has been proposed[14] to explain why caloric restriction reduces the incidence of cancer.

5.3. Disorganization of Connective Tissue: Skin, Artery, Eye, Cartilage

Another major feature of aging with which we are all familiar is the loss of elasticity in the skin. This is much more pronounced in sun-exposed skin than elsewhere, indicating that damage is probably caused mainly by ultraviolet light; but that does not explain why it accumulates. The skin's elasticity derives from the fibres of connective protein in the lower

* *The replacement is in fact followed by a rearrangement that moves the hydrogen of the hydroxyl group onto a nearby nitrogen.*

layers of the skin, called the dermis. These fibres are made mainly from two proteins, collagen and elastin, which are secreted by specialised cells (fibroblasts) and linked into a network outside the cells. This network is not subject to the rapid turnover that intracellular proteins undergo. But, contrary to what is commonly stated, it is (in most tissues, anyway) amenable to **some** turnover—its half-life in rats is a few months.[15] Fibroblasts are able to secrete an enzyme called collagenase, which breaks collagen down and allows fibres to be replaced. This happens in response to ultraviolet damage, in fact.[16] Unfortunately the repair process is not very precise, so that the network of fibres becomes progressively less organised. This matters, because the regularity of the fibrous network is essential for its elasticity.

Again, the point to focus on is not that this damage occurs but that it worsens as we get older. In this case, the worsening is because the ability of fibroblasts to perform the turnover process diminishes with age. This diminution seems to be due to three cellular changes. The first is that, as with DNA repair, the ability of all cells (fibroblasts included) to synthesise protein gradually declines with age. The second is that fibroblasts (along with other skin cell types) diminish in number throughout life.[17] The third, rather more curious, reason is that older tissues destroy a large proportion of newly-created, so undamaged, collagen:[18] this may be due to excessive collagenase production by a few cells that are approaching replicative senescence.[19a] Elastin (sometimes—see below) and the other two classes of extracellular matrix protein, proteoglycans and structural glycoproteins, are also slowly recycled.[19b]

Osteoarthritis is another disease of this type. There, the material whose turnover is inadequate is again collagen, but this time it is the collagen in the cartilage of our joints. As in the skin, it seems that the main reason why collagen turnover diminishes with age is that the fibroblasts which produce and recycle it diminish in number and also in efficacy.[20]

A very similar process happens in the lens of the eye, and also in the walls of our major arteries. Both of these are elastic, and need to remain so in order to do their job. They undergo the same sort of damage as UV causes in the skin—random, extra cross-linking of the elastic protein (elastin in the arteries, crystallin in the lens). In this case, though, the reason why the process accelerates with age is one step more indirect. These two materials are not just recycled very slowly, they aren't recycled at all; thus there are no recycling-responsible cells to blame for the decline. Rather, the cross-linking accelerates because it is caused not by ultraviolet light but by a chemical, and that chemical is increasingly inefficiently removed from the blood. The chemical is simply glucose, and this damage done by too much circulating glucose is called glycation.* Levels of glucose in the blood are controlled by the extremely rapid release of insulin in response to glucose arriving in the blood from the gut. That response diminishes with age, because the uptake of glucose in response to elevated blood insulin becomes increasingly sluggish (especially in inactive or obese individuals);[21] this is probably, once again, a change brought about by (a) reduced function of the glucose uptake process and (b) a reduction in the number of relevant cells. The pathological manifestation of this effect is late-onset (Type II) diabetes.

This group of symptoms of aging, therefore, seems to have two underlying driving causes: functional decline of cells, and decrease of cell number due to non-replacement of cells that die. The non-replacement is superficially somewhat curious, since fibroblasts are perfectly capable of cell division—and indeed perform it very rapidly in response to wounding. One very plausible theory is that the avoidance of division in response to the very slow loss of cells that occurs with aging is a defence against cancer: non-division is tolerable (albeit only for a lifetime), whereas each and every cell division carries a risk of DNA replication error that could, if it happened in the wrong gene, initiate a cancer that would kill us sooner. This is not a complete explanation of the phenomenon, however: we

** Note that glycation is more wide-ranging than this; we will return to it in Sections 5.6.3 and 6.5.6.*

must understand not only why cells that die are not replaced, but also why they die in the first place. But there is no reason to seek a single reason for this low-rate, steady cells death—the most obvious theory, that they die of a wide variety of causes due to simply living for a long time in a relatively unstable environment, is enough. Also, cells become more sensitive to stress as we age:[22,23] liver cells isolated from elderly individuals are more prone to die from a given degree of stress than cells of the same type isolated from younger people.*

Does this group of symptoms of aging have anything in common with atherosclerosis and cancer? Indeed it does. The efficacy of the various cellular functions touched on above will be diminished if the proteins that perform them are damaged, and LECs can damage all these proteins, just as they damage those that perform DNA maintenance. The ability of a cell to survive stress of various kinds is, likewise, dependent on its ability rapidly to synthesise the proteins that protect it; if many of these proteins (or those involved in their construction) are damaged, that ability is correspondingly reduced.

5.4. Blindness

We saw in the previous section that cells recycle their internal protein and lipid quite rapidly, in contrast to the rather slow and imperfect recycling of extracellular proteins. Much of the intracellular recycling is done in lysosomes, which are the generic garbage collector of the cell. One of the first age-related changes to be discovered inside cells was that lysosomes gradually accumulate a fluorescent pigment, which is called lipofuscin.[26,27] Lipofuscin proved rather hard to characterise chemically, but there is now a consensus that it comprises highly heterogeneous polymers of protein and lipid, together with a substantial concentration of metal ions which were presumably once the cofactors of some of the proteins. It is believed to result from a feature of LEC-mediated oxidation reactions: namely, that the products of oxidation are enormously varied (see Section 3.8). Lysosomes contain a huge variety of destructive enzymes, capable of breaking down all manner of protein and lipid molecules (and their modified forms resulting from peroxidation) into their small-molecule constituents, but it is simply not possible to have an enzyme for everything. Some of the molecular knots tied by peroxidation will thus be too Gordian for lysosomes to unravel. Lipofuscin has just the characteristics that would be expected of this indigestible remnant: a granule in which such molecules are packaged up out of harm's way, and never need to be properly disposed of. Since the indigestible products are very rare, resulting only from particularly complex sequences of reactions, they never reach levels that cause the cell any inconvenience.**

Except, that is, in the retina. There, protein turnover is probably just as efficient as anywhere else—that is, the proportion of protein and lipid that lysosomes are unable to digest is just as small—but what is different is the amount of protein and lipid that must be recycled. The absorption of light by rhodopsin is an extremely protein-damaging process,

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** It should be noted that there are also reliable reports of the opposite effect: of cells becoming progressively* ***less*** *prone to die when stressed.[24,25] But there is in fact no conflict of data, because this resistance to death is a feature of cells in culture that have replicated many times more than they are ever required to in the body and have become unable to replicate any more—have reached replicative senescence. This happens because the type of cell death that these stressed cells undergo—apoptosis—is not simply atrophy: it is a program, very rigorously controlled by a complex series of genetic interactions, and replicative senescent cells have lost not only the ability to divide but also the ability to perform some of these interactions. Put simply, they want to die but they can't. We will come back to replicative senescence in Section 7.3 and to apoptosis in Chapter 14.*

*** It is not in fact certain that lipofuscin is as harmless as this. One possibility[28,29] is that it harms us passively, by "distracting" lytic enzymes in lysosomes. In vivo evidence for this is still lacking, however.*

such that the discs of rhodopsin which form the light-sensitive part of rod and cone cells are recycled more than once a week.[30] The lysosomes in the pigmented epithelium, the cell layer behind the rods which does the recycling, eventually become so full of lipofuscin that they cease to function and the cells begin to lose integrity, leading to gradual loss of vision, technically termed macular degeneration. This process is exacerbated by light-induced rupture of these lysosomes.[31]

But what determines the rate of accumulation of lipofuscin in other cell types? An answer is suggested by the description of its composition given above. Oxidative damage increases the incidence of damaged protein and lipid in cells, and that translates directly into more lipofuscin. And indeed, the cells elsewhere than the eye which accumulate the most lipofuscin are those which are non-dividing, so cannot dilute it away, and among non-dividing cells the worst affected are those (such as cardiomyocytes) which use the most energy, so create the most LECs and suffer the most oxidative damage.

5.5. Other Macroscopic Changes

The preceding sections have summarised only a selection of the major symptoms of aging, though they have covered most classes. Many other cell types diminish in number with aging; these include ones that are incapable of regeneration by division, such as neurons, glomeruli, and the sensory hair cells of the inner ear, as well as ones which can divide but generally do not. Various hormones diminish in concentration with age, for this reason and others. A similar process happens to the immune system: this becomes progressively less robust with age due to loss of cells in the thymus.[32] Another major change that more indirectly involves loss of a specific cellular function is bone loss, leading to osteoporosis; this is thought to be a response to a general failure of calcium regulation in many cell types, something which can be detected histochemically as excessive calcium uptake.

5.6. Feedback, Turnover and Oxidative Stress

5.6.1. Negative Feedback

We would certainly not live as long as we do if not for our ability to react to, and recover from, adverse physiological conditions. At the subcellular level, this response comprises rapid regulation of all our systems for biological homeostasis, including our antioxidant systems.

This may seem vacuous, but in fact a remarkable deduction can be made from it. It tells us that, despite (as discussed in the preceding sections) being pro-oxidant in nature, the processes which drive aging are simply not challenged by our antioxidant defences—otherwise, aging would not progress inexorably as it does. They proceed, slowly but surely, impervious to antioxidants. This is further confirmed by the repeated failure of antioxidant therapy to increase maximum lifespan of mammals,[33,34] an observation which will be discussed further in later chapters.

This is a useful point because, on closer analysis, it dramatically narrows the field of choices for the driving force behind aging. As we get older, there is a steady increase in the levels of proteins which have suffered oxidative damage but not been recycled. Some such proteins are themselves pro-oxidant, so they are part of the problem. But they cannot be a driving part, because recycling is tunable: all other things being equal, the recycling machinery would simply be up-regulated to match the increased levels of damaged proteins, thereby lowering them again. This is the sort of negative feedback that maintains stability in the body and in the cell in the face of exogenous challenges, such as disease. The same logic applies to any component of cells—or of the extracellular space—that is recycled, whatever the rate of that recycling (unless that rate is so slow as to be comparable with our lifetime).

If everything that is oxidized were recycled, aging due to pro-oxidant damage simply could not proceed indefinitely: it would asymptotically approach a maximum level but never exceed it.*

Therefore, in seeking the root cause of the observed, inexorably rising pro-oxidant levels, we can restrict ourselves to material that is not recycled at all. They are hearteningly few in number:

1. some connective proteins (elastin, crystallin, maybe some others)
2. tissues that lose (and fail to replace) cells
3. nuclear DNA
4. mitochondrial DNA, maybe
5. lipofuscin

All of these except mitochondrial DNA are known, as described in the preceding sections, to be intimately involved in one or other aspect of aging. Mitochondrial DNA is not immediately implicated, but, as will be explained, it is also a prime suspect.

5.6.2. Oxidative and Reductive Stress

In 1985, Helmut Sies defined** the term "oxidative stress" as "a disturbance in the prooxidant-antioxidant balance in favor of the former."[37] His use of the word "balance" was, I think, both insightful and unhelpful. It was insightful because it emphasises the fact that pro-oxidants and antioxidants participate in a complex network of reactions, which are broadly maintained in a stable equilibrium. If this equilibrium is disturbed, such as in disease, it is restored once the disease has been overcome. But conversely, this emphasis on the stability of the system gives a misleading impression of the possible role of oxidative stress in aging, in which there is no such equilibrium; rather, there is an inexorable rise in the levels of pro-oxidants, which is not balanced by a corresponding rise in antioxidants and which in fact progressively diminishes those antioxidants' efficacy.

There is another problem with the term "oxidative stress" which relates to the chemical reactions involved. I stressed in Chapter 3 that LECs are generally more reactive than non-LECs; they can be thought of as taking the initiative to undergo reactions of classes a and b in Table 3.3. "Oxidation" is a term usually reserved for reactions in which the more reactive molecule is the electron acceptor, but, as Table 3.3 shows, there are many important reactions related to free radical chemistry in vivo in which the more reactive molecule is the electron donor. Reactions of this sort are normally termed "reduction"; thus, a situation in which there is an excess of LECs that are inclined to donate electrons would much better be called "reductive stress"—a term which, though introduced as long ago as 1987,[38] has not found widespread use. This is the situation at the earliest stage of LEC production by mitochondria, where the ubisemiquinone in the respiratory chain donates electrons to molecular oxygen to form superoxide. It is also the situation that I have proposed[39] to exist at the cell membrane of respiration-deficient cells, as will be discussed in Chapter 9.

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* *This logic has clear implications for interventions that might retard aging. Many groups have investigated the ability of certain antioxidant dietary supplements to reduce the steady-state levels of oxidative damage to material that is recycled; for example, a recent investigation[35] of the effects of combined supplementation with acetyl-L-carnitine and R-lipoic acid was particularly impressive in restoring youthful metabolic performance in rat liver. But we do not yet have lifespan data on these rats; I predict that their maximum lifespan will not be much greater than controls.*

** *The term had, however, been in use in broadly this sense since at least 1970.*[36]

5.6.3. Positive Feedback

One of the major lines of thought that will be discussed in the next chapter is known as the "vicious cycle theory." This name is rather unfortunate, because the theory in question was a very specific proposal for the mechanism of age-related mitochondrial decline, whereas "vicious cycle" is an extremely general term describing any system which undesirably accelerates its own rate of change—that is, which exhibits positive feedback. The vicious cycle theory did indeed hypothesise such a system, but many other systems are also vicious cycles.

More to the point, many aspects of aging can be so described. A prominent example is atherosclerosis, in which the lesion grows increasingly rapidly as the surrounding tissue resorts to ever more drastic measures to control it. At the molecular level, there is an interplay between oxidation and glycation,[40] which makes glycation harmful over and above its effects on unrecycled tissues (discussed in Section 5.3). For example, LDL can undergo glycation; this does not of itself make LDL toxic, but it does make it more prone to undergo oxidation and thereby become toxic, with the result that diabetics are unusually prone to atherosclerosis.[41] This interplay also goes the other way—oxidation exacerbates the deleterious effects of glycation, because the irreversible cross-links (such as pentosidine) that were discussed in Section 5.3 require an oxidation reaction following the glycation process. For this reason, pentosidine and its relatives are sometimes called glycoxidation products.[42] This dependency may help explain why birds can sustain such high blood glucose (see Section 6.5.6); biochemical investigation of glycation in birds has so far been restricted to relatively short-lived poultry, however.[43]

5.7. An Interim Conclusion: The Overinterpretable Pleiotropy of Human Aging

5.7.1. Biochemical Pleiotropy

This chapter has reviewed only rather superficially the major changes that occur in our bodies as we age, but it suffices to remind us, if we needed reminding, that they are intimidatingly many and varied. Perhaps it is that, more than any other characteristic, which has caused gerontology to be much less popular as a field of study than non-biologists might expect it to be.

It should also be apparent, however, that the more deeply one explores these many processes, the more they reveal themselves to have in common. This commonality is most evident not in the mechanisms that initiate these processes, but rather in the microenvironmental features that determine the rate of that initiation. Oxidative stress has a strong claim to being the main accelerating factor for all of them. Moreover, the root causes of increasing oxidative stress cannot be all that numerous, since they are restricted to the unrecycled materials listed in Section 5.6.1. And even among those, there is no reason to suppose that all make the same contribution; equally possible, a priori, would be that one is the primary culprit.

There is thus a very real possibility that the continued exploration of aging will follow very much the same pattern as did the exploration of cells' energy utilisation. It was appreciated for many decades that energy was used in a huge number of biological processes, and similarly that it could be extracted from a huge number of different nutrients. Now, however, we know that the link between energy extraction and energy utilisation involves just one molecule, ATP. Lonely electrons are shaping up to fill the role in aging that ATP does in metabolism.

5.7.2. Pathological Pleiotropy

Another way of looking at the pleiotropy of aging is from the evolutionary perspective. Natural selection has determined our lifespan, just as it determined everything else about our biology; moreover, it seems to be able to vary it remarkably easily, since (to take just one example) we live more than twice as long as our nearest relatives, chimpanzees. The environmental factor that most centrally determines maximum lifespan is now widely believed to be a species's rate of extrinsic (age-independent) mortality, which in turn is mainly determined by its position in the food chain; this "disposable soma theory" will be described in Section 6.5.2. Here we will examine just one aspect of it: how many things (genes, systems) have to be varied.

The answer from evolutionary theory is clear: everything—that is, all of the irreversible changes listed in Section 5.6 and any change to anything else that is not actively recycled. All of these degenerative processes can be slowed, but only at a metabolic cost, since maintenance requires energy and hence consumes nutrients. Therefore, none of these changes will be retarded "unnecessarily" assiduously, because that would be a waste of energy. An organism's lifespan will not be increased by slowing one or two of these changes, only by slowing all of them. Thus, in well-tuned organisms (which is what evolution creates), all of those changes will reach macroscopically pathological proportions at about the same age (though perhaps only in one organ, as for lipofuscin in the retina)—which is exactly what we see, as surveyed in this chapter.

But that pleiotropy is also overinterpretable. This chapter has drawn attention to the highly uneven distribution of some of these changes—that lipofuscin only does harm in the retina, for example; others, such as cancer and atherosclerosis, can happen in many places but don't happen everywhere at once. This is a large part of the reason why such phenomena are popularly—and inaccurately—thought of as "not really aging", but rather as diseases. But it has a profound implication for the practicality of extending lifespan by medical intervention: that all of these "particulate" pathologies are treatable by correspondingly particulate interventions, such as surgery. Therefore, the irreversible molecular changes that we should consider as the heart of aging from an interventionist viewpoint are the ubiquitous ones, which happen steadily throughout the body. As we shall see, this criterion points the finger very plainly at damage to mitochondrial DNA.

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Chapter 6

History of the Mitochondrial Free Radical Theory of Aging, 1954-1995

This chapter, unlike the rest of the book, is structured explicitly as a narrative. The theoretical and experimental advances which have given rise to the mitochondrial free radical theory of aging are many and varied, and their contributions to the overall theory are liable to be lost in a purely descriptive account. Here, then, is a roughly chronological summary of how the theory approached its current state.

6.1. Precursors of the Mitochondrial Free Radical Theory

Gerontologists of the 19th and early 20th century performed many studies aimed at gaining an understanding of what biological properties underlie longevity. Biochemistry was insufficiently advanced to be of use, but by the 1920s it was possible to perform quite sophisticated experiments on live animals. The foremost example, which is often cited to this day, was the work of Raymond Pearl, culminating in his book "The Rate of Living."[1] In this and prior work, he established detailed correlations between the longevity of fruit flies and certain genetic and environmental factors: in particular, he found that conditions which made the flies less active, due to either temperature or genetic inability to fly, increased their lifespan. He concluded from these studies that, "In general the duration of life varies inversely as the rate of energy expenditure during life." He was not, however, in a position to hypothesise in any detail how this relationship might come about.

6.2. The First Mechanistic Idea

The theory whose modern version will be described in the coming chapters was born in early November, 1954. The man who had the initial idea was then a 38-year-old research associate at the Donner Laboratory of Medical Physics in Berkeley, named Denham Harman. He had spent seven years at Shell Oil working on the chemical properties of free radicals, followed by a course in biology at Stanford. With the help of this cross-disciplinary background, he realised that the reactions which are initiated by free radicals could—in theory—be the driving force for the increasing macromolecular disorganisation that seemed to underlie the aging process. The most amazing thing, to me, about Harman's idea was that he thought of this before it was known that toxic free radicals existed in the human body at all.* He based the idea only on the fact that oxygen exists in the human body, and on his knowledge that, at the temperature and in the chemical environment of the human body, it might be prone to initiate such reactions.

** It had just been established[2] that one well-known and altogether unreactive component of the human body, melanin, is a free radical, and also that their formation was implicated in the toxic effects of hyperoxia and X rays,[3a] but that cannot really be considered a hint that any highly reactive ones are constitutively present.*

The Mitochondrial Free Radical Theory of Aging, by Aubrey D.N.J. de Grey.

Harman published this idea as a paper entitled "Aging: a theory based on free radical and radiation chemistry", which came out first as an internal University of California Radiation Laboratory report, in July 1955, and the following year as an article in the Journal of Gerontology.[3b]

6.3. Suppressiveness

A phenomenon which would, much later, be revealed as another clue to MiFRA was discovered in the laboratory of Boris Ephrussi in the early 1950s, and first discussed in print in 1955.[4] For some time it had been realised that baker's yeast, whose full scientific name is *Saccharomyces cerevisiae*, was an excellent organism for the study of really elementary biological processes. It is unicellular, easy and cheap to culture, and there was even money in it on account of its commercial relevance. It also has the feature of existing in either a haploid or diploid state; sexual reproduction occurs by the fusing of two haploid cells to form a diploid one, but both haploid and diploid cells also undergo asexual division. In 1996 it became the first eukaryote whose genome was completely sequenced.[5]

Ephrussi and his colleagues had reported previously[6,7] that colonies would constantly arise in yeast cultures which grew more slowly than the rest of the culture, and were therefore named "petite colonie", usually shortened to petites. This property was shown to result from loss of aerobic respiration, and in particular from "the lack of several enzymes (including cytochrome oxidase) bound, in normal yeast, to particles sedimentable by centrifugation." (These particles were, of course, mitochondria.) But in their 1955 paper, they reported something much stranger and more exciting. They found that many of the mutants which arose in this way exerted a dominant effect when they underwent sexual reproduction. That is: when a haploid petite cell was mated to a haploid wild-type cell, forming a diploid cell with a hybrid (heteroplasmic) mitochondrial genotype, the culture that that diploid cell gave rise to (by subsequent asexual reproduction) was more prone than average to contain petite colonies, even though that original diploid cell exhibited no phenotype itself. This was shown to be a characteristic carried in the cytoplasm of the petite cells, and was named suppressiveness. It is now known to be caused by spontaneous deletions or point mutations of the mitochondrial genome.

6.4. Mitochondria as Free Radical Victims

Gradually, over the next 15 years, more and more evidence came to light—much of it due to Harman's experimental work—indicating that his basic precept was absolutely right. Reactive free radicals—LECs—were indeed discovered in living cells, and in 1969 an enzyme was isolated which destroyed one.[8] The LEC was superoxide, which was already thought to be likely to mediate oxidative damage, and the enzyme was SOD, superoxide dismutase.

In 1972, Harman made a further great theoretical contribution[9] to the hypothesis he had originated. He had established, through many experiments over the previous 15 years, that the most obvious choice (if his theory was correct) for a type of chemical that might extend lifespan was only partly successful.[10-12] Such chemicals—antioxidants—act to soak up LECs, so intake of (otherwise non-toxic!) antioxidants should lower the levels of LECs in the body. If the free radical theory was correct, that should retard aging. Harman found that he could indeed raise, quite considerably, the average lifespan of mice by feeding them various antioxidants. What he could not do, however, was raise the maximum lifespan. All that happened was that the distribution of mortality became more "rectangular": most mice lived nearly as long as the longest-lived. It seemed that there were some degenerative processes which were non-universal, i.e., which predisposed some mice to die younger than others; the exogenous antioxidants were successfully retarding these non-universal degenerative processes, but not retarding the universal processes that were central to aging.

Harman suggested[9] that this might be because some crucial component of cells, whose rate of decline dictated the overall rate of aging, was not benefiting from the antioxidant therapy. Since mitochondria were very likely to be the primary source of LECs in cells, and since LECs (being so reactive) are likely to be very short-lived—in other words, to react with something very nearby their site of creation—he reasoned that mitochondria were also very likely to be the principal **victims** of LEC-mediated damage. He proposed, on this basis, that aging was driven by the decline of mitochondria, caused by self-inflicted damage. This was, therefore, the first statement of the mitochondrial free radical theory of aging, i.e., MiFRA.

This idea had little impact for several years, but was carried forward with great effect during the 1980s. The human mitochondrial genome was sequenced in 1981,[13] facilitating the isolation of mutant mitochondrial DNA and eventually its quantification. These experiments strongly confirmed that mtDNA becomes increasingly damaged with age.[14] Further support came from the finding[15] that, broadly, the tissues which exhibited the most mtDNA damage were the ones that consumed the most energy per unit volume and/or generated the most reactive molecules: there was more in the substantia nigra (which makes a lot of hydrogen peroxide) than in the cerebellum, for example,[16] and more in the extraocular muscles (which are almost constantly active) than in the limbs.[17] (The correlation was not perfect—in fact, an even better correlation was with regard to the rate of cell division of the various tissues, a fact which became comprehensible only some time later: see Section 8.5.3.) This correlation brought MiFRA strongly into line with the "rate of living" theory that high metabolic rate shortens lifespan.

Moreover, many early hypotheses attempting to link the rise of oxidative stress with the self-inflicted damage to mitochondria became testable and thereby fell by the wayside. One such idea[18,19] was that mitochondria suffer damage to their DNA that inhibits their ability to replicate it; thus a cell's ability to synthesise ATP will progressively diminish with age, because it cannot make enough of the mitochondrially encoded proteins. It was some time before experimental techniques became able to test this idea—and to establish, as it turned out, that it was wrong: mitochondrial numbers and mtDNA levels do not significantly decline with age.[20] In fact, cells that carry a large quantity of mutant mtDNA appear to **over**-replicate their mitochondria in a (futile) attempt to compensate for low OXPHOS function.[21,22]

6.4.1. The "Vicious Cycle" Theory

Another proposal which arose during this period survived for much longer. As will be discussed in Chapter 11, it is generally found that production of superoxide rises with age, and is also unusually high in the affected tissues of sufferers from genetic defects of the respiratory chain. Since LECs are able to damage all classes of macromolecule, including nucleic acids, it seemed clear that the rate at which mtDNA mutations occurred would rise as the production of superoxide rose. (Oxidative damage to DNA does not directly cause mutations, but it is known to cause inaccurate replication by the mitochondrial DNA polymerase.)[23] But, as we have seen, the free radical theory (by this time) already implicated mtDNA mutations as the **cause** of the rise in superoxide production with age. If they were both its cause and its consequence, one had a vicious cycle which would cause exponential increase in both (see Fig. 6.1)—which was exactly what was seen.

This theory was persuasive for all the right reasons: clear-cut, simple, and in absolute accordance with the data. But, beginning in 1989, new and more detailed studies have conclusively refuted it. In an effort to adhere at least vaguely to a chronological account, I shall delay the description of that work until Section 6.6.

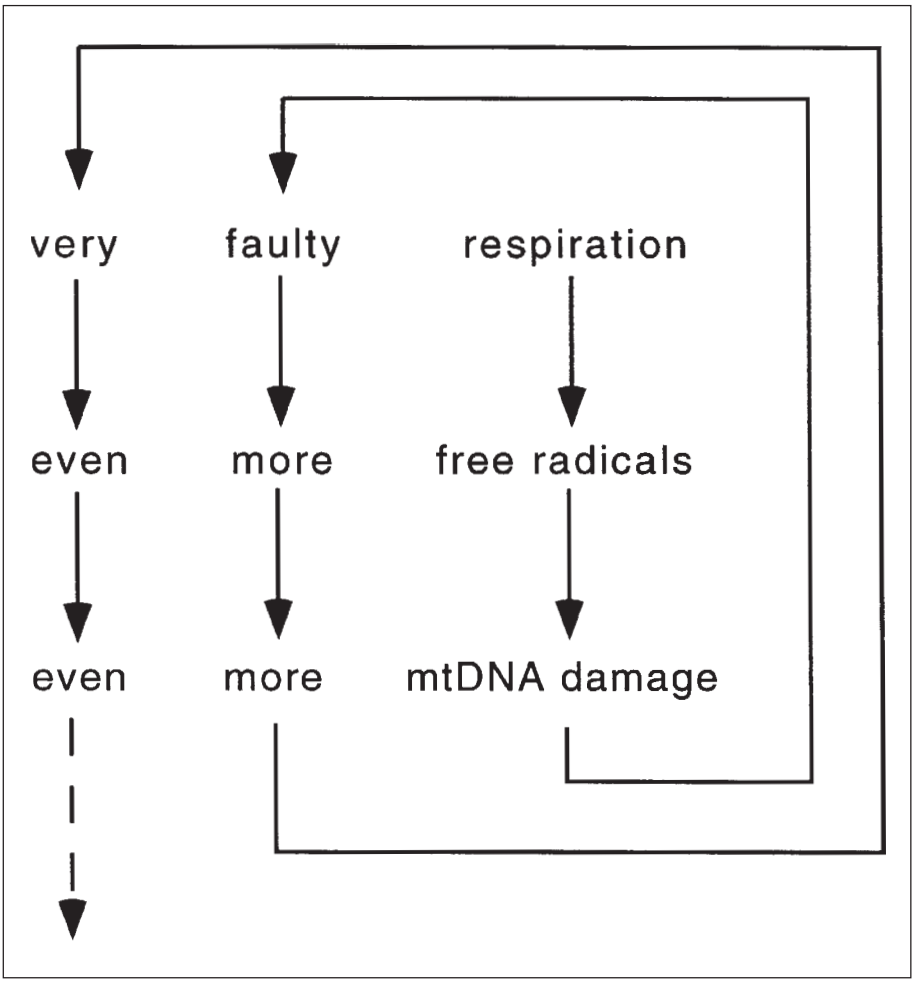

Fig. 6.1. The vicious cycle theory (with emphasis on its instability).

6.5. Comparisons with Other Species

The past decade has seen a series of advances in understanding aging of lower organisms. The three that have been studied most are the ones one would expect—the fruit fly *Drosophila melanogaster,* the nematode *Caenorhabditis elegans* and the yeast *Saccharomyces cerevisiae*—and they have been attractive for the usual reasons that their husbandry is convenient, their generation time short and their genetics and molecular biology very well characterised over many years. However, they feature only occasionally in this book. The phenotype of aging in a single-celled organism is a rather contentious concept in the first place, as exemplified by yeast: in *Saccharomyces cerevisiae* aging is defined in terms of the number of times a cell can "bud off" a daughter cell, but in other yeasts which divide by symmetrical fission there is no such definition, so there is deemed to be no aging! In multicellular organisms with a clear distinction between germ line and soma there is no difficulty of definition, but there is good reason to suspect that the processes of

macromolecular degradation which determine lifespan are not the same as in warm-blooded animals (see Section 10.5).

Nonetheless, it is undeniable that comparing and contrasting the details of a process in multiple different species is a hugely useful approach in biology. Its role is very similar to that of mutational analysis, where the ultimate effects of a genetic alteration—that is, a comparison of a mutant organism with a wild-type one—can be used to demonstrate, for example, that the product of gene X stimulates or inhibits the expression of gene Y. Comparisons between vertebrate (and very long-lived invertebrate) species have been used extensively, especially in recent years, in an attempt to gain insight into what controls the rate of aging. Several relationships have emerged as being of particular importance, and they will be discussed in turn here.

6.5.1. Longevity and Specific Metabolic Rate

An extremely obvious inter-species correlation with regard to longevity is that bigger animals tend to live longer. Unfortunately this is not (on the face of it) a very valuable insight, since not only does it fail to suggest any mechanisms, but also it is a necessary consequence of the fact that growth—cell division, in particular—entails a complex and intricate series of chemical reactions, and therefore takes time. If an animal is capable of reproduction when it is only a few millimetres long, it can tolerate living only a few days; if it cannot reproduce until it is a few metres long, it needs to live a lot longer.

One can, however, make more progress if one examines the relationship of these two variables with a third: body temperature. This differs very greatly between warm-blooded animals (homeotherms) and cold-blooded animals (poikilotherms). All homeotherms maintain about the same body temperature, and doing so obviously requires the conversion of nutrients into heat, which consumes oxygen. An animal's oxygen consumption (which is easy to measure, unlike, for example, its heat output) is thus a measure of the rate at which it is then using nutrients—its metabolic rate. An animal's metabolic rate varies—it increases when the animal is physically active, and decreases during sleep—so it is usual to measure standard metabolic rate, which is defined to be that when the animal is awake but at rest. From that one establishes the animal's specific metabolic rate by dividing its standard metabolic rate by its mass. This is the interesting number, because it is a measure of how hard the average cell is having to work to keep the animal warm. Since smaller animals have a higher ratio of surface area to volume, and hence of surface area to body mass, they end up needing a higher specific metabolic rate in order to maintain the same body temperature.*

So the question is: is there a correlation between specific metabolic rate and longevity? Do animals of similar sizes but different specific metabolic rates have different longevity? Indeed they do. A poikilotherm of a given size generally lives much longer than a homeotherm of the same size.[25] Unlike the early observation that lifespan varies with size, this need not necessarily be so. Furthermore, the same relationship applies to poikilotherms kept at different temperatures: fruit flies live only about half as long at a given temperature as at 10° C cooler (within their range of good viability, of course).[26] This is also a measure of specific metabolic rate, since for poikilotherms specific metabolic rate varies with temperature just like any chemical reaction.

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* *Intriguingly, the relationship between mass and specific metabolic rate that is seen is not precisely the obvious one. One would naturally predict that the rates of animals whose body temperatures were the same would vary as the inverse 2/3 power of their mass, since our surface is two-dimensional and our bodies are three-dimensional. In fact it is almost exactly the inverse 3/4 power.[24] This may not sound like much of a difference, but it has kept eminent comparative biologists in business for many years.*

Unfortunately, though this correlation certainly exists, it is not a fantastically good correlation. Primates, birds and bats are probably the most extreme classes of homeotherm that live a great deal longer than would be predicted by their sizes and specific metabolic rates. The search has thus continued for other variables which, when added into the equation, give a more universally accurate prediction of species lifespan.

6.5.2. How Does Lifespan Evolve?

The search for such factors has been greatly aided by evolutionary biology, so I shall digress for this section into a summary of current theory regarding the evolution of lifespan. I mentioned in Section 5.7.1 that lifespan seems to be rather easily adjustable by evolution; since civilisation has found it so very hard to adjust by medical intervention, we may reasonably ask "why?"

Well, first of all: why not? Evolution, of course, works by selecting between slightly different sequences of DNA based on their phenotype, which largely derives from the three-dimensional structure of the proteins that the DNA encodes. The mechanism(s) whereby genetic makeup determines longevity is no different, in this respect, from any other aspect of our physiology: it involves chemical reactions, and those reactions involve macromolecules—mainly proteins. Thus, small changes to those proteins' three-dimensional structure, due to changes in their sequence, will affect the rates of the reactions in which they participate.

So, here is the current evolutionary explanation for both the general "rate of living" correlation and the major exceptions to it.* Fundamental Darwinian logic tells us that all species gravitate, by natural selection, to a lifespan which maximises their chance of producing offspring and raising them to maturity, and that this maximum is a balance between the avoidance of predators and the optimising of the environment in which the offspring will mature. An animal which is at a severe risk of death by predation will be best advised to live its life fast, ensuring that it has some offspring before it succumbs. One whose risk of predation is very low will, conversely, be best advised to wait until the environment is particularly favourable for procreation—for example, until there is a particularly plentiful food supply. And in order to wait, it must invest in better self-maintenance so as to avoid dying of old age. Now, the simplest way of avoiding predation is to be large: this explains why, in general, large animals live long. But that is not the only way to avoid predation: one of the other good ways is to be able to fly, so this also explains why bats and birds live so long. Finally, an even better way to live a long time is to be highly intelligent; that is what gives primates (and, among them, humans) their advantage. This general idea, that the main determinant of lifespan is one's position in the food chain, is the ecological description of current thinking on the evolution of lifespan.[27] It has also been strikingly confirmed by intraspecies comparisons.[28] Another way to say essentially the same thing is to note that, while the cells of an organism's germ line must be maintained in a pristine state in order to give rise to viable offspring, the rest of us (our somatic cells) need only be maintained well enough to stay just about working throughout the optimal lifespan that our ecological niche determines. This is, naturally, called the disposable soma theory.[29]

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* *This theory is the culmination of a century of analysis of the evolution of lifespan. It and its predecessors, due primarily to Weissmann, Haldane, Medawar and Williams, are discussed in detail in, for example, ref. 43.*

6.5.3. Molecular Correlations with Longevity

In this book, however, the major topic of discussion is not why species have the lifespan they do but how. In other words, we want to identify the molecules whose reaction rates determine the rate of aging, and which therefore vary in detailed structure from one organism to the next in a manner correlated with longevity. Two very strong molecular correlations have indeed been discovered, and they both provide strong circumstantial evidence for the controlling role of oxidative stress in aging.

6.5.3.1. Longevity and Membrane Oxidizability

It is difficult to measure directly the rate at which the membranes in a tissue undergo oxidation, because this damage is constantly being put right by turnover of various sorts.* Relatively easy, however, is to measure the composition of a tissue—or of a chemical category extracted from it, such as its lipids. Better still, one can first fractionate the tissue into its cellular components and then determine the lipid composition of a single fraction, such as the mitochondria.

When this is done, a very great difference is found between the membranes of short-lived and long-lived animals of similar specific metabolic rate. The same difference is found when comparing rats to pigeons as when comparing horses to humans.[31,32] In both cases, the fatty acid side chains of the phospholipids in the mitochondrial membranes are much less unsaturated on average in the longer-lived animal. The degree of unsaturation of a fatty acid is the number of carbon-carbon double bonds, C=C, in the chain; a fully saturated fatty acid has no C=C bonds, only C-C bonds. This is thus very suggestive of the importance of oxidative stress in aging, because only regions between two C=C bonds of fatty acids are susceptible to oxidation by LECs (see Section 3.9).[33]

6.5.3.2. Longevity and Superoxide Production

The second correlation, found in some of the same studies, also strongly supports the idea that oxidative stress is central in setting the rate of aging. Longer-lived animals apparently generate less superoxide.[31,34] Their respiratory chain enzymes seem, simply, to be more fastidious about not fumbling electrons. The likelihood that an electron will become detached from a respiratory chain component and annexed by oxygen is likely to be very dependent upon the exact shape of the enzyme in question, which is of course determined by the amino acid sequences of its various subunits.** These sequences are highly conserved in evolution, but differences certainly exist, and they may well cause significant variations in how often an electron can be fumbled.

6.5.4. Longevity and Calorie Restriction

During this time, it was also becoming increasingly accepted that calorie restriction (CR—also, unfortunately, often termed DR, ER or FR, for diet, energy and food) could reliably and significantly increase the lifespan of many species, notably laboratory mice: this had in fact been discovered some decades previously.[35,36] Calorie restriction is achieved by

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** However, there are measures of the rate of repair of some molecules. DNA is an easy one, because many DNA repair processes involve removing a damaged nucleotide and discarding it into the blood stream, and thence into the urine.[30] The quantity of nucleotide derivatives in urine is one of the best measures of the rate of increase of DNA damage with age.*

*** The non-protein ubisemiquinone is the molecule believed to do most of the fumbling, but recall from Section 2.3.3.3 that it exists only bound to Complexes I and III, so their exact shape is the relevant variable under evolutionary control.*

giving mice a diet which is sufficient in protein, vitamins and minerals of all types, but is perhaps 40% lower than normal in fats and carbohydrates. Crucially, it increases mice's maximum lifespan as well as the average, in contrast to antioxidants which appear only to "rectangularise" the distribution of mortality by raising the average lifespan but not the maximum (see Section 6.4). This phenomenon was largely ignored for nearly 40 years after its discovery, but once real interest in it began (in the 1970s) people rapidly observed that the association of calorie restriction with longer life might be at least broadly explained by MiFRA, since the rate of turnover of nutrients determines the rate of transfer of electrons along the respiratory chain, and would thus be expected to dictate the rate of production of superoxide.[37] Initial calculations of specific metabolic rate based on caloric intake[37,38] showed that, indeed, the calorie-restricted mice had lower metabolic rate proportional to their increase in lifespan, in line with the rate-of-living theory. This was actually found to be in error when metabolic rate was measured explicitly,[39] but more recent work has shown that the detailed biochemical predictions of MiFRA (such as that repair enzymes will be upregulated by CR) are indeed seen, in line with the inter-species molecular differences noted in the previous section. Moreover, these same biochemical changes have been identified in the ongoing studies of primate CR.[40-42]

6.5.5. Longevity and Indeterminate Growth

In recent years, some interest—though still far less than seems motivated—has arisen in the apparently very great longevity of several species of cold-blooded animal who continue growing throughout their lives. Extreme examples include lobsters and certain fish; there are also amphibian and reptile examples. A thorough discussion of these species was provided by Finch.[43] These animals seem to live much longer than would be predicted by the inter-species metrics discussed in the preceding sections.

Not only that: statistical measurements of the rate at which they age indicate, within the accuracy available from the sample size, that they may not be aging at all—a phenomenon termed negligible senescence. This is so dramatic a result that it is worth going into the details of exactly what is being measured. There is only one basic requirement here: a way to identify accurately how old an organism is. It turns out that some of the bones and other hard tissues in these species are ideal for this, because they exhibit annual oscillations of chemical composition, which build up from the outside like tree rings.[44] This means that one can simply catch* a large number of a given species and plot their ages. A species that does not age at all will show a curve in which the number of individuals of age N is always the same fraction (95%, say) of the number that are age N-1, for any N above the age of maturity; a species exhibits negligible senescence if its age distribution is statistically indistinguishable from that. A species that is detectably aging shows a different curve, in which this fraction is progressively smaller for larger N. The larger the study, the more accurately one can distinguish between gradual and non-existent senescence, but all studies are of finite size; the existence of non-senescing species, as opposed to a continuum of rates of senescence, is therefore very hard to prove. The studies performed so far are not really large or numerous enough to establish that any animal senesces (that is, the fraction defined above increases) more than about twice as slowly as humans. I will discuss some options for further work in this area in Section 12.3.

One must also choose the species of study with some care, because some species grow throughout their life but die at a fairly young age anyway. Salmon are an example.[43]

* *"Catch" is not really the right word, in fact, because in the wild the rate of extrinsic mortality (death not caused by old age) is so high that* ***most*** *organisms cannot be observed to age. Thus, such studies must usually be done in captivity.*

(The mechanisms underlying this are programmed: this can be inferred from the very rapid senescence that occurs at the end of a salmon's life, in contrast to only gradual aging before that, and in fact the neuroendocrine mechanisms that trigger it, shortly after spawning, are now somewhat understood.)[45] Also, many fish—including some that grow throughout their lives—exhibit an intermediate behaviour, senescing gradually just like mammals.[43] The same range of types of aging is seen in amphibians and reptiles; there are even a few cases of programmed senescence in mammals,[43] though no negligibly senescing mammals or birds are known. Thus, indeterminate growth definitely does not confer negligible senescence,[46] as was originally[47] proposed. A much more difficult question to answer, with current data, is whether negligible senescence requires indeterminate growth; some very longevous fishes such as rockfish appear to grow extremely slowly if at all, so this may also not be the case.

A principal reason why species with indeterminate growth may be of great relevance to MiFRA is that, in order to continue growing, they may continue cell division of all tissues, including muscles and nerves, and they may thereby be escaping the mitochondrial decline that overtakes other animals' postmitotic cells (see Sections 6.4 and 8.5.3). It is possible, alternatively, that the indeterminate growth is achieved purely by making the existing muscle fibers and nerves longer, but this seems not to be what happens: certain amphibian species have been shown to perpetuate neurogenesis and myogenesis throughout their lives.[48] Also, lizards and snakes appear not to accumulate lipofuscin with age in spinal neurons,[49] suggesting that there may be neuronal turnover. We will return to these topics, too, in Section 12.3.

6.5.6. Some Instructively Unexpected Non-Correlations

Most species of bird are much longer-lived for their metabolic rate than the average mammal; this was the main motivation for the studies mentioned in Sections 6.5.3. The natural prediction would be that **every** contributor to age-related degeneration will be down-regulated in birds relative to mammals: not only will superoxide production be lower and lipids less oxidizable, but also (for example) antioxidant enzymes will be present at higher levels, and there will be less glucose in the blood so as to retard glycation. Curiously, however, in both cases the reverse is seen. One early study reported a positive correlation of superoxide dismutase with longevity,[50] but this was done using flightless mammals only and was derived by factoring out specific metabolic rate—that is, the correlation was between longevity and (SOD levels divided by oxygen consumption per unit mass). Such a "correction" may seem well-motivated, but it is arbitrary: equally justifiable would be, for example, to divide by the square of the metabolic rate. More recent studies compared birds and mammals of similar metabolic rates, so no such correction was needed; these studies showed an unambiguous negative correlation between most antioxidant enzyme levels and longevity.[51,52] Similarly, birds have at least twice the blood glucose levels of mammals.[53a]

This tells us that, in some way, antioxidants and low blood glucose are not important for longevity. How can that be so? My answer brings us back to Harman's 1972 insight that the central determinants of the rate of longevity might be in some way inaccessible to dietary antioxidants. They might also be inaccessible to **enzymatic** antioxidants, in which case no positive correlation would be expected. We can go further and explain the negative correlation by noting that, as explained in Section 5.7.2, no maintenance or defense system is likely to be unnecessarily good. Thus, if an organism achieves longevity by low LEC production (and thus low LEC-mediated damage to material inaccessible to antioxidant enzymes), there will be the side-effect of low LEC-mediated damage to material that **is** accessible to them. This allows the organism to lower its investment in the production of those enzymes (see Fig. 6.2). A plausible compartment that lacks antioxidant enzymes is the mitochondrial intermembrane space (see Section 11.2.3).[53b,53c]

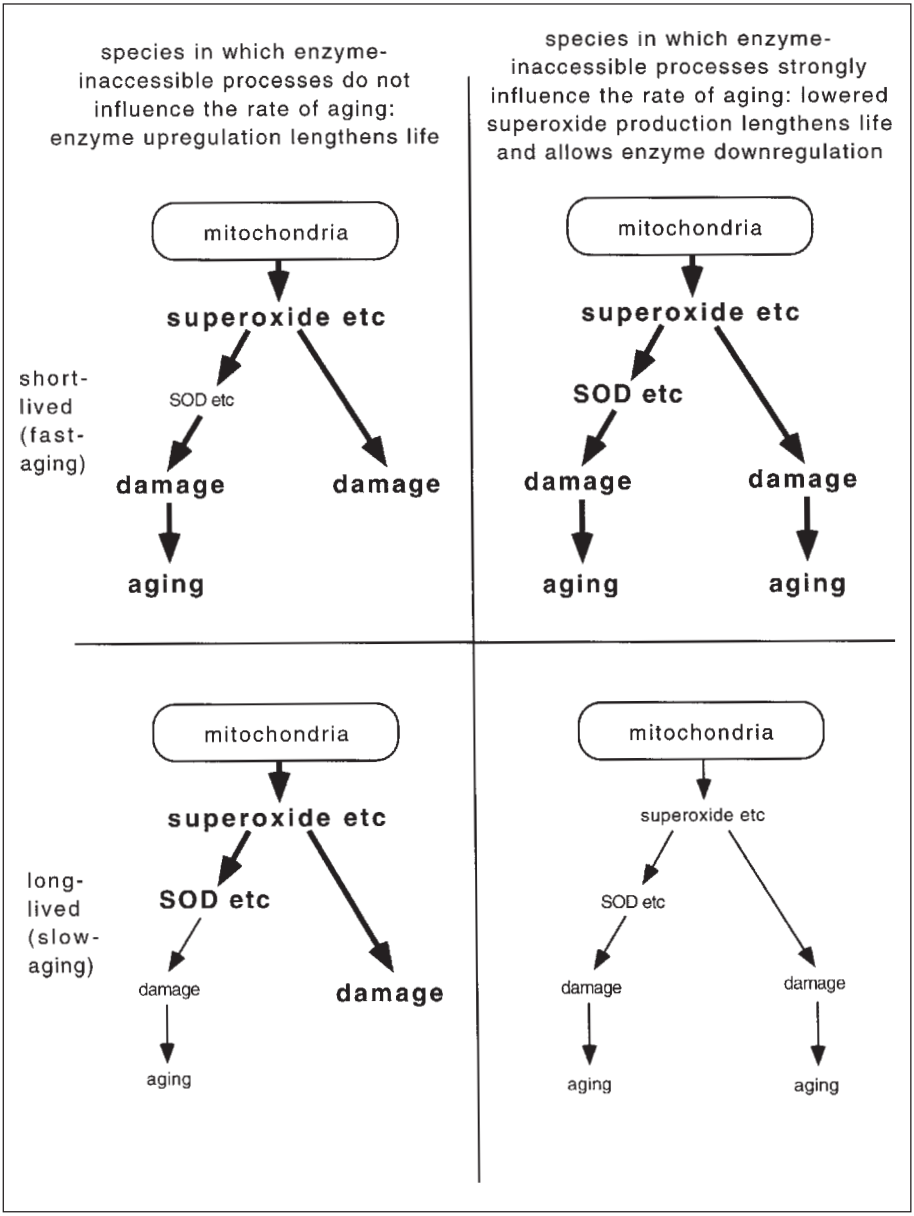

Fig. 6.2. A hypothesis for why longer-lived homeotherms make less enzymatic antioxidants.

6.6. Amplification of Mutant mtDNA; Demise of the Vicious Cycle Theory

The last of the experimental breakthroughs which underpin the modern mitochondrial free radical theory of aging was due to a number of researchers; foremost among these was Josef Müller-Höcker, working in Munich. He concentrated on studies of intact tissue samples,

most often muscle, which he analyzed in various ways—with DNA probes, with antibodies, or with enzymatic assays—in order to identify the distribution of certain mitochondrial defects. He made two pivotal discoveries. The first of these was that the loss of mitochondrial function which others had detected was not distributed evenly across the tissue, but was localised in just a few cells or muscle fibres, and that these fibres (actually, as it turned out, short segments of them) were totally devoid of aerobic respiration.[54] Even stranger was that he only occasionally detected fibres in which the level of respiration was reduced but non-zero.[54,55] This showed that, if the vicious cycle theory was correct, then (a) it went on in each cell independently of its neighbours, and (b) it was very, very vicious. Cells independently, for whatever reason, reached a point where the cycle took off; after that, they must plummet to aerobic oblivion in (at most) a matter of months, or else we would catch more of them in the act of plummeting.*

The second, and even more important, observation came when he assayed the same sample of muscle with two different mtDNA probes.[57] He found that the cells which were completely lacking in respiration were lacking for genetically different reasons. In one such cell there would be almost complete loss of hybridisation to one probe but absolutely normal reaction with the other, while in another cell it would be the opposite way around. Some cells had lost affinity for both probes; some had lost neither (indicating a mutation elsewhere). This meant that the vicious cycle theory had to be radically revised. It was no longer possible to say that stress caused more mtDNA mutations causing more stress, because a cycle like that would necessarily give a virtually identical spectrum of mutations in each and every affected cell, irrespective of which ones were present first in a given cell. We see the exact opposite—each cell taken over by, ostensibly, just one mutation. (A single mutation was able to remove both probed regions in Müller-Höcker's experiments, by being a large deletion or a complete loss of the whole mtDNA (mtDNA depletion, of which more in Section 10.11); in histochemical assays the same effect would also result from mutation of a mitochondrial tRNA gene.) This meant that there was not an intracellular vicious cycle—at least, not at the level of mtDNA mutation. Rather, the cell was somehow allowing its mitochondrial population to be taken over by copies of a single initial mutation: the mutant molecule was somehow experiencing a selective advantage.

It is appropriate—and salutory—at this point to take a brief leap back in time. In 1974, just two years after Harman had suggested the involvement of mitochondria in the free radical theory, Comfort[58] identified the existence of mitochondrial turnover as a serious challenge to that possibility. He reasoned that damaged mitochondria would obviously be got rid of and replaced by functional ones, so damage could never accumulate. He was wrong, but his point that mutant and wild-type mitochondria have independent propensities to be maintained in the cell—they compete, effectively—was key. In hindsight, it is highly unlikely that the two genotypes will be exactly equally matched in this context, so one or other will inevitably win out; since we see an accumulation of mutant mtDNA, we should have immediately expected that it would be caused mainly by clonal amplification. Yet this idea was not picked up on for twenty years.

**Another way, not involving intact tissue samples, in which it was established that mutations are not distributed evenly across all cells is to analyse the DNA of only a few cells. If a homogenate of many thousands of muscle fibres is analysed, it is found always to contain the "common deletion" (see Section 2.4.5) at levels around 0.1%. If the same sample is divided into small bundles of only ten fibres, however, nearly all the bundles show no evidence of the deletion whereas one or two show high concentrations of it.*[56]

The amplification phenomenon has since been studied in some detail in a variety of systems.[61-62c] Several subtleties have emerged which have a large impact on the search for a plausible mechanism; the rest of this chapter deals with these details in turn.

6.6.1. Segmental OXPHOS Deficiency in Muscle

Müller-Höcker's studies on muscle all involved staining of a sample of muscle that was cut perpendicular to the direction of the fibers, so that each fiber appeared as a disc. (This is termed a transverse section of the muscle.) A different approach is to dissect a sample longitudinally: to extract an entire fiber (or a substantial length of it) from a sample and stain it all the way along. This technique[59-61] reveals that the loss of, for example, cytochrome c oxidase activity does not extend throughout the fiber, but is localised to short segments, almost always under 1 mm in length. (The same result can be obtained by studying transverse sections if a series of consecutive sections are lined up so that the slices through a particular fiber can be identified.)[62a] The obvious question arises: if the mutation is clonally amplified within part of the fiber, why not within all of it?

This distribution is not so curious, however, when one considers the structure of muscle fibers. They are syncytial, comprising a single cytosolic compartment, but they have nuclei dotted along their entire length. Thus, it is theoretically possible for each nucleus to maintain its own mitochondrial population independent of the other nuclei to either side. One might imagine that mitochondria would randomly move along the fiber, into neighbouring nuclei's fiefdoms, so that a mutant with a selective advantage would march along taking over successive sections of fiber. This may indeed happen, since the regions very near to an affected segment are unusually prone to morphological changes,[61] but if so then it is clearly very slow, or else much longer mutant segments would sometimes be observed. A simple explanation can be seen, however, from the way that muscle fibers arrange their mitochondria. They do not float free in the cytosol, but are attached to the contractile filaments to which they supply energy.[63] They must necessarily be free for short periods after mitochondrial division, but once fixed in place they may stay put effectively forever. The tight packing of mitochondria may also impede the diffusion of cytosolic ATP along the fiber.

6.6.2. Rate of Acquisition of Selective Advantage

A shortcoming of in situ assays of tissue is their low sensitivity. One can detect a mtDNA deletion by in situ hybridisation to a mtDNA segment within that deletion only if the deletion is present in large quantity in a cell, so that the wild-type mtDNA (which is the molecule to which the probe hybridises) is perceptibly depleted. It is theoretically possible to design probes that bind selectively to the deleted mtDNA, so allowing detection down to lower levels: such probes identify the new junction between the ends of the deletion, which is not present in the wild-type molecule. But in practice this technique is rather inexact, largely because the most common deletions are formed by illegitimate recombination between direct repeats (see Section 2.4.5), so that the new junction is in fact a sequence that **is** present—twice—in the wild-type molecule.

Much lower levels of mtDNA mutations can be detected, however, if the mtDNA is extracted and subjected to the polymerase chain reaction (PCR).[64] Moreover, by using a relatively new variation called long PCR,[65] which is capable of amplifying the whole mtDNA in one go, one can identify most of the different mtDNA deletions present in a tissue sample, in contrast to the original technique which could only detect a small proportion of sequence variants.[66,67] When this technique is applied to one or a small number of muscle fibers,[56,62] it is found that, though the proportion of fibers with high levels of any deletion is comparable to that seen by in situ hybridisation, a much higher proportion—perhaps as high as 4%[56]—carry some deletion at very low levels.

This is something of a paradox, since we know (from the rarity of fibers with reduced but non-zero OXPHOS function—see Section 6.6) that the selective advantage of mutant molecules is very great. One might therefore predict that these 4% of fibers with low levels of mutant mtDNA would become completely OXPHOS-deficient within months, whereas in fact even very elderly individuals never exhibit such levels.

The answer explains the title of this section. When a mtDNA mutation first occurs, it probably has no impact whatever on its host mitochondrion's function, because that mitochondrion also possesses several wild-type genomes. Accordingly it is a matter of pure chance, unbiased by any selective pressure, whether that mitochondrion divides (and its mtDNA is replicated) or is destroyed by lysosomal digestion (see Section 2.4.6). If it does divide, then it is again a matter of chance whether the two copies of the mutant molecule segregate into the same daughter mitochondrion or one into each. Cumulatively, therefore, the chance that a given spontaneous mutation will end up homozygous in some mitochondrion is rather low, since it involves several mitochondrial divisions going the right way. It is probably not until then that the mutation will have any significant effect on its host mitochondrion's OXPHOS functionality. In fact, that functionality is likely to be largely retained for a couple of mitochondrial generations even after homozygosity is achieved, since some of the host mitochondrion's membrane proteins are inherited from previous generations when it still had some wild-type genomes.

This means that, on average, a given mutation must have survived for a considerable while by pure luck in order to exert a phenotypic effect, and thus in order to begin enjoying a selective advantage. Most mutations will be snuffed out (by chance, not by any selective pressure) before this. But many of those that are snuffed out will progress part of this way, being randomly replicated up to five or ten copies. And that sort of level, among a total of perhaps 10^4 or 10^5 molecules total in a sample, is enough to be detected by PCR. This means that the high proportion of fibers which harbour detectable levels of mutations are not a paradox after all: most of those mutations were not destined to take over their cellular environment anyway.

6.6.3. Cybrids

An in vitro technology was developed in the 1980s[68,69] which has had a dramatic impact on, among other things, the study of selective advantage of mtDNA species. King and Attardi developed a method for removing all the mtDNA in a human cell culture and yet keeping the culture alive. A summary of the technique appears in Section 9.2; its relevance to this chapter is in how these cells (termed ρ^0cells) have been used to study mtDNA selection. The value of ρ^0 cells is that one can repopulate them with mitochondria of one's choice. Thus, for example, if one has a patient suffering from a mtDNA-linked disease, whose cells contain some mutant mtDNA and some wild-type, then one can take platelets[70] (which have mitochondria but no nucleus), or other cells whose nucleus has been artificially removed, and fuse them with ρ^0 cells. The resulting fusion cells are called cytoplasmic hybrids, usually contracted to cybrids. Then one can follow the relative abundance of two species of mtDNA in a culture of these fused cells; and, crucially, one can repeat the experiment with a different source of mtDNA, from a different patient, but with the same ρ^0 line, thereby eliminating complications of interpretation due to differing nuclear genotypes. It was quickly demonstrated[71] that the mutant mtDNA genotype of a well-studied mutation often took over such cultures.

Unfortunately, since then the picture has become altogether muddier. A study[72] which essentially repeated this experiment found that some such cultures behaved as reported,[71] but others exhibited the opposite behaviour—mitochondria of the mutant genotype dwindled in number. Moreover, multiple cultures created from the same recipient (ρ^0) cell

line tended to behave the same way, indicating strongly that the nuclear genotype had a strong influence on the relative selective advantage of the two genomes.

I personally feel, in the light of this and other studies, that the ability of this technique to tell us about what happens in vivo in aging is less than many people claim; however, a very recent variation on the technique shows promise of changing that pessimism completely. The main challenge to the relevance to MiFRA of cybrid mtDNA segregation is that cybrids are rapidly dividing, and we have long known that rapidly-dividing cells tend to accumulate far less mutant mtDNA than non-dividing cells. Thus, any major selective advantage of mutant mtDNA in cybrids is necessarily due either to some curiosity of the particular mutation—which is in fact quite likely for those mutations that cause inherited diseases (see Section 6.6.5)—or to some non-physiological aspect of the cultured cells' environment.

The recent advance[73] is the use of cybrid technology on long-lived, non-dividing cells—muscle fibers. It has long been known that muscle precursor cells—myoblasts—can form into muscle fibers in culture but atrophy shortly afterwards. It is also long established that this atrophy can be prevented, and the fibers kept alive for several months, by coculturing them with neurons, particularly from the spinal cord.[74] What has now been achieved is the combination of this technique with the cybrid technique. Muscle precursor cells are first divested of their endogenous mtDNA, then repopulated with the mtDNA mixture of interest, and finally induced to fuse and form muscle fibers, which are kept alive by the neurons. It is thus now possible to study mitochondrial segregation in a population of genetically well-defined postmitotic cells. I have high hopes that this will lead to rapid progress, not least in the testing of the details of MiFRA that I will describe in later chapters.

6.6.4. Do mtDNA Point Mutations Accumulate?

Naturally, the discovery of clonal amplification led to a search for a plausible mechanism whereby mtDNA mutations might derive their selective advantage. It was widely—and reasonably—felt that this might be easier if there were more information about which mutations accumulate during aging and which (if any) do not. Efforts to acquire such information were hampered, however, by the absence of a reliably accurate technology for measuring the concentration of a particular DNA sequence relative to another: standard PCR technology can only identify molecules of a different length than normal, so it cannot pick up point mutations. Also, even among deletions it was very clear that a great many mutations were possible, and the available techniques (which until recently did not include long PCR) could only assess the level of a few mutations per experiment. It was established quite quickly that particular mutations tended to be present at very low concentrations—0.1% or lower—even in postmitotic tissues of very aged individuals; but it was not possible to extend this to an estimate of the total mutation "load" in such tissues, because one never knew how many other, untested mutations were present at similar, or higher, levels or not at all.[75] It eventually became possible to gauge the overall levels of mtDNA deletions, due to the development of long PCR; but that still left open the question of the overall levels of point mutations.

In due course, however, various refinements of PCR were developed which could identify point mutations, and several were demonstrated to accumulate with age in the same tissues.[76,77] But then, just as a consensus seemed to be forming on this point, it became controversial again as a result of experiments in the laboratory of Eric Schon, which established very convincingly that, in one particular section of the mtDNA, point mutations seemed never to occur at all—or, if they did, then they were not preferentially amplified in what others were coming to think of as the usual way.[78] Schon's experiments used a PCR variant which was only applicable to a few places in the mitochondrial genome, but which, in those regions, was likely to give exceptionally accurate measurements of the real

concentrations of mutations. He therefore concluded that, most probably, point mutations never accumulate at all, and the contrary findings that had been published by other labs were artifacts of their experimental techniques. The other possibility, of course, was that there was something unusual about the particular region of the mtDNA that Schon had assayed (within the gene for ATPase subunit 6); but nothing of that kind was apparent, so this formal possibility was not—at that time—considered.

6.6.5. Inherited mtDNA-Linked Diseases; The Threshold Effect

Just as it is difficult to extrapolate confidently from observations in rapidly dividing cells in vitro to the situation in non-dividing cells in vivo, so there are pitfalls in extrapolating from the phenotypes caused by genetic abnormalities to those that exist in normal aging. It is very tempting—and by no means always fruitless—to do this when the macroscopic phenotype of the disorder resembles accelerated aging, such as in Werner's syndrome. It is equally tempting when the similarity is at a microscopic scale, and, indeed, several disorders exist in which mutant mtDNA appears to accumulate in a way similar to that in normal aging, but faster, often leading to early mortality. The similarities are great: the same tissues (non-dividing ones, particularly those with high energy demand) are most affected, and the levels of activity of mt-coded enzymes (such as cytochrome c oxidase) exhibit the same mosaic, "all-or-none" distribution with some cells (or fiber segments) having no activity while the rest have normal activity[79]

The differences are also rather striking, however, and immediately give reason for caution. One great potential value of these diseases for the study of mtDNA dysfunction is that they are caused by the presence of the same mutation in every affected cell, so that the total level of mutant mtDNA in a tissue sample can be assessed quite accurately, in contrast to the situation in normal aging discussed in the previous section. But when this is done, patients are often found to have very large amounts of mutant mtDNA—sometimes over 50% by the time their symptoms have become severe.[80,81] The shortcomings of quantitative PCR (especially of point mutations) are considerable, but histochemical assays of enzymatic activity (which of course detect point mutations just as well as deletions) had by the early 1990s clearly excluded the possibility that the total mtDNA mutation load reached such levels in normal aging. This could be (and indeed has been: see Chapter 9) seen as a challenge to the relevance of mtDNA decline in aging, since the survival—albeit in poor health—of people with lots of mtDNA damage surely meant that the low levels seen in normal aging were essentially harmless. But the alternative interpretation is that these inherited mutations are not knockouts, but in fact only reduce the OXPHOS activity of cells by a rather small degree. Most spontaneous mutations, by contrast, would be severe,* and would therefore have a phenotypic effect when present at much lower levels.

This interpretation makes sense of the curious observation that all known inherited, disease-causing mtDNA mutations in protein-coding genes are missense mutations, which change one amino acid, as opposed to deletions or nonsense mutations, which remove or truncate the encoded protein and are therefore generally more severe. (This is in contrast to sporadic, non-inherited mtDNA-linked diseases, which exhibit similar symptoms and are often associated with mtDNA deletions.)[82] These mutations (and those in tRNA genes) are known to inhibit OXPHOS severely in vitro,[81] but again one must be cautious, because this

** If most spontaneous mutations are deletions then this is certainly true, but if most are point mutations then it needs some defending, since typical amino acid substitutions in typical genes do not completely abolish their function. But it must be taken into account that all the mt-coded proteins are subunits of complex multimeric enzymes; proteins of this sort are more sensitive to amino acid changes than average.*

was measured in rapidly dividing cells, which need more energy for biogenesis than a non-dividing cell needs just to survive—and which, incidentally, do not accumulate high levels of these mutations in vivo! Presumably null mutations, if present in the oocyte, are sufficiently debilitating to cause early termination of embryogenesis or even failure of ovulation; this is discussed further in Section 10.3.2.

Nevertheless, these inherited diseases may still be useful: after all, there is no a priori reason why a mild mutation should behave qualitatively differently (in terms of selective advantage) to a null mutation, which most spontaneous ones are likely to be. Irritatingly, however—or perhaps, in the end, instructively—it seems clear that they *do* behave differently. The "all-or-none" distribution of cytochrome c oxidase activity in normal aging is shown, by in situ hybridisation,[57] to be caused directly by a similar, though not quite "all-or-none", distribution of a mtDNA mutation. But in the inherited diseases, in situ hybridisation reveals many muscle fiber segments with intermediate levels of the mutation. This goes a long way towards explaining how the overall levels in tissue can be so high—cells have a substantial surplus of mitochondrial capacity,[81] so a cell (or fiber segment) with 50% or less mutant mtDNA would show very little loss of performance—but it raises two big questions. The first is: why is the cytochrome c oxidase activity all-or-none when the DNA is not? This has become known as the threshold effect—when the level of wild-type DNA falls below a threshold level, something happens to the expression of the nuclear-coded OXPHOS machinery which abruptly eliminates all cytochrome c oxidase activity—but we have no idea of the mechanism. But the second question is: why don't these mutations get rapidly amplified all the way to nearly 100%? Again we have no idea, though the existence of the threshold effect may be a hint—if the selective advantage in normal aging is due to loss of OXPHOS function of individual mitochondria (which seems reasonable, and see Chapter 8 for a detailed hypothesis), and if nuclear factors disable OXPHOS in the residual wild-type mitochondria, then the selective advantage disappears. But whatever the reasons, the fact that they don't rise to the same levels—which, again, may have a lot to do with why they can be inherited at all (see Section 10.3.2)—means again that they are a very dubious model for mtDNA decline in normal aging.

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Chapter 7

The Status of Gerontological Theory in 1995

The central motivation of this chapter is a fact that may surprise, if not appall, non-biologists: that theoretical biology has a bad name. Theoretical gerontology, moreover, has a particularly bad name: it is considered to be a magnet for sloppy thinkers with half-baked ideas. As a theoretical gerontologist, I am therefore obliged to dissuade the reader from prejudging what I have to say; this I hope to do in the following sections.

7.1. What Is a "Theory of Aging"?

In gerontology, as in any field of science, the development of a hypothesis involves a perpetual oscillation between creative and analytical thinking. Advances of understanding are rarely achieved by purely deductive analysis of existing data; instead, scientists formulate tentative and incomplete generalisations of that data, which allow them to identify which questions are useful to ask by further observation or experiment. (In this respect, gerontology differs from most other scientific disciplines only in how little we have so far progressed along the path to that understanding.) The further any such path is travelled, the more complete and detailed are the hypotheses under active investigation.

The above is, in fact, so universally accepted as a cornerstone of the scientific method that some may wonder why I have chosen to belabour it. I have three reasons.

The first is that there is a long-standing and widespread tendency, in the study of aging, to over-sell a hypothesis. Many of the physiological or molecular changes that occur in our bodies as we age can be proposed, with reasonable plausibility, to influence the rates of other changes—and, in some cases, their own rate. But there is a huge difference between proposing that a process influences the rate of aging and proposing that it is the dominant influence. This difference has too often been glossed over: the process under consideration is presented as being the dominant determinant of the rate of aging, when the supporting arguments actually only suggest that it makes a non-zero contribution. This is all the more regrettable because hypotheses which propose a non-zero contribution by a particular process are perfectly legitimate stepping-stones to more ambitious hypotheses; the only requirement is to keep the two types of hypothesis distinct. A recent review of the free radical theory[1] took care to stress the distinction between the weak statement: "oxidants contribute significantly to the process of degenerative senescence" and the strong statement: "oxidants determine maximum lifespan potential"; the field will benefit greatly if this example is widely followed.

My second motivation is to dispel a view which has gained some popularity recently:[2,3] that aging is, broadly, already understood. The foundation of this view is that we have discovered most, if not all, of the major mechanisms by which our macromolecular organisation gradually breaks down, so that all that remains is to flesh out the finer details

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of those mechanisms. From this is inferred, among other things, that the design of higher animals is incompatible with indefinite survival, and therefore that "the goal of extending human lifespan...—at least in extreme form—is an illusion."[2] This logic is, in my view, unsound. My difficulty with it is that it assumes that the discovery of these unknown finer details will not markedly increase our ability to improve our design. That assumption is based on an over-zealous reaction to the over-selling that I mentioned above. It is illogical to assume that if a process influences the rate of aging then it is the main influence; but it is equally illogical to assume that, if many processes have some influence, then none has a dominant influence.* And if some process does indeed have a dominant influence on the rates of all the otherwise unavoidable changes that comprise aging, and discovery of its finer details were to proceed to the point where (with the medical technology of the time) it could be largely subverted, our lifespan potential would indeed be greatly increased. (It would not be rendered indefinite by that single advance, but the practical difference between the two is rather slight, as will be discussed in Chapter 17.) These are big "ifs," to be sure.

My third reason ties the others together and explains why I work in theoretical gerontology. I maintain that the framework presented here is a true theory of aging, avoiding both of the logical shortcomings described above. I do not claim that this hypothesis is proven, but I will explain in the next few chapters that it is now substantially more detailed and experimentally supported than the very incomplete hypotheses with which gerontology has hitherto struggled. This is double-edged, though: the very fact that MiFRA is now so detailed means that it is virtually certain to be partly wrong, and to be easily demonstrated to be so. I regard this as a good thing, because falsification is the basis of scientific progress; moreover, when one component of a coherent theoretical framework is falsified, the remainder of that framework serves as a valuable foundation for identifying a replacement. Though it risks sounding over-glib, I like to say that I prefer to be wrong nine times out of ten than zero times out of zero.

7.2. What Causes What?

In case Chapter 6 gave the impression that, by 1995, the case was closed (or, at least, closing) that mtDNA decline was the predominant culprit in mammalian aging, I will now return to the shortcomings of that view. In common with some of the other popular lines of thought, it was relatively unchallenged by the facts; its difficulties lay in what it did **not** say. It contained two large and serious gaps—one intracellular, one intercellular—which undermined the precept that mtDNA mutations are the main originating cause of human aging.

It is perfectly normal, of course, for a developing hypothesis to contain gaps—sometimes, large gaps. This is not necessarily a huge barrier to acceptance of the hypothesis as being "very probably on the right lines." But in aging it matters a great deal, because of the massive interdependence of degenerative processes that was discussed in Chapter 5. Virtually any of them can plausibly be proposed as the cause and/or the effect of any other. This is the main reason why so many theorists have fallen foul of the "over-selling" problem that I mentioned in Section 7.1: in order to avoid it one must find a way out of this pervasive, mutual causality. In particular, MiFRA was still vulnerable to the complaint that all the observed characteristics of mtDNA decline might also result if it were only a downstream phenomenon, driven by oxidative stress that was predominantly generated by some other means.

** This is especially so in view of the clear evidence that these various processes act synergistically to accelerate each other, as was discussed in Section 5.6.*

7.3. The Two Big Mechanistic Gaps in MiFRA'95

The first mechanistic gap was that no one had identified a plausible mechanism to account for the preferential amplification of mutant mtDNA at the expense of wild-type DNA, nor for the fact that this amplification occurred to a far greater extent in non-dividing cells than in rapidly dividing ones. We knew that this happened, but without a simple and free-standing model for how it happened one was haunted by the lingering doubt that it was not an autonomous process at all. Perhaps, instead, it was merely a subtle and relatively harmless downstream effect of oxidative stress, and the dominant source of that oxidative stress was unrelated to it.

The other gap was that, however one counted them, very few cells ever actually suffered this OXPHOS collapse at all. Estimates based on quantitative PCR varied widely, and it was tempting to dismiss them as methodologically flawed; but histochemical analysis left no real doubt that the proportion of cells affected was under 1% even at death, and in most tissues much less than that. It was therefore necessary, if mtDNA decline really were the main driving force, to identify a mechanism whereby this tiny proportion of sick cells engendered stress to mitochondrially healthy cells. On the face of it, such transmission seemed highly implausible, because the ATP synthesis capacity of the huge majority, the mitochondrially healthy cells, would only need to increase by that 1% or less in order to supply the energetic needs of the anaerobic few.

Both these gaps in the mitochondrial free radical theory of aging may now be bridged. They will be dealt with in turn in the next two chapters. But first, in order to see why—despite these problems—many workers remained convinced that mtDNA decline had a major role, we must also briefly consider the alternatives.

7.4. Some Other Theories and Their Difficulties

I finished Chapter 6 at (roughly) 1995 mainly for a personal reason: it was in that year that I became involved in theoretical gerontology. The next two chapters will resume the story, with emphasis on my contributions; thus it is appropriate to conclude this chapter with a brief review of my impressions of the subject at that time. The question that, necessarily, confronted me first was where to begin—which of the many lines of thought that gerontologists had promulgated hitherto was, by my intuition, the most promising and hence the most attractive to try to extend.

Just as some variants of the mitochondrial free radical theory had fallen by the wayside due to experimental refutation, so some of the other mechanisms that had previously been proposed to underlie aging became increasingly unsustainable. One prominent example was Orgel's error catastrophe theory.[4] This theory was based on the knowledge that there was a non-zero error rate in both transcription and translation, so that some proportion of protein in all cells will have the wrong amino acid sequence even though the gene coding for it is correct. Orgel realised that, while a small proportion of incorrect protein would do no harm in most contexts, there was one potential exception—the proteins that actually perform transcription and translation. An erroneous protein involved in either of those processes might very well increase the chance that more errors would be introduced into subsequently constructed proteins. In particular, it could cause errors in the construction of new copies of itself. Above a certain threshold level, this would become a vicious cycle—a catastrophe—whereby eventually the cell would cease to be able to construct any correct protein and would thus die. This theory received widespread acclaim at first, on account of its undeniable elegance and simplicity. Now, however, essentially no one believes it, simply because it has become possible to determine whether there is in fact a significant buildup of incorrect proteins (that is, ones with incorrect amino acid sequence rather than with oxidative damage) with age, and there is not.[5]

Some other prominent theories of aging have come under circumstantial challenge because of our ability, by dietary supplementation or depletion, to influence the rate of accumulation of the supposed causative agent, and the finding that this is not accompanied by changes in the rate of aging in general. For example, vitamin E deficiency markedly accelerates the accumulation of lipofuscin in lysosomes,[6] but does not accelerate aging; conversely some chemicals are known (particularly centrophenoxine, also known as meclophenoxate) which seem to dissolve lipofuscin[7,8] but do not seem to retard aging (except perhaps by inducing voluntary calorie restriction[9a]—see Section 10.4). This is not a proof that lipofuscin is unimportant in aging, because the dietary modulation may not be affecting the "important" lipofuscin in vivo, but it does constitute circumstantial evidence. (However, both the vitamin E and the centrophenoxine findings have been contradicted in more recent studies.)[9b-9d] Similar interventions are being developed (in rodents) to retard and reverse glycation of long-lived proteins;[10] likewise, as yet there are no reports of lifespan effects. (On the other hand, the deleterious effects of these changes will surely increase to life-threatening levels if given significantly more time. Thus, if we were to identify and thoroughly subvert the major driving force(s) in aging, it would become correspondingly more necessary also to subvert many currently peripheral phenomena. Such work is therefore an unarguably vital contribution to life extension research.)

The theory that was perhaps the most similar to MiFRA in terms of its compatibility with known facts and the size of the gaps that remained in it was the telomere theory. This is based on the long-established finding[11] that vertebrate cells, with the exception of cancer cells, cannot be grown indefinitely in culture: their rate of division gradually slows, until eventually they stop dividing altogether, a phenomenon termed replicative senescence. The telomere theory of aging is founded on a proposed mechanism for replicative senescence: it suggests that the rate of aging is predominantly determined by the progressive loss of DNA from the ends of chromosomes of dividing cells, a process that was first postulated by Olovnikov in 1971[12,13] and independently by Watson.[14] This is proposed not as a challenge to the evidently central role of oxidative stress, but rather as a process which—by an as yet undetermined mechanism—brings oxidative stress about. The fact that oxidative stress appears to affect non-dividing cells more than dividing ones is proposed to result from the dependence of these non-dividing cells on material (maybe proteinaceous, maybe hormonal) secreted by the dividing ones, and in particular from the inability of the non-dividing cells to cope with changes in the level and/or nature of these secretions which result from the shortening of the dividing cells' telomeres.

The telomere theory also ranked well by the theoretical and experimental criteria discussed earlier. On the theoretical side, telomere shortening can readily be fitted into a positive feedback loop, which is necessary in order to explain why aging accelerates as it progresses (see Section 5.6.3). In this case, then, we seek a mechanism whereby telomere shortening might be accelerated by oxidative stress. A possible mechanism is that DNA damage from oxidative stress is not repaired when it occurs in telomeric DNA, but instead is allowed to develop into a full-blown chromosome break, following which the segment of DNA beyond the break is lost. Turning to the experimental criteria: accelerated telomere shortening in oxidative stress was confirmed experimentally.[15,16] It was in due course also confirmed (but see Section 10.15) that human telomeres do shorten with age;[17] it was moreover found—at least in cell culture—that cells with very short telomeres did indeed undergo changes of gene expression,[18] and finally in 1998 that maintenance of telomerase expression allowed normal (that is, non-cancerous) cells in culture to avoid replicative senescence.[19-21] The main gap in this hypothesis, as of 1995, was the failure to identify any relevant secreted substance whose changing levels might harm non-dividing cells (or to identify the relevance of secreted substances that **have** been identified, such as collagenase

or β–galactosidase). That gap remains to this day—though it may, of course, be bridged tomorrow.

So, which theory was the most believable? I do not by any means mean to insinuate that the gaps in all these other proposed mechanisms were "clearly" more serious than those in the mitochondrial free radical mechanism. In my view, it was completely impossible to make a purely logical scientific case one way or another. One simply had to rely on intuition. My intuition at that time was that the gaps in the mitochondrial free radical theory seemed more bridgeable than those in any other theory, so that was where I devoted my energies.

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Chapter 8

The Search for How Mutant mtDNA Is Amplified

This gap in the mitochondrial free radical theory was a particularly inviting challenge to a newcomer in the field. Superficially, it seemed that all the information was available to lead rapidly to a detailed mechanism for how mutant mitochondria out-competed wild-type ones; but in fact, only a few possibilities had been proposed, all of which had serious difficulties.

8.1. Does Deleted—Shorter—mtDNA Replicate Faster?

An early suggestion[1] was that the mitochondria which were amplified were specifically those that had suffered a large deletion in their DNA. The idea was that, having less DNA to replicate, they could get it done more quickly and so out-replicate the normal mitochondria. Such mutations had been found,[2] and it was clear that they take over cells. Unfortunately, we now know that many point mutations are also amplified,[3] so this cannot be the whole story. Moreover, it has since been established[4] that some mtDNA duplications are amplified: thus, any selection there may be for small molecules must be easily overridden by some other factor. Also, the tiny size of the mtDNA molecule means that, on average, it need be replicated only at a rate of under ten base pairs per hour in order to keep up with the observed rate of mitochondrial turnover. This is so hugely slower than the typical DNA replication rate of about 2000 bases per minute[5] that it is hard to imagine mtDNA replication being rate-limiting. In fact, replication of the whole molecule takes 1-2 hours.[10]

A variant on this model[6] avoids this problem, but has one of its own. It was proposed that certain parts of the mtDNA molecule act as inhibitors of mtDNA replication, only allowing replication to proceed when more mitochondria are needed. These sequences were hypothesised to be deleted or otherwise inactivated in mutant mtDNA, so that such molecules engaged in runaway replication. This proposal faces the major difficulty that no such **cis**-regulatory sequence has been identified, and that its existence becomes progressively less plausible with every new mutation that is found to accumulate in vivo.

8.2. Do Perinuclear Mitochondria Trigger Their Own Replication?

A highly ingenious idea[7] was inspired by the discovery[8] that the cell does not replicate all its mitochondria at once. Nor is replication a constant process, however: the truth seems to be somewhere between. Mitochondria are replicated in pulses: a few percent of them are replicated simultaneously, then none for a while. This allows the possibility that the signal which tells the cell to replicate some mitochondria is somehow biased towards the replication of mutant ones. We do not know what this signal is, but a perfectly plausible candidate is a simple shortage of ATP in the nucleus. This may preferentially occur when there are (randomly) a lot of mutant mitochondria around the nucleus. Now, if the replication signal goes out from the nucleus and only gets to a few percent of the mitochondria in the cell, then the beneficiaries are most likely to be exactly the ones near the nucleus, namely the

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mutant ones that sparked the replication pulse in the first place. A remarkably neat idea; and moreover, it was recently shown by direct visualisation[9-11] that, at least in vitro, mtDNA replication indeed occurs mainly around the nucleus. Unfortunately, though, this proposal is fraught with statistical difficulties. Firstly, such amplification could not begin until quite a lot of mutant mitochondria had arisen without selection, all of which would be equally amplified thereafter; this seems incompatible with the apparent takeover of cells by copies of a single mutation. Secondly, once there are very many mutant mitochondria, their random movements will tend more and more exactly to cancel each other out, so that variations in the energy supply near the nucleus would become negligible, as therefore would the replicative advantage of mutant mitochondria. Finally, once there are debilitatingly few respiring mitochondria, the cell would be replicating mitochondria at full speed all the time, irrespective of mitochondrial distribution (because there would be a perpetual ATP shortage in the nucleus), so mutants would have no advantage at all.

8.3. A Clue From Yeast

I mentioned in Section 6.3 that suppressiveness, the dominant deficiency of respiration discovered by Ephrussi in the 1950s,[12] was much later to become a clue to MiFRA. It will already be clear that there are similarities between suppressiveness in yeast and amplification of mutant mtDNA in humans. But the big mechanistic clue from suppressiveness turned up in the 1980s,[13] and was only identified as such in 1996. It was a study of the rate of mtDNA replication in the context of suppressiveness. The experimenters found that, for most suppressive strains, the heteroplasmic diploid cells made by fusion of a suppressive cell with a wild-type one did not replicate their mutant mtDNA any more often than the normal mtDNA.

This was, with hindsight, a sensational result. If, as one would naturally predict, this characteristic of suppressiveness was also true of human cells that amplified mutant mtDNA, then the search for a mechanism in human cells was starting from a false assumption: that preferential amplification resulted from preferential replication.

The same paper[13] also reported an observation which supported the hypothesis that suppressiveness and human mtDNA decline were driven by similar mechanisms. This was that starvation of the culture in which heteroplasmic cells were formed, causing a temporary suspension of cell division, markedly increased the levels of suppressiveness which the culture then exhibited, independent of genetic factors. This bore a striking similarity to the finding that non-dividing human cells are much more susceptible to mtDNA decline than dividing ones[14] (see Section 2.4.6). Moreover, suppressiveness had also been found[15,16] in *Neurospora* and *Podospora*, filamentous fungi which, like a muscle fiber, are syncytial. A subsequent observation[17] fitted the same scheme: that even amoeba exhibit suppressiveness if they are kept with the cell cycle arrested for a time.

8.4. Proton Leak: Boon or Bane?

Oxidative phosphorylation relies, as explained in Section 2.3.4, on the inner mitochondrial membrane's being impermeable to protons. But of course this is not an all-or-nothing condition: if the membrane is just slightly leaky, so that the occasional proton gets through it, the only harm done is that the respiratory chain must work a little harder in order to make the required amount of ATP. Membranes are, after all, only a few nanometers thick—and indeed they have to be that thin, since (among other reasons) otherwise they would be dysfunctionally rigid—so it is reasonable to expect a trade-off between impermeability and other membrane properties.

And in fact, mitochondrial membranes do exhibit an easily measurable amount of this "proton leak."[18] Research has been done on it for many years, but its biological role is still highly controversial. It has been popular[19] to seek reasons why proton leak is valuable to the

organism, on the basis that if it were disadvantageous then it would have been evolved away. This has led to the proposal that it increases the potential for regulation of metabolism: organisms may benefit from being able to vary rather rapidly the rate of OXPHOS within a cell, and this is achieved by the presence of "futile cycles" (of which proton leak would be one) which act as extra loci of control on the steady-state rate. This is sound in principle as an explanation for having **some** leak, but it does not appear to explain why some animals have far more leak than others. Another proposal,[20] which I believe to be logically flawed, is that proton leak exists as a safety valve to dissipate the mitochondrial proton gradient at times when the cell is at rest, i.e., using little ATP; the idea is that a high gradient causes greater production of superoxide, which is undesirable. The flaw in this is that the gradient can be controlled in other ways that do not involve the wastage of nutrients which leak does: these include the permeability transition pore, which is available as an emergency safety valve, and control of the rate of the precursors to the respiratory chain (the TCA cycle, etc.) which would have a longer lag but are less drastic. This challenge applies equally to a third proposal: that leak exists to maintain the cellular NAD^+/NADH ratio at a value compatible with various biosynthetic reactions such as amino acid synthesis. Cells have a variety of systems to use for this purpose, including the reversible conversion of pyruvate to lactate, which likewise do not waste nutrients in the way leak does.

This compels one to revisit the precept upon which the search for such uses of leak was founded: that if it were genuinely a bad thing it would have disappeared during evolution. The alternative viewpoint is that it really **is** a bad thing, but that it is a side-effect of some vital process, and evolution has not found a way to maintain that vital process without having leak too. A candidate for the vital process in question is readily to hand: OXPHOS itself. It is generally accepted that one side-effect of OXPHOS—superoxide production—is a bad thing, so evolution already has an imperfect track record in this regard.

But how could leak be a side-effect of OXPHOS, let alone an inevitable one? Remarkably, a very direct answer to this question has been available in the literature for over 25 years. LEC-mediated damage affects all macromolecules: in particular, it affects lipids, the molecules that make up the mitochondrial membranes. The only missing link, therefore, is whether membranes that have suffered LEC-mediated damage are leakier to protons. If they are, then we have an explanation for proton leak as a deleterious, but inevitable, side-effect of mitochondrial function. And indeed, lipid peroxidation does make membranes leakier.[21,22]

A couple of very recent results confirm this in different ways. If one isolates the lipids from a preparation of mitochondria and reconstructs them as vesicles—usually termed liposomes—then one can measure their proton leak and compare it to that of intact mitochondria. The remarkable finding is that liposomes prepared in this way exhibit only about 5% of the leak of intact mitochondria.[23] It is hard to see how this can result from the absence of the non-lipid components of mitochondrial membranes (transmembrane proteins and coenzyme Q), since they are also highly hydrophobic so should repel protons; but if most leak is caused by peroxidation then a simple explanation emerges. Peroxidation proceeds as a chain reaction (see Section 3.7), so the products of peroxidation in an intact mitochondrion will be concentrated around the occasional point at which a reaction was initiated. These locally very high levels of membrane damage will constitute "pin-pricks" in the membrane, through which protons will flow rapidly. Synthesis of liposomes, on the other hand, will homogenise the lipid, spreading the peroxidation products evenly through it; thus they will have a uniformly low concentration which will only negligibly ease the passage of protons.

The second recent study showing the effect of peroxidation was an X-ray diffraction analysis, which allowed the detection of peroxidation-induced changes in membrane

structure at submolecular resolution.[24] The membrane becomes substantially thinner as its internal organisation breaks down and the fatty acids from the two layers become interdigitated. It is no surprise that this increases proton leak.

8.5. Less Degradation, Not More Replication

The story outlined in this chapter and the two preceding it summarizes the state of the mitochondrial free radical hypothesis of aging in late February,1996, when I made my initial contribution to it. My idea was published a year later.[25] It claims to provide the detailed mechanism for amplification of mutant mtDNA that had eluded the field hitherto.

In seeking a mechanism whereby mutant mtDNA is amplified, I tried also to address two enigmatic features of mitochondrial turnover that were noted in previous chapters. Firstly: Why is the amplification so much more severe in postmitotic cells than in dividing cells? And secondly: why does mitochondrial turnover in postmitotic cells happen at all—what drives it?

The breakthrough came when I saw that the field had been unwittingly making an unfounded assumption about the amplification process. Just as Mitchell's colleagues had been held back by their commitment to a chemical nature of the intermediate between the respiratory chain and the ATPase (see Section 2.3.4), so in this case people were presuming that preferential amplification of mutant mtDNA must occur by preferential replication. In fact, in postmitotic cells at least, there was another option—"anti-preferential" degradation. That is, the replication machinery may be completely unbiased with regard to whether it acts on mutant or normal mitochondria, but the lysosomal degradation machinery, when given the choice, may select normal ones in preference to mutant ones. This would have exactly the same ultimate effect. Moreover, it matched the observations of Chambers and Gingold in yeast (see Section 8.3).

Of course this initial idea is merely a paradigm: it doesn't constitute a mechanism. In principle, it might have been just as hard to come up with a detailed mechanism based on biased degradation as it had been to find one based on biased replication. As it turned out, however, this was not the case: a detailed mechanism was rapidly apparent. It provides an explanation of all three of the observations under consideration: the existence of turnover, the amplification of mutant mtDNA in non-dividing cells, and its non-amplification in dividing cells. And as a bonus, it made a prediction which was in line with Schon's report from the same year[26a] (see Section 6.6.4): that a particular class of lesion, mutations affecting only the ATPase subunits, would not be amplified, even in non-dividing cells. Detailed discussions of this hypothesis have since appeared.[26b-26e]

8.5.1. The Mechanism Driving Turnover

The concept begins from the realization that mitochondrial turnover in non-dividing cells is unavoidable, because mitochondria do themselves harm by LEC production. Most importantly, the peroxidation and polymerisation of the lipid molecules of the inner mitochondrial membrane by LECs is substantially not repaired. A respiring mitochondrion will therefore accumulate such damage to its inner membrane. In due course, if nothing is done, the membrane will become unable to perform its main function, which is the maintenance of the proton gradient created by the respiratory chain. If and when this becomes severe enough, the mitochondrion will engage in runaway (and futile) consumption of oxygen and nutrients, since the proton gradient is the brake on the respiratory chain. This wastage of oxygen and nutrients is unarguably bad for the cell and the organism, so the offending mitochondrion must be destroyed without delay. The cell has a system for this: lysosomal autophagocytosis and degradation of the mitochondrion, a phenomenon which has been

directly visualised under the electron microscope.[27,28] Thus, it seemed clear that the lifetime of a mitochondrion must necessarily be finite.

This was all purely conceptual though (apart from the existence of autophagocytosis): there was no direct evidence that self-inflicted damage was the trigger for turnover. On the other hand, it was plausible: lysosomal detection of a damaged mitochondrion could realistically be mediated by way of a proteinaceous signal that is triggered by either the oxygen depletion or the rise in temperature around the affected mitochondrion.

But this logic forces us to consider how the cell retains any mitochondria at all in the long term. Somehow it must avert the above process by maintaining the degree of contamination of its mitochondrial membranes at a stable level. Superficially, this might look like a cast-iron refutation of the role of self-inflicted damage, but there is a way out: stability of damage can be achieved by mitochondrial replication. This works because the new membrane lipid and protein that is added to the parent mitochondrion, in order to bring it to a size ready to divide, has not been exposed to LECs so is pristine. Replication of a mitochondrion thus acts to dilute its existing membrane damage, by roughly a factor of two.

Accordingly, my first proposal was that mitochondrial turnover in non-dividing cells is driven by this membrane damage (see Fig. 8.1). Mitochondria accumulate damage until they become poisonous, and are then digested. This randomly happens to some mitochondria sooner than others, so the number of mitochondria in the cell steadily falls. Mitochondrial replication occurs when the cell detects a shortage of ATP, caused directly by the diminished numbers of mitochondria. Of necessity, the mitochondria that are replicated are those that have not already been digested. The cell would probably not tolerate a loss of half its mitochondria before initiating replication, so the pulse of replication* would be "sub-saturating"—as proposed in the model described in Section 8.2, it would target only the mitochondria near the nucleus.

8.5.2. Survival of the Slowest, or SOS; Amplification of Mutant mtDNA

This situation is stable while all mitochondria are genetically functional. At some point, however, a mtDNA mutation may occur that lowers the respiratory capability of its host mitochondrion. That mitochondrion's lower level of respiration results (eventually—see Section 6.6.2) in a smaller proton gradient across its inner membrane. That, in turn, will translate into a lower concentration of harmful LECs in its immediate environment.** This will result in a slower accumulation of damage to its inner membrane than is occurring in properly respiring ones. Such a mitochondrion will, therefore, preferentially still be intact when many of the cell's non-mutant mitochondria have succumbed to the degradation process hypothesised above. Thus it will be preferentially replicated (see Fig. 8.2). Repetition of this cycle will rapidly divest the cell of all its properly respiring mitochondria. I have termed this process "survival of the slowest," or SOS.

8.5.3. Why SOS Doesn't Happen in Dividing Cells

Dividing cells must replicate their mitochondria at least once per cell division, so as to maintain the number of mitochondria per cell. This replication is proposed to be driven by exactly the same mechanism as in non-dividing cells, namely shortage of ATP. But that

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** In principle it actually need not be a pulse: if the regulation of replication rate is precise enough, it could tick over at a broadly constant rate.*

*** The whole of Chapter 11 is devoted to a defence of this sentence, which rests on a challenge to some central principles of textbook bioenergetics.[29]*

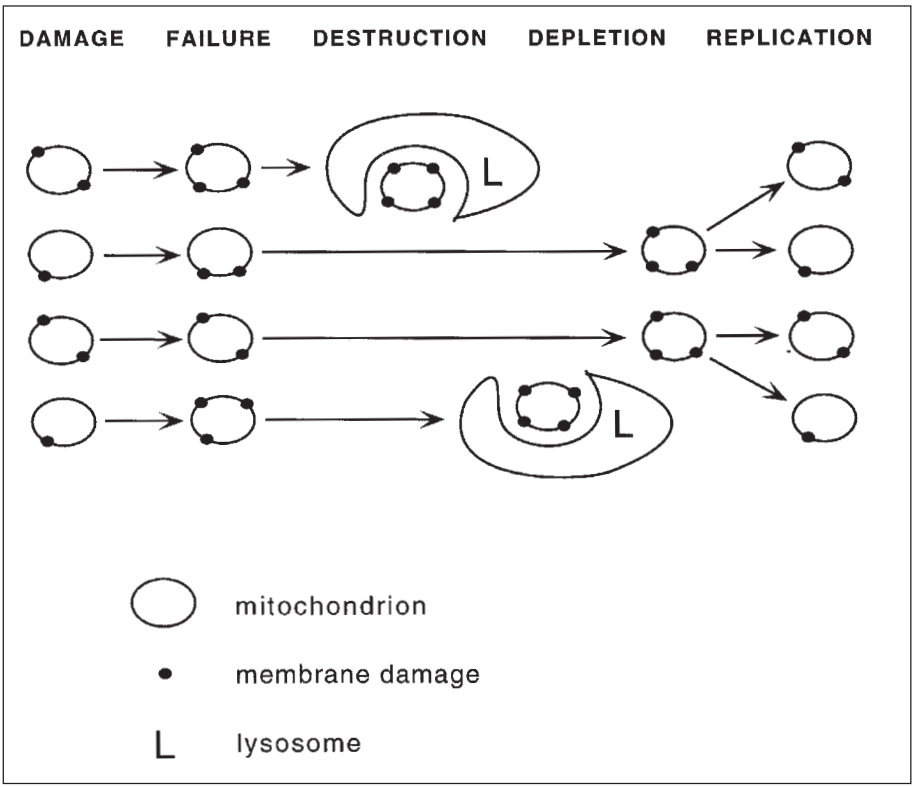

Fig. 8.1. Membrane damage drives turnover.

means that, if cellular division is rapid enough, the SOS cycle will be short-circuited (see Fig. 8.3). There will be no significant digestion of mitochondria, because membrane damage is diluted as fast as it is inflicted. Mutant mtDNA will therefore not accumulate by this mechanism in tissues composed of such cell types. Only in non-dividing or rarely-dividing cell types will there be an accumulation of respiration-deficient mitochondria.

8.5.4. Why SOS Doesn't Happen with ATPase Point Mutations

Mutations that will be amplified according to SOS are ones that reduce the proton gradient. All mutations that abolish the function of respiratory chain will do this, whether they affect individual proteins or whether they mutate tRNAs and thus eliminate all 13 proteins. But mutations that affect only the ATPase subunits will have the opposite effect: they will have no effect on the creation of the gradient, only on its dissipation, so in fact the gradient would be predicted to rise slightly. Such mutations would thus be destroyed by SOS, rather than amplified.

8.6. Division as Autonomous Repair?

The phenomenon of self-inflicted proton leak gave rise in 1990 to one more possible mechanism for preferential replication of mutant mtDNA, which is a great deal more consistent with the observed data than any of the models described earlier (Sections 8.1 and

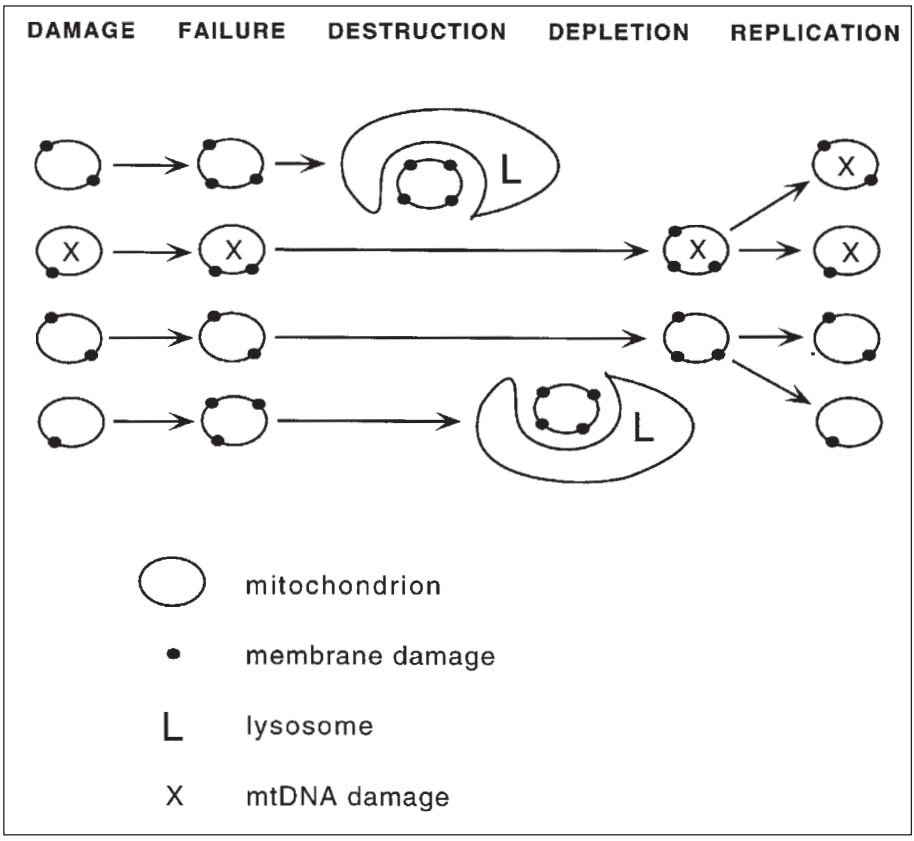

Fig. 8.2. Mutant mtDNA preferentially evades destruction.

8.2). Furthermore, it shares with SOS the additional advantage of providing an explanation for why mitochondrial turnover happens at all. I present it here, rather than with the other preferential replication-based models (see Sections 8.1 and 8.2), partly because I was unaware of it at the time I formulated SOS: it was proposed[6] only negatively, as a circumstantially unlikely alternative to the cis-regulation model. It must still be considered a candidate for the actual mechanism of clonal amplification of mutant mtDNA: there is some experimental evidence against it, but not enough (in my view) to rule it out quite yet.

The essence of this idea is that the individual mitochondrion, not the cell, is the entity that controls the timing of its replication. There is proposed to be a pool of raw materials for mitochondrial replication available in the cytosol, which a mitochondrion imports when triggered to do so by some aspect of the intramitochondrial environment—most simply, shortage of matrix ATP. Shortage of ATP can be caused by self-inflicted proton leak, so mitochondrial replication in non-dividing cells will result. The role of lysosomal degradation is then at the tail of the causal chain, as the random destruction of mitochondria (irrespective of their integrity) when the cell simply has too many. In rapidly-dividing tissue, a mechanism for homeostasis is also available: cell division requires cell growth, which dilutes cytosolic ATP; intramitochondrial ATP is thus exported more rapidly, so intramitochondrial ATP

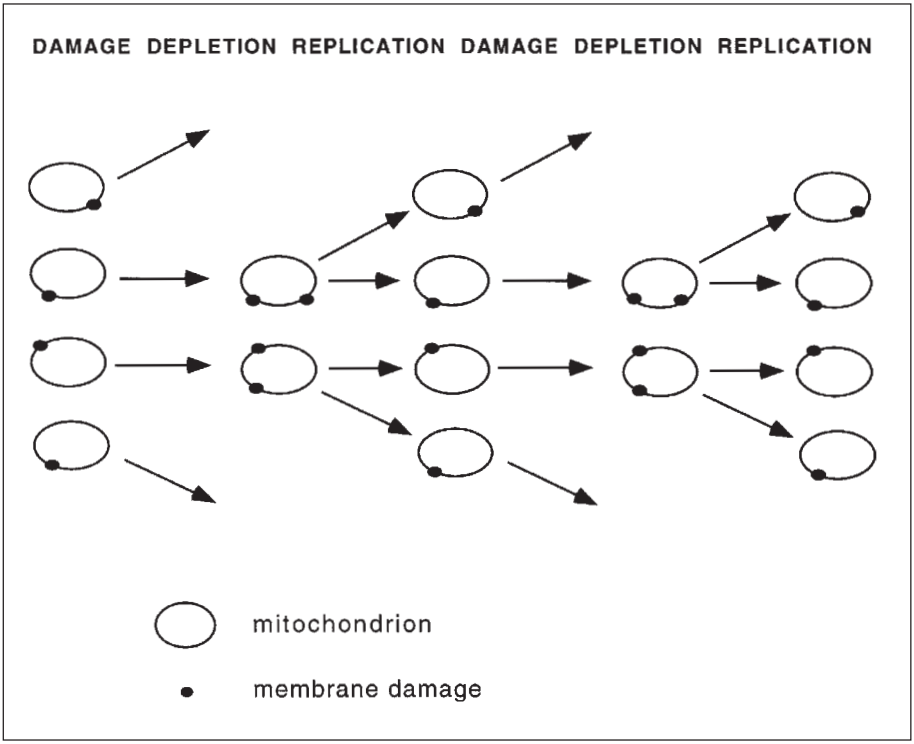

Fig. 8.3. Cell division short-circuits the mechanism by pre-empting degradation.

concentration falls; this again triggers mitochondrial replication. Finally, we can consider the case of the mutant mitochondrion: it can make some ATP and import more as needed, but will not have the same ATP concentration as a wild-type mitochondrion so engages in runaway (or at least accelerated) replication.

This extremely neat model suffers, as noted above, only from rather inconclusive inconsistency with the evidence. The results of Chambers and Gingold[13] (see Section 8.3) are a direct refutation of preferential replication, but they have not been repeated in mammalian cells. Point mutations in ATPase subunits should be amplified by this mechanism just like other lesions, since the consequently greater proton gradient will cause faster membrane damage so trigger more frequent replication; but the evidence that such mutations do not accumulate is still restricted to only one report.[26] A third possible challenge derives from the segmental distribution of mutant mitochondria in muscle—the removal by lysosomes of supernumerary mitochondria in muscle fibers must be very assiduous, in order to prevent rapid propagation of a mutation along a fiber—but since we have no knowledge of this mechanism we cannot say that it is not so assiduous. A fourth is the report[8] that mitochondria undergo replication independent of the time since their previous division: this is incompatible with damage-driven replication because damage would be continuous, so mitochondrial generation time should be fairly uniform. This too is only weak evidence, though, because the experiment in question used dividing cells in culture, whereas a role for damage in mitochondrial replication is proposed only for non-dividing cells. Perhaps the best test would be to establish whether the replication of mitochondria in non-dividing

cells is perinuclear, as SOS predicts, or occurs throughout the cell, as this model predicts; there is evidence that replication is perinuclear in vitro[9-11] but there is as yet no conclusive in vivo information on this point.

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Chapter 9

The Search for How So Few Anaerobic Cells Cause So Much Oxidative Stress

The apparently low level of mutant mtDNA even in very elderly individuals was perhaps the most powerful argument, in 1995, against the idea that mtDNA decline is central to aging. This was stressed in a number of articles at the time; a representative study from 1992[1] drew particular attention to the fact that mitochondria undergo a functional decline far in excess of the level of mutation, and inferred that mtDNA damage could therefore not be the cause of this decline. Looking at it another way: we knew[2] that cells can survive quite well with only a fairly small proportion of functioning mitochondria. Therefore, the natural inference was that a similar level of overcapacity would exist on a larger scale: that a tissue would be able to function perfectly well even if a significant proportion of its cells had undergone OXPHOS collapse. The overcapacity would not be expected to be so great as at the intracellular level, because ATP does not diffuse freely between cells as it does within cells; thus any mechanism whereby aerobic cells supported their anaerobic neighbours would presumably have to involve some kind of active exchange of metabolites. But it certainly seemed unreasonable to suppose that the body should encounter much difficulty if, say, only 1% of its cells are anaerobic, unless there is some other source of oxidative stress. Thus, the question of just how few cells were anaerobic in elderly people seemed to be an acid test of MiFRA—as it stood then.

9.1. How Few?

There has been a great deal of dispute with regard to what proportion of the cells in a given tissue actually suffer OXPHOS collapse by the time we die. By and large, the disagreement has been about the absolute, rather than relative, levels of mutant mtDNA: that is, there is broad consensus about which tissues accumulate more and which less. Moreover, so long as one only considers postmitotic cell types, there is a reasonably good correlation between the levels of mutant mtDNA and the energetic load of the tissue: the tissues that make a lot of LECs, like the substantia nigra of the brain and the extraocular muscles in the eye, are more heavily affected than the cerebellum or the average skeletal muscle.[3,4]

The first obfuscating factor in assessing the absolute levels of a given mutation is technological. Variants of PCR have been developed which identify point mutations (see Section 6.6.4), but most such methods are of controversial sensitivity. It is therefore still extremely difficult to measure, with the degree of accuracy that we would like, the relative levels of two DNA sequences that differ in only one base pair when they are present in a ratio exceeding 1:100. Unfortunately, this is exactly what is required in order to test for the accumulation of mutant mtDNA with aging, because there are so many possible mutations that (unless a few were far more common than the rest) each one would necessarily occur only in a small minority of cells. This would be less of a problem if it were proven that point

The Mitochondrial Free Radical Theory of Aging, by Aubrey D.N.J. de Grey.

mutations are much rarer than deletions, because quantitation of deletions is much more accurate with existing technology (though still by no means ideal);[5] but there is no evidence that this is so, other than the study of a region of ATPase 6[6] which SOS anyway indicates that we should discount (see Section 8.5.4).

The second is methodological. There are potentially about 50,000 possible point mutations in the mitochondrial genome, since each of the 16,569 base pairs can mutate to any of three others. PCR, unfortunately, can only search for particular ones,* rather than all at the same time. It would be something of a shame, therefore, if one designed an experiment to search for a mutation which was of no consequence to the function of the "mutated" gene—was phenotypically silent, in other words. In order to avoid this possibility, most workers have chosen to look for mutations that are known to cause severe diseases (see Section 6.6.4). Quite a few such diseases are known; they are not very hard to identify, because they exhibit maternal inheritance (see Section 2.4.2). This certainly protects against the possibility that the mutation being sought is silent. Unfortunately, however, it also almost certainly eliminates the possibility that it is null—a complete loss of function of the gene in question. This is because any mutation which has a really severe effect cannot be identified as a (maternally) inherited defect, because it won't be inherited at all—any embryo unlucky enough to be carrying it will die long before birth, as will be discussed further in Section 10.3.2. Thus, experimenters who focus on disease-causing sequence variants are probably limiting themselves to extremely mild mutants, which may therefore not tell the whole story. See Section 6.6.5 for evidence that they do indeed behave qualitatively differently to null mutations.

In view of this, it would be attractive if we could identify the level of mutations by some other means. Luckily there is one. The discovery that entire cells are taken over by copies of a single mutation allows one to examine them not only for their mtDNA composition, but also for their enzymatic activity. A number of laboratories have analyzed non-dividing or rarely-dividing tissues—usually muscle—by assaying for either the presence (by antibody staining) or the activity (by histochemistry) of certain proteins encoded by the mtDNA at the level of the individual cell.[7,8a] This gives a value towards, but not beyond, the high end of the range that was estimated by sequence analysis: around 0.1% to 1% at most.**

Is this as complete a show-stopper for the mitochondrial free radical theory of aging as it seems? The fact that this book was written answers the question, of course. In mid-1997 I formulated a hypothesis that appears able to reconcile the low levels of anaerobic cells with the idea that those cells are the main promoters of oxidative stress; it was published in February 1998.[9] I began, as with my work leading to SOS, with consideration of a related paradox, in the hope that the two problems would provide clues to each other's solution.

9.2. The Creation of mtDNA-Less Human Cell Lines

Given our utter reliance, as organisms, on a steady supply of oxygen, it might be guessed that each individual cell is similarly reliant on it. That is, that cells which have—for whatever

** Some variants of PCR, such as that used by Schon and coworkers,[6] can identify a dozen or so potential point mutations, but there is no way to identify more than that in one experiment.*

*** Recently there has been a spate of reports of extremely high levels of mtDNA deletions in the elderly,[8b-8d] resulting in much controversy.[8e] The main criticism that has been levelled against these studies is that they used long PCR, whose quantitative accuracy is still in grave doubt. But the argument given here — that clonal amplification of mtDNA makes serious intracellular heteroplasmy for deletions impossible, since it would not be stable — is a much more cast-iron argument that these recent findings[8b-8d] must be methodologically flawed.*

reason—lost the ability to make use of oxygen would rapidly die. Yet, as noted in Sections 6.4 and 6.6, it seems that such cells in fact accumulate. How? Well, the alternative possibility is that they do not really survive—that they struggle on for a short while and then die. But if that is indeed what happens, we're back to a turnover problem (see Section 5.6): the only reason why we see more of them in older individuals must be because they are occurring more often and/or being killed off more sluggishly, and this itself would need an explanation in terms of a prior cause of oxidative stress.

But the alternative of indefinite survival of these cells has its own problem: it seems, on the face of it, necessarily to require them to exchange some metabolite with still-aerobic cells, via the extracellular medium. But then, we would expect to find this metabolite in plasma, at levels that increase with age. No substance has been found which fits this criterion. For example, the most obvious candidate—a simple exchange of lactate, allowing the anaerobic cell to maintain glycolysis without any ancillary process—appears to be ruled out because plasma lactate was not observed to rise with age.[10]

However, a breakthrough was made a decade ago which showed that this option—that cells with no OXPHOS functionality nevertheless survive indefinitely—was still a possibility after all.

I mentioned in Section 6.3 that various fungi have, since the 1950s, been observed to undergo spontaneous "suppressive petite" mtDNA mutations which take over their cells. The cells that suffer this phenomenon are totally incapable of aerobic respiration, relying purely on glycolysis, and they grow much more slowly than other cells. But they do grow. This is because the fungi in question are facultative aerobes, which can survive without oxygen (that is, without OXPHOS) if they have to. Human cells in culture, by contrast, are obligate aerobes and die rapidly when divested of OXPHOS function, even in the presence of unlimited glucose.

In 1989, however, King and Attardi succeeded in creating a viable human tissue culture cell line which had no mitochondrial DNA.[11] Such cultures are termed "ρ^0" lines. This was a major advance, because until then the only cells that were able to grow without fully functioning mtDNA were ones (like yeast) which can grow without oxygen anyway. It was fairly easy to remove the mtDNA—low concentrations of ethidium bromide, a chemical that inhibits mtDNA replication but not nuclear DNA replication,[12] achieved this without killing the cells—but in order to keep the cells alive and dividing indefinitely they had to find the right nutrients, and, indeed, glucose on its own was not adequate. Two further nutrients turned out to be absolutely vital. One was uridine, which is an intermediate in nucleotide synthesis and was needed because one enzyme involved in its synthesis uses the respiratory chain.[13] The other was the one of most relevance here, though: it was pyruvate.

Why on earth pyruvate? The cells were being given plenty of glucose, and were clearly breaking it down to make ATP; pyruvate is the product of this breakdown, so obviously it was in plentiful supply already. Yet, with glucose but no pyruvate, the cells rapidly died.

The answer lies in the cofactors. When glucose is broken down to make two molecules of pyruvate, simultaneously—and unavoidably—two molecules of NAD^+ are turned into NADH (see Fig. 2.3). There is no escaping this: pyruvate has two fewer than half the electrons in glucose, so there are four electrons to lose. However, pyruvate can easily be turned into lactate; splendidly, lactate has two electrons more than pyruvate, so the NADH can be restored to NAD^+. The net conversion of NAD^+ to NADH that results from the conversion of glucose to lactate is thus zero. This is convenient in the short term, since it means that cells can briefly engage (during strenuous activity, for example) in faster energy expenditure than is allowed by the oxygen supply: they can burn glucose and make lactate at a rate not limited by the supply of oxygen. Lactate is toxic though, so after a short time we have to

cease activity and allow the oxygen supply to catch up, so that the lactate can be turned back into pyruvate and its mitochondrially-mediated derivatives.

But the toxicity of lactate is not the reason OXPHOS-less cells in culture die: it is not present in nearly high enough quantities to be toxic, since after excretion by the cells it is diluted out in the culture medium. The problem is the NADH. It turns out that a net zero conversion of NAD^+ to NADH is not good enough for the cell: it relies on OXPHOS for a significant conversion the other way. This is because certain vital cellular processes, such as the synthesis of serine (and consequently various other amino acids), turn NAD^+ into NADH.[14] Other equally vital processes do the opposite (usually with NADP rather than NAD), so it is actually a rather close-run thing whether the cell can balance the books; facultative aerobes, such as the fungi that exhibit suppressiveness (see Section 6.3), can balance them when pushed, so they can survive (albeit with severely retarded growth) with the net zero recycling of NADH that glycolysis provides.

Now it should be clear why pyruvate worked (see Fig. 9.1). The pyruvate created from glucose was being turned into lactate and excreted; but now, the exogenous, imported pyruvate could **also** be turned into lactate and excreted. This supplied the cell with the net conversion of NADH to NAD^+ that it needed in order to go about the rest of its business.

9.3. The Plasma Membrane Oxidoreductase

This was the state of knowledge until 1993. In that year, experiments in the laboratory of Alfons Lawen discovered a variety of other chemicals which could substitute for pyruvate: that is, they could keep ρ^0 cells alive without pyruvate, so long as the medium also contained glucose and uridine.[15] They were eager acceptors of electrons, so they were presumably working by taking electrons from intracellular NADH, thereby recycling it. But the really curious thing about these chemicals—ferricyanide, for example—was that they were **impermeant to the cell membrane.** Since the NADH is inside the cell at all times (being also impermeant), there must be some intermediate system in the membrane itself, which transfers electrons across it.

It was no accident that Lawen investigated such chemicals. Many years previously, a system had been discovered[16,17] which potentially has the capacity to achieve, for anaerobic cells, the same effect as the exchange of lactate and pyruvate with their environment. It has been found in all cell types yet examined; it is called the Plasma Membrane OxidoReductase, or PMOR. This system is able to accept electrons from intracellular NADH—thereby regenerating the all-important NAD^+—and export them out of the cell, as long as some chemical is present in the extracellular medium which can then absorb them. Evidently, despite presumably operating only at low rates in aerobically respiring cells, it is able to pump electrons at a sufficient rate in anaerobic cells that there is no need for a pyruvate/lactate exchange with the culture medium.

9.4. Getting the Most ATP Without Oxygen

There is, however, a very big difference between a pyruvate/lactate exchange and a PMOR in terms of the anaerobic cell's energy supply. One of the enzymes that Müller-Höcker and many others since have tested for in muscle fibers[18,19] is succinate dehydrogenase (SDH), otherwise known as Complex II. They have assayed for it because it has important similarities to, and differences from, the proton-pumping respiratory chain enzymes, so is a good "control" for the validity of the experimental technique. Like the proton pumps, it is embedded in the mitochondrial inner membrane; but unlike them, it is entirely encoded by nuclear genes. Müller-Höcker and others found that, in line with MiFRA, it was never affected in the anaerobic fibers they examined. Moreover, they found that it is actually present at

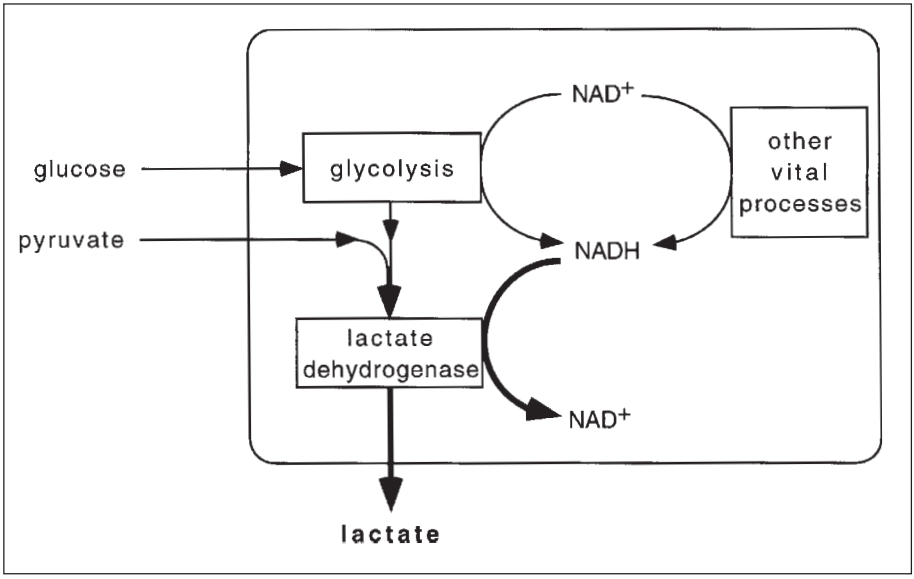

Fig. 9.1. How pyruvate sustains anaerobic cells.

increased levels in these fibers,[20a] even after allowing for the often-seen increase in the number of mitochondria per fiber,[20b] which strongly indicates that it is active.

The activity of SDH in anaerobic fiber segments is of enormous relevance here. Recall that succinate dehydrogenase is not only a component of the respiratory chain: it is also a component of the TCA cycle. Like three of the other components, it extracts electrons from a three- or four-carbon molecule and delivers them (via a small carrier molecule) to the respiratory chain. Unlike those other three, though, the carrier molecule is not NAD but FAD, which resides inside the succinate dehydrogenase enzyme itself and hands the electrons on directly to ubiquinone. But the crucial point is that the TCA cycle is a cycle, so that if the SDH step is happening then all the other steps must also be happening.* One of those steps phosphorylates a molecule of GDP to GTP, which is then used to make a molecule of ATP (see Section 2.3.3.2). Thus, by maintaining the TCA cycle, the cell is doubling the amount of ATP per glucose molecule that it would get from simple glycolysis; moreover, it is able to metabolise fatty acids productively too. All in all it is far better off than if it were just turning glucose into lactate.

Another observation is worth mentioning with regard to ρ^0 cells. The stoichiometry of glycolysis and OXPHOS is well understood, so it is a straightforward matter to calculate how many times more glucose a ρ^0 cell would have to metabolise in order to grow at the

** In fact this does not strictly follow, since enzymes can function in either direction. Thus it is logically possible that the activity of SDH in the matrix is compensated by the opposite reaction (succinate synthesis from fumarate) elsewhere in the cell, in the same way that, for example, the malate/aspartate shuttle involves malate dehydrogenation in the matrix and malate synthesis in the cytosol. But examination of SDH's location in the TCA cycle shows that, in this case, such a shuttle is not an option, since it would require the import of succinate, for which there is no available mechanism. A more elaborate shuttle, involving some but not all of the other parts of the TCA cycle, is also prohibited because it would entail the reversal of the step that makes GTP: that is, no net ATP synthesis would be occurring.*

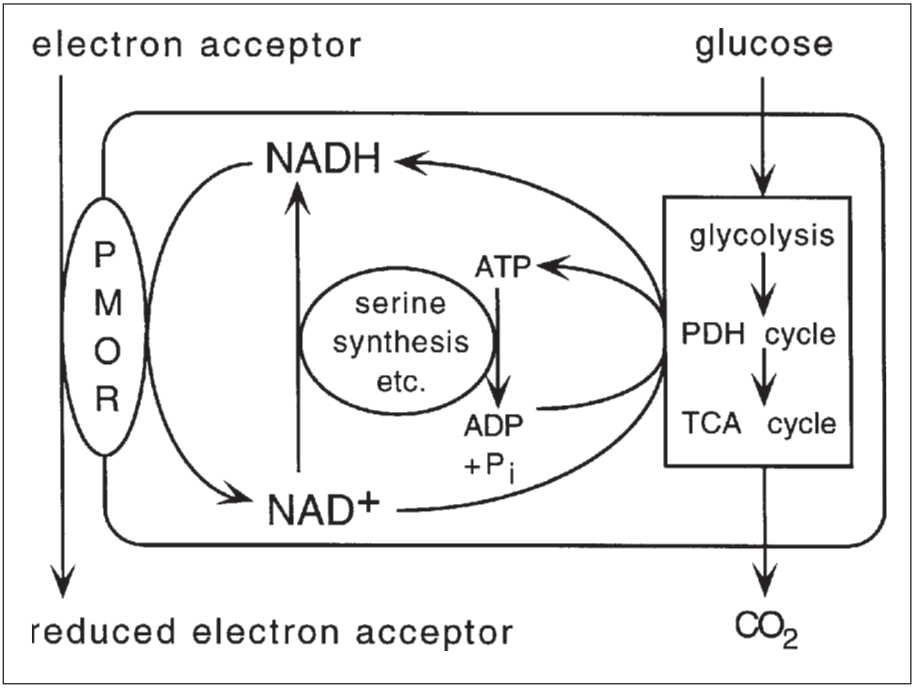

Fig. 9.2. Electron export allows NAD^+/NADH stability without aerobic respiration.

same rate as an aerobic cell: namely, 19 times. Some ρ^0 cell lines do indeed grow as fast as wild-type cells of the same type,[11] and the ratio of glucose consumption is actually only about **2.5!**[21] The maintenance of the TCA cycle provides a simple explanation for a large fraction of this disparity—perhaps for all of it, since it may well be that the **exogenous** pyruvate, as well as that derived from glycolysis, is introduced into the TCA cycle, and it may also be that the progenitor ρ^+ (non-ρ^0) cells exported some lactate naturally. Some early studies of cell lines with reduced, but non-zero, OXPHOS function[22,23] found inhibition of the TCA cycle, but this has not been demonstrated for ρ^0 cells; moreover, only some ρ^0 cells grow as fast as their progenitor, OXPHOS-competent cells, whereas other do not grow nearly as fast. Therefore, these early studies do not necessarily indicate the in vivo situation; more measurements of these aspects of ρ^0 cell metabolism are clearly needed.

There is a big side-effect, however, for a cell whose mitochondria are performing the TCA cycle but not OXPHOS. It is creating NADH many times faster than if it were only doing glycolysis, and the NADH is not handing on its electrons to the respiratory chain, since that is broken. But, by sufficient up-regulation of the PMOR, the situation in these anaerobic cells can be kept stable and the cycle can continue (see Fig. 9.2). The only other prerequisite is reversal of the malate/aspartate shuttle, which is readily achieved by a sufficient shift in the matrix and/or cytosol NADH/NAD^+ ratios.

Finally it should be noted that this NADH recycling is sufficient for TCA cycle maintenance, despite the fact that succinate and fatty acid oxidation both generate $FADH_2$ which must also be recycled. This $FADH_2$ passes its electrons to ubiquinone in the normal way, forming ubiquinol, but then the ubiquinol can pass them back to NAD^+ forming NADH, by reverse operation of, most simply, the **s,n**-glycerophosphate shuttle (see Section 2.3.2.4).

9.5. PMOR: The Solution, or the Problem?

Lawen's group did not extend this work beyond these in vitro experiments, in which the impermeable electron acceptor was provided in sufficient quantity that it never ran out. If such a system is functioning in the body, however, something more must be going on. The electrons must become attached to some molecule in the plasma. If that molecule were an antioxidant, that would be fine.... but there are other options. The most dangerous one is that they could reduce transition metal atoms, such as iron and copper. The reduced ("ferrous" and "cuprous", as opposed to "ferric" and "cupric") forms of these atoms are the ones with the capacity to cause "branching" of lipid peroxidation chain reactions, which are probably the most powerful amplifiers of oxidative stress (see Section 3.7).

It should now be evident why the PMOR is relevant to the paradox of such low numbers of anaerobic cells. It is clear that cells whose mitochondria are healthy nevertheless suffer considerably increased oxidative stress in old age, disproportionate to the overall energy shortfall of the tissue. The levels of the various markers of oxidative stress rise with age in all cells, including rapidly-dividing ones.[24] Thus, either these mitochondrially healthy cells must have an independent intracellular source of oxidative stress—i.e. the mitochondrial free radical theory of aging, if stated strongly enough to be a real theory of aging (see Section 7.1), is wrong—or the toxicity of the mitochondrially mutant cells must somehow be getting greatly amplified. If the PMOR is "hygienic" in disposing of electrons in vivo, donating them only to antioxidants and thus generating no toxic extracellular products, then it becomes difficult to see how anaerobic cells can be blamed for systemic oxidative stress. But perhaps, just perhaps, this flow of electrons out of anaerobic cells might be the first step in a toxicity-amplification process, since it might not be hygienic: those electrons might become lonely and create LECs in the blood, which might initiate chain reactions as described in Section 3.7. This, then, would resemble the situation at the mitochondrial inner membrane—"reductive stress," as it would most appropriately be termed (see Section 5.6.2).

9.6. Systemic Consequences: The "Reductive Hotspot" Hypothesis

So far so good; but, just like the idea of biased destruction rather than biased replication as a means of proliferation of mutant mtDNA, this speculation is not of much use unless and until it can be fleshed out into a reasonably detailed mechanism. I thus sought such a mechanism: in particular, one which allowed the possibility that peroxidation chain reactions might result at some stage, since that might plausibly provide the required degree of amplification, enabling the observed small number of anaerobic cells to generate the observed high levels of age-related oxidative stress.

An important, and powerfully antioxidant, electron acceptor in blood plasma is ascorbate (vitamin C). As was summarised in Section 3.5, it mainly acts by donating an electron to a lipid radical (generally via tocopherol, vitamin E) so as to terminate a lipid peroxidation chain reaction; in doing so it becomes ascorbate radical. Pairs of ascorbate radicals rapidly react, undergoing disproportionation, which gives one molecule of ascorbate and one of dehydroascorbate. The latter has no antioxidant capacity, and must be turned back into ascorbate by the addition of two electrons. It had been proposed[17] that this last step is a physiological role of the PMOR; no other physiological extracellular electron acceptor for the PMOR* has been identified.[25] So at first, it seemed that no avenue existed for electrons to find their way into toxic LECs.

** The enzymes of the PMOR do appear to interact with membrane-bound species, including ubiquinone and protein disulphides.[26] But, because these are membrane-bound, it is necessary to regard them as components of the PMOR, rather than substrates.*

But plasma also carries high levels of one very undesirable potential electron acceptor: oxygen. It has been shown that the PMOR has lower affinity for oxygen than for nonphysiological electron acceptors such as ferricyanide;[27a,17,28] but not necessarily low enough to prevent any superoxide production in the plasma surrounding an anaerobic cell. (A functionally related enzyme, which oxidises NADPH rather than NADH, is found in leucocytes, and its role is the **deliberate** production of superoxide outside the cell to act as a bactericide.) Furthermore, three recent reports[27b-27d] show that extracellular superoxide can indeed be created by cell surface NADH oxidases, in some circumstances.

This superoxide would not be directly problematic. One of our three variants of superoxide dismutase is specific to the extracellular medium; it will scavenge most superoxide generated in this way, particularly since it is known to be present at very high levels in the artery wall.[29] The hydrogen peroxide that is thereby produced will, similarly, be converted to water by extracellular glutathione peroxidase and/or catalase (see Section 3.5).

Some superoxide, however, will inevitably evade this defense. Superoxide is a relatively unreactive radical, and cannot autonomously initiate lipid peroxidation; but it has a high affinity for ferric iron (Fe^{3+}), which it reduces to ferrous (Fe^{2+}). Ferrous iron, in turn, participates in Fenton reactions: it can react either with hydrogen peroxide, creating the highly reactive hydroxyl radical, or else with lipid hydroperoxides, creating a lipid alkoxyl radical. This last reaction is particularly worthy of consideration, because it effects the "branching" of lipid peroxidation chain reactions, which is the main reason why they propagate so rapidly[30] (see Section 3.7).

Since iron is an essential component of many enzymes, it must be provided to all cells after extraction from the diet. This is of course done via the blood stream. But such iron is maintained in the ferric state, almost certainly protected from reduction by superoxide, by its carrier protein, transferrin,[31] except possibly during cellular uptake.[32] Another major iron-carrying plasma protein, ferritin, probably also has a low affinity for superoxide because of the protective effect of ceruloplasmin, which also binds virtually all plasma copper.[33] A third major source of iron in plasma is haemoglobin, which is released into plasma by rupture of red blood cells, especially at sites of inflammation; but it is both removed by haptoglobin and (according to a recent report)[34] detoxified by haemopexin whenever it assumes the more unstable ferric state, methaemoglobin. A fourth source, however, appears to have less such protection. It is haemin.

Haemin is the non-protein component of haemoglobin, composed of an iron atom in a porphyrin ring. Haemin becomes detached from methaemoglobin at a significant rate and is prone to desorb from its host red blood cell, becoming free in plasma. Once free, it is probably not a significant pro-oxidant, because it is assiduously bound by albumin and haemopexin, the latter of which transports it to the liver for destruction.[35] Recent work,[36] however, has firmly established that haemin which is still suspended in the red cell membrane also binds—albeit transiently—to low-density lipoprotein (LDL) particles. Crucially, these studies took care to assess the binding affinities in physiologically realistic conditions. The authors concluded that haemin may be heavily involved in LDL oxidation in vivo. This is the reason why the Fenton reaction of ferrous iron with lipid hydroperoxides is so likely to be important: most of the lipid hydroperoxides present in plasma are in LDL.[37]

Adding all this together, it begins to look as though a pathway really does exist whereby anaerobic cells can be highly toxic to aerobic ones (Fig. 9.3). LDL uptake is not something that cells can forgo; it is their source of cholesterol, without which their membranes would lose fluidity and break down, with rapidly fatal consequences to the cell. So if the LDL available in the blood is becoming increasingly contaminated with pro-oxidants such as lipid hydroperoxides, the cell has no choice but to import those impurities, whatever the consequences.

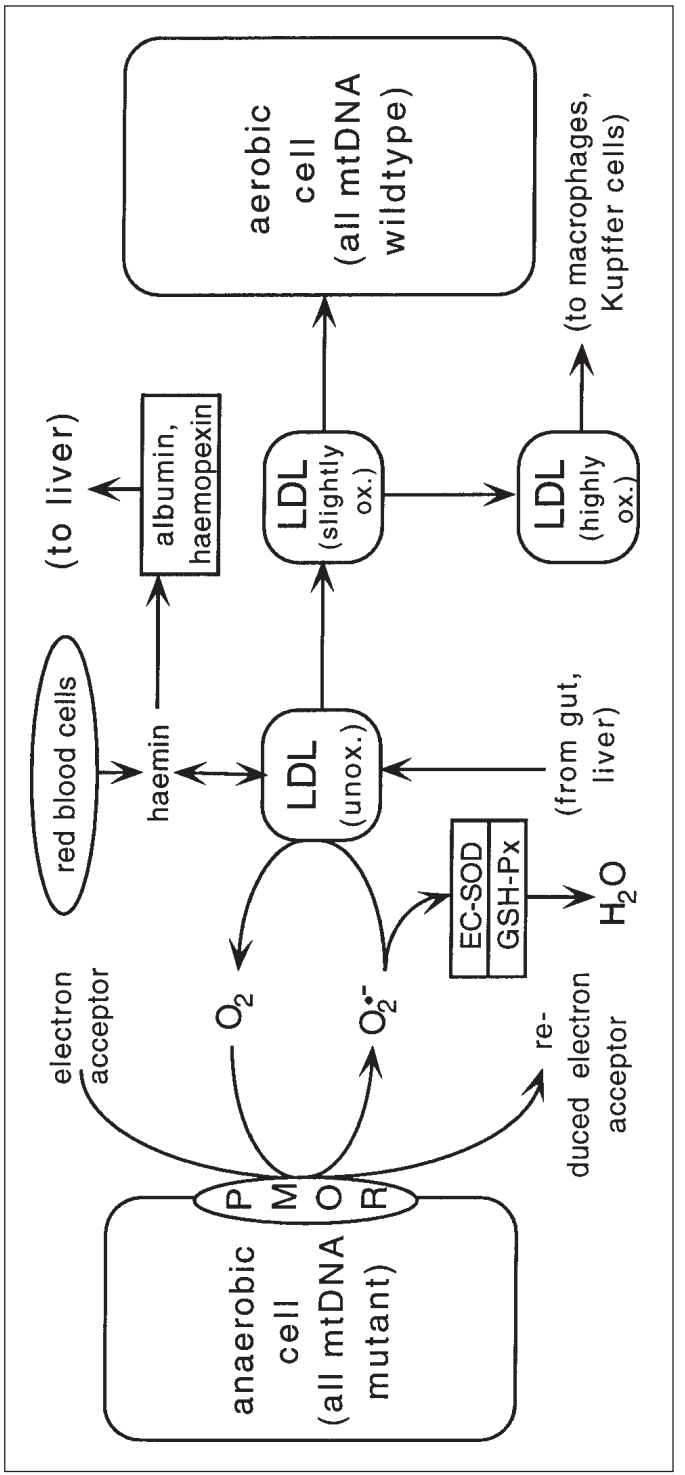

Fig. 9.3. Scheme for the transmission and amplification of oxidative stress.

Furthermore, the involvement of LDL (or other material transported in the blood) gives us a preliminary explanation for why the cells whose function is most affected by aging are not exactly the same ones as those whose mtDNA mutation load is highest. In particular, it is found that muscle fibers accumulate more mutant mtDNA than most other tissues,[38] but old muscle fibers can work just as well as young ones—the loss of strength associated with old age is almost entirely due to reduced exercise.[39] Similarly, the failure of young muscle to thrive when transplanted into old mice, and the converse success of old muscle transplanted into young mice, has been shown to derive from the old mice's reduced ability to re-enervate the transplanted tissue, rather than from any reduced capability of the muscle fibers themselves.[40] A reasonable conclusion is that nervous tissue is the main victim of mtDNA-mediated oxidative damage, but muscle is the main toxin.

9.7. Alternative Pathways

The pathway proposed in the previous section is quite complex, and is unlikely to be unique. It is therefore worthwhile to examine some possible variations.

It is possible that some electron efflux from anaerobic cells is effected by a lactate/pyruvate couple, rather than via the PMOR. However, there is evidence against an age-related increase of either lactate or pyruvate in plasma,[10] so this seems likely to play at most a minor role. On the other hand, it could be argued that the rise in lactate or pyruvate levels would be very slight and might be outweighed by secondary factors; therefore, a much better test would be to double-stain muscle tissue asking whether cytochrome c oxidase inactivity colocalises with hyperactivity of lactate dehydrogenase (LDH), the enzyme that converts pyruvate to lactate. Histochemical quantification of LDH activity is routine,[41] so this is a relatively straightforward experiment.

The next step is the transfer of electrons from the PMOR to oxygen, forming superoxide. It is possible that electrons might move directly from the PMOR to haemin, with no intermediate, or alternatively via some other intermediate. The involvement of some intermediate seems likely, because it serves as a reservoir which can be filled and tapped asynchronously; it thereby allows haemin-bound iron to receive electrons when at some distance from the anaerobic cell. This permits a greater throughput of electrons than if physical adjacency of the two were necessary. Oxygen was identified above as the likely major intermediate, since it is present in much greater amounts in plasma than any other plausible electron acceptor/donor; it has also been implicated in LDL oxidation,[42,43] though this is not certain.[44] There have been suggestions that ascorbate itself can act as a pro-oxidant electron donor, but this has been clearly shown not to be the case in physiological conditions.[45]

The role of haemin as the supplier of iron is also probably not unique. I presented arguments above for why transferrin and ferritin are less prone to be involved, but this should not be construed as proof that they are not involved at all. Free iron and copper are hardly present in plasma, due to the activity of ceruloplasmin,[46] but may also play a minor part.

Finally, LDL is not the only oxidisable substance imported by cells; they also import free* fatty acids from the plasma. This indeed constitutes the most important pathway of fatty acid import, since the amounts of phospholipid or cholesteryl ester imported in LDL are regulated only by the cell's need for cholesterol.[47a] But plasma contains a powerful enzymatic defense against the oxidation of free fatty acids: a selenium-dependent phospholipid hydroperoxide glutathione peroxidase, which reduces lipid hydroperoxides to alcohols, which cannot participate in chain reactions.[47b] This means that the levels of free lipid hydroperoxides in plasma are extremely low, thus protecting cells from importing

* *Strictly, not free but bound to albumin until import.*[46]

them.[48] This glutathione peroxidase can probably also act on phospholipids at the surface of LDL particles,[48] but, crucially, most of the oxidisable material in LDL is deep inside the particle and inaccessible to most antioxidants. (Lipid peroxidation must begin at the surface of the particle, but it will then rapidly undergo chain reactions and other molecular rearrangements that spread the damage into the cholesteryl ester core.) The protein component of LDL is also prone to undergo oxidation[49]—indeed, there is evidence that oxidation of that protein is the feature that the standard LDL receptor uses in order to avoid importing heavily oxidised LDL (see Section 4.3)—and protein hydroperoxides thus formed can stimulate further LEC production after import into cells, just like lipid hydroperoxides.[50]

For these reasons, it seems quite probable that the particular pathway described earlier, via the PMOR, oxygen, haemin and LDL, is the primary route transferring oxidative stress from anaerobic cells to aerobic ones. Extensive evidence supporting this mechanism has since emerged.[51]

9.7.1. Mechanical or Electrical Pathways

The fact that muscle fibers become anaerobic in short segments allows a radically different mechanism for the propagation of oxidative stress to mitochondrially healthy regions. I do not think, on current evidence, that it is likely to be correct; however, the history of science is so littered with failures of such intuition that it would be remiss not to mention it. This caution is particularly germane in the context of mitochondria, since it was just such a failure of intuition that induced bioenergeticists to cling for so long to the belief in a chemical intermediate in OXPHOS (see Section 2.3.4).

The function of a muscle fiber depends critically on two features which are properties of the fiber as a whole, rather than of each section of it independently. They are that it must be able to transmit both electrical potential and tensile force along its length. The electrical potential (the action potential) is necessary because it is the stimulus, starting from the motor neuron attached to the fiber, that induces each contractile unit of the fiber (termed a sarcomere) to contract. The tensile force is necessary because, ultimately, a muscle can only work if it pulls together the two things that are attached to its ends, and (like any chain) it is only as strong as its weakest link.

Now: it is likely that an anaerobic segment of a muscle fiber undergoes many changes of cellular chemistry in order to adapt to the absence of OXPHOS. There will probably be changes to many fundamental aspects of the cytoplasm and the organelles, such as the NAD^+/NADH ratio, the ubiquinone/ubiquinol ratio, and maybe even the pH. There is no known way in which such changes could affect the fiber's conductivity—its ability to transmit the action potential—but the possibility cannot be absolutely discounted at this point. Similarly, the fiber's tensile strength would not obviously be affected in an anaerobic region, since it derives from highly stable intracellular proteins which should be impervious to such changes, but again there may be vulnerabilities which we have not yet discovered.

Let us then suppose, for a moment, that a muscle fiber does indeed suffer with regard to either its electrical or tensile integrity. What might that cause? The fiber is quite tightly attached to its neighbours, so tensile strength is shared; however, the attachments between fibers might be over-stressed in this situation and weaken. Consequently it is conceivable that quite large regions of mitochondrially healthy fibers suffer unusually large tensile forces as a result of small anaerobic segments nearby. This may have consequences for turnover of muscle protein; that, in turn, may conceivably contribute to oxidative stress. The gaps in this theory are clearly extremely large, but that is its only serious shortcoming; hence it would be premature to dismiss it out of hand at this stage.

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Chapter 10

Frequently-Asked Questions

The theory discussed in this book is a particularly easy topic on which to give seminars: not so much because it is easy to explain, but because it inspires so many lines of thought in the listener, so allowing one to tailor the presentation much more accurately to the target audience than would otherwise be possible. The questions I most often encounter are addressed in this chapter.

10.1. Why Is Carl Lewis Still Alive?

Or, to put it less succinctly: "Surely this theory predicts that higher than average energy utilisation would create more LECs, cause more rapid mitochondrial mutation and turnover, and thus cause faster aging? But athletes live quite as long as anyone else—and, indeed, their mtDNA appears to undergo slightly **slower** damage with age than average."[1]

The big flaw in this logic is that it overlooks the effect of training. It is correct that high energy utilisation would cause high production of LECs. It is also correct, according to the theory set out here, that if we went out today and were a great deal more athletic than usual then we would age more rapidly as a result. Today. But if we were to make a habit of it, there would be a highly relevant adaptive response: we would get fitter. In particular, our respiratory capacity would rise, due to an increase in the number of mitochondria in each fiber of the muscles that we were training. This would mean that the rate of respiration per unit surface area of our inner mitochondrial membranes would be no greater than it was before we began this athletic activity. And it is that, not the total respiration rate, which (according to SOS) determines the rate of turnover of mitochondria. Each individual mitochondrion is no worse off than before.

10.2. Why Haven't We Evolved Our Mitochondrial DNA Away?

It should first be stressed that the continued existence of mitochondrial DNA does not in any way challenge MiFRA, nor even the general idea that mtDNA decline is substantially to blame for aging. This is because there is absolutely no reason why evolution should select for longevity—indeed, ecological factors can just as effectively select the other way (see Section 6.5.2). The survival of the species, not of the individual, is what evolution promotes.

Nevertheless, this is a highly interesting question for another reason. As was summarized in Section 2.1, mitochondria originally had many more genes encoded in their own DNA—all those needed for existence as independent prokaryotic cells. Many of these genes were lost because they were redundant with ones in the nucleus, but many had to be retained. Of those, however, most have been transferred into the nucleus. Just 13 protein-coding genes, plus the rRNAs and tRNAs needed to turn their transcripts into protein, remain.[2] The retention of these 13 in the mitochondria is highly "inconvenient," since it necessitates the retention of masses of mitochondrion-specific machinery, such as a special DNA polymerase and special ribosomal proteins. It is also, admittedly, a lot of trouble to maintain the protein import machinery that transports nuclear-coded proteins into the mitochondrion; but the

The Mitochondrial Free Radical Theory of Aging, by Aubrey D.N.J. de Grey.

same machinery is used for all proteins, so one would think it could easily have been extended to handle those last 13.

Moreover, apart from one very recent report[3] there is no evidence that any genes have ever moved the other way. There is evidently a selective advantage associated with having one's genes in the nucleus, albeit not enough of one to have pushed the transfer process to completion. We do not know what this advantage is, but it could be as simple as that such genes then benefit from Mendelian inheritance, which seems to be evolutionarily desirable since it allows the gene to escape from "Muller's ratchet."[4,5]

There are probably two main reasons why we retain these 13. The first one goes some way towards explaining why it is precisely those 13, rather than some others. They are all subunits of the OXPHOS enzymes, which means they all exist embedded in the inner mitochondrial membrane. Transmembrane proteins have an amino acid composition that tends to push them into solution in lipids (such as membranes) and out of solution in water. This is called, for obvious reasons, hydrophobicity, and it can be quantitatively calculated from the amino acid sequence as a property of the whole protein or of a region of it. It turns out that almost all the 13 mt-coded proteins have several regions of high hydrophobicity, whereas imported proteins have fewer (though some have many regions with moderate hydrophobicity).[6] So, one reason why these 13 genes are still encoded by the mtDNA may well be because the protein import machinery is not good enough at handling proteins which have this sort of sequence.

The second reason requires some introduction. It seems that all but these 13 were transferred to the nucleus a very long time ago. Phylogenetic analysis tells us that all but one of them moved before the animal kingdom diverged from fungi, and that last one—ATPase subunit 9—must have moved quite soon after that, because it is in the nucleus of all animals that have been studied, though it is still in the mitochondrion in most fungi.[7] The only difference that has been found so far in the complement of genes in different animals' mtDNA is that nematodes and some mussels may have transferred one more gene, ATPase 8, to the nucleus.[8,9] (I say "may" because all we know at present is that ATPase 8 is not encoded in these species' mtDNA: it has not yet been found in the nuclear genome either, so it may not be retained at all.) It thus seems that evolution was getting along reasonably well in transferring genes, and that then suddenly everything froze.

An explanation can be seen by comparing animals with plants. In plants, the transfer process seems not to have frozen quite so solid. For example, the gene for cytochrome c oxidase subunit 2 is in the mitochondria of almost all plants (and all animals) but is in the nucleus of some legumes such as *Vigna radiata.*[10] Since legumes form a relatively small taxon in the plant kingdom, this transfer must have happened fairly recently. A similarly recent transfer of cytochrome c oxidase subunit 3 has been identified in the club moss *Selaginella elegans.*[11]

The basis for this difference is not known for sure, but it is very likely to be because the mtDNA of animals uses a slightly abnormal genetic code. Almost all eukaryotes use exactly the same genetic code in their nuclei; so do almost all bacteria, and it is virtually certain that at the time of the original endosymbiotic event the primordial mitochondrion also used that code. Since so many genes would be affected by any change in the code, there is a huge selective pressure to eliminate any mutation causing such a change in a cytoplasmic tRNA. But mitochondria have their own tRNAs, which are the molecules in which the genetic code is defined. As the number of protein-coding genes in the mtDNA steadily diminished, the pressure against drift in the code correspondingly became less and less. Eventually, some time after the divergence of plants from animals (but probably before the divergence of animals from fungi) there was a switch in just one codon. This switch thus affected fungi and animals but not plants. The trinucleotide UGA, which in the standard code means "stop," changed so that in mitochondria it meant "tryptophan."

The effect of this in mitochondria was slight enough to be survivable. A few mitochondrially-encoded proteins would have ended up having a few extra amino acids tacked on their ends, and that happened not to do any real harm.* So the change stuck. But the effect on gene transfer to the nucleus was immense. Now that UGA coded for tryptophan, it was possible for base pairs in the mitochondrial protein-coding genes to mutate silently to create UGA codons where there had previously been UGG. Such mutations would be silent in the mtDNA, because UGG codes for tryptophan already, so the encoded protein would have an unaltered amino acid sequence and the mitochondrion would be unaffected. In a very short time (by evolutionary standards), roughly half of the tryptophans encoded in the mtDNA would have come to be specified by UGA rather than UGG. Now consider the protein product of a mitochondrially-coded gene which is transferred to the nucleus after this short period. Rather than the minimal effect of having a few extra amino acids tacked onto its end, it will have the opposite experience: it will be brutally truncated at the first UGA, since the cytoplasmic translation machinery still interprets UGA as "stop." A successful transfer would thus require reversal of all the UGG-to-UGA changes that had accumulated in the transferred gene, without any other deleterious mutations being introduced in the meantime: a phenomenally unlikely scenario.

It is therefore no surprise that animals have failed to transfer any more genes except ATPase 9 and maybe ATPase 8. It is also no surprise that those two are the ones that have been moved. They are extremely small genes: ATPase 8 is only 50 to 70 amino acids long, and ATPase 9 less than twice that, compared to 300 or more for the average protein. The number of amino acids in a protein determines, on average, the number of base pair substitutions that would have to occur in a transferred gene in order to restore its consequent amino acid sequence to what the mitochondrial translation machinery would produce; since they must all be done if the gene transfer is to succeed, the difficulty of that transfer is thus an exponential function of the length of the protein. The shortest genes have had the easiest time.

It should be noted that the UGA switch was not the end of the story. The mitochondrial genetic code has remained under only this relatively slight pressure to remain the same since that time, and many other drifts have taken place, so that different animal phyla have different codes, and for example our mitochondrial code differs in four codons from the universal one[7] (see Table 10.1). This fact has been cited as a challenge to the argument outlined above: the logic is that if the code drift mostly happened recently, but the gene transfer froze much longer ago, then code drift can't have been the reason gene transfer froze.[12] But this is wrong, since it is not the average timing of the code drift which matters, but the timing of the **first** drift. This is particularly true because that first drift was a change from STOP to coding, causing truncation of any transferred proteins as explained above; a change from coding to STOP or from coding to coding would have the much weaker effect of changing or appending some amino acids to the transferred protein, which might often not destroy its function[13] (see Table 10.2).

Similarly, one might argue that it still seems to be extremely hard to get genes across, even in plants, given that only two recent cases (the *Vigna* cytochrome c oxidase subunit 2 and the *Selaginella* cytochrome c oxidase subunit 3) are known. Yes, it probably is hard, given the apparent difficulty in overcoming these proteins' hydrophobicity. But also, this

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** Plants probably retain the standard code largely because they still have upwards of 20 mitochondrially-encoded genes, including many ribosomal proteins. A few extra amino acids may usually be harmless, but not always: it can quite easily do harm to a protein's functionality. The more proteins are extended, the less is the chance that all the extensions will be harmless.*

Table 10.1. Deviations from the standard code of various taxa's mitochondrial genetic codes

Taxon	UGA = Trp?	AUA = Met?	AGR = Ser?	AGR = stop?	CUN = Leu?
All vertebrates	yes	yes	no	yes	no
Most invertebrates	yes	yes	yes	no	no
Saccharomyces	yes	yes	no	no	yes
Most other fungi	sometimes	no	no	no	no
Plants, most algae	no	no	no	no	no
Most primitive eukaryotes	yes	no	no	no	no

See ref. 7 for more detail and primary references. R stands for "A or G"; N stands for "any base."

overlooks the fact that far fewer plants than animals have yet had their mitochondrial genomes sequenced,[14a] so it is quite possible—in fact, many would say absolutely certain—that more such cases will be found.

Another confounding factor is that, judging from the situation with *Vigna,* such transfers may be very easy to overlook. There are two stages to a transfer: the introduction of the DNA into the nuclear genome and its loss from the mitochondrial one. The functional transfer occurs when the nuclear copy is "turned on" and the mitochondrial one turned off; this must occur very soon after the DNA arrives in the nucleus, since otherwise it would undergo random mutations that would render it useless. But the inactive mitochondrial copy can, in theory, stay put indefinitely, since it is inactive. One might guess that it would usually be deleted rather quickly; but if we recall that there is a lot of "junk DNA" in plant mitochondria (as against essentially none in animals), perhaps this is not so obvious. And in fact, Nugent and Palmer found when they examined other legumes that the entire bean taxon uses a nuclear-coded cytochrome c oxidase subunit 2, even though only *Vigna* has lost the mitochondrial copy! This means that the inactive mitochondrial copy has been retained for up to 100 million years in most beans. The implication for discovery of other transfers is clear: simply probing the mtDNA by in situ hybridisation with genes from other organisms will not necessarily detect a transfer. The only reliable assay is more laborious: Northern blotting of fractionated samples, to detect whether the messenger RNA is localized in the mitochondria or the cytoplasm.

In closing this topic, it should also be mentioned that a number of other proposals have been put forward to explain why we retain our mtDNA. These reasons are potentially relevant to the possibility of retarding aging by mitochondrial gene therapy, which is the subject of Chapter 15. Discussion of these will therefore be deferred to Section 15.8.

Table 10.2. Consequences for gene transfer of different types of change in the mitochondrial genetic code

Code change	Human example	Newly silent substitutions	Amino acid sequence of subsequent transferee	Function of that transferee
coding to STOP	AGA	UGA to AGA	C-terminal extension	slightly impaired
coding to coding	AUA	AUG to AUA	conservative changes	fair to middling
STOP to coding	UGA	UGG to UGA	early truncation	nil

A change from STOP to coding allows silent mtDNA mutations that cause subsequently transferred genes to encode truncated products with no activity, so inhibits successful gene transfer far more powerfully than other types of mtDNA code change.

10.3. How Does the Germ Line Avoid mtDNA Decay?

This question also has two answers, but in this case that is because it is really two questions. The first is "How does mtDNA survive from one generation to the next?" and the second is "How does mtDNA survive in the ovum until fertilization?" So:

10.3.1. How Does mtDNA Survive from One Generation to the Next?

Though the adult body is mainly composed of non-dividing cells, each generation goes through a period in which all cells are dividing rapidly: early embryogenesis. In Section 8.5.3 we saw that SOS will only matter in tissues composed of non-dividing—or at least rarely-dividing—cells, because if cells are dividing rapidly then they will replicate their mitochondria before any have had time to "go critical," so the SOS mechanism is short-circuited. In fact such tissues are even better off than that. Consider a cell which carries some mutant mitochondria and some wild-type ones. When it divides, the mitochondria will be randomly partitioned between the daughters. Thus, the chances are that one daughter cell will receive slightly more mutant mitochondria than the other. The one with fewer normal mitochondria and more mutant ones will (at least initially) have less capacity to make ATP, so it will grow more slowly, so it will take longer to grow to a size ready to divide again. The same will apply to its remoter descendants, since they have more mutant mitochondria to partition in later divisions. Thus its descendants will be out-replicated by those of the daughter that received more normal mitochondria and fewer mutants. This therefore constitutes a strong selection against mtDNA mutants, resulting from intercellular competition.

A word should be added about the manner in which this process can run to completion. The selective pressure described above will reduce the amount of mutant mtDNA in the early embryo, but when its level becomes very low, so that a typical cell has only a few mutant mitochondria and hundreds of working ones, the selective pressure against that cell will be negligible. At this point, however, another phenomenon will step in to finish the job: genetic drift. Genetic drift is not a biochemical pathway but a statistical phenomenon. If a dividing cell has only a few mutant mitochondria, and its mitochondria are randomly distributed between the daughters, there is a significant chance that all of them will be segregated into one daughter; this chance of course rises as the number of mutant

mitochondria falls. This acts as a selective pressure against the mutants, because it is unidirectional: once a cell has no mutants, its descendants stay that way unless and until there is a new mutation event, whereas a cell that retains a few mutants can give rise at any future time to mutant-free descendants. See Section 10.7 for more about genetic drift.

A difficulty with the above explanation is that genetic drift will only exert a significant pressure to eliminate mutant mtDNA once its copy number is very low, and the typical cell has a surplus of bioenergetic capacity, so the pressure due to intercellular competition may not get the number of mutant molecules low enough for drift to take over. A better explanation was provided recently:[14b] that the necessarily slower protein import of mutant mitochondria, though probably irrelevant in non-dividing cells due to the time available (see Section 10.6), will select against the mutant mtDNA in dividing cells where SOS is absent.

10.3.2. How Does mtDNA Survive in the Ovum Until Fertilization?

This is harder—in fact, it is still a flourishing research topic. Since the ovum is a non-dividing cell until fertilization, it is potentially susceptible to SOS, and it has to avoid it for dozens of years. An age-related increase in oocyte mtDNA deletions has indeed been found.[14c]

Three explanations have been explored, and—unusually—they are probably all correct. The first is that ova are extremely quiescent until ovulation.[15] They have almost no energy requirements. Thus they probably generate hardly any LECs, so their mitochondria probably have very little chance to mutate; also, if a mutation arose through replication error, it would be only very slowly amplified. This is probably sufficient to deal with the case of mutations that arise in the ovum, rather than being already present when it was formed, since those few mutant mitochondria would be so diluted out during early embryogenesis that they would be easy prey to replication disadvantage and genetic drift (see above).

The second explanation deals with the opposite end of the spectrum: the case where a substantial proportion of the mtDNA in the ovum was mutant when it was formed. In such cases, SOS will ensure that there is no normal mtDNA left by the time of ovulation. A substantial burst of energy output on the part of the ovum is demanded during its ovulation and rapid early divisions, which is impossible without working mitochondria. If this energy is not forthcoming, subsequent cell division of that embryo fails, so no gestation will occur. The mother may have to wait another month, or have a litter one offspring smaller, but that is cheap in evolutionary terms.

What is really expensive in evolutionary terms is the third, intermediate situation: when there is some mutant mtDNA in the ovum at formation, but not quite so much that SOS can cause failure of early embryogenesis. That circumstance allows the possibility that the ovum would be fertilised, but that during embryogenesis a proportion of cells would retain enough mutant mitochondria that genetic drift would not eliminate them. This would mean that the embryo could develop normally, but its aging process would have a head start. Thus, the affected ovum would get all the biological investment needed to get it as far as birth and perhaps some way beyond, but it would not get far enough to have its own offspring, so in evolutionary terms all that investment (of parental care, etc.) would have been wasted. This is a plausible explanation for the very rare occurrence of sporadic (non-inherited) mtDNA-linked diseases such as Kearns-Sayre syndrome.[16]

It seems that evolution has found an extremely ingenious way to minimise the chance of this last scenario.[17] In the average cell there are at least 1000 mtDNA genomes, but in the ovum there are about 100,000. The simple way to get from 1000 to 100,000 is to engage in about seven rounds of mitochondrial replication; but that is not what happens. Instead, the mitochondrial population that is destined to inhabit the ovum is first depleted! It is forced

through a bottleneck of probably fewer than 100 genomes—some researchers think as few as 10—and is then increased to the required 100,000. The benefit of this is that, unless there is an error of mtDNA replication, the ovum will contain either no mutant mtDNA or else at least 1000 copies of a mutant. The idea is that 1000 copies of a mtDNA mutation at the end of oogenesis is enough to ensure that SOS will prevent that ovum from progressing beyond very early embryogenesis, whereas 100 or fewer (as would have been allowed with the simpler approach) is not.

10.4. Why Aren't Dietary Antioxidants More Beneficial?

First of all it is necessary to expand on exactly how beneficial they are. In *Drosophila*, combined overexpression of two important antioxidant enzymes causes a substantial increase in maximum lifespan;[18] but,

1. enzymatic and dietary antioxidants may differ in efficacy in regard to aging, and
2. we must be careful in extrapolating from this result to humans, for reasons that will be elaborated in Section 10.5.

In mammals, it has repeatedly been found that antioxidant supplements have virtually no effect on maximum lifespan, though they do have a substantial effect on average lifespan.[19,20] (These studies are complicated by the danger that a supplement added to the food will make the food less palatable and induce voluntary (depending on one's point of view!) calorie restriction. Only one supplement, deprenyl, has so far been shown to increase any mammal's maximum lifespan without reducing caloric intake.)[21] This has been interpreted to mean that the central processes which dictate the rate of aging are not being affected by these drugs, but that there are other, ancillary processes which can affect the rate of aging, and which are slowed by antioxidants. Another thing we can deduce is that the rates of these ancillary processes must vary substantially from one individual to the next in the absence of the antioxidant drugs, whereas those of the processes that are central to aging do not vary nearly so much.

Harman proposed in 1972 that (self-inflicted) damage to mitochondria was the main driving force in aging, and in the same paper he acknowledged the failure of antioxidant drugs to slow aging. He suggested that mitochondria might somehow be failing to gain access to the extra antioxidant resource.[22] In the context of SOS, and of the wealth of information that has become available since then, there is an answer which, though different in detail, approximates Harman's suspicion.

Mitochondria are, in a sense, really very sloppy. Assays of the activity of various antioxidant defense mechanisms have estimated that fully 1-2% or more of the oxygen we consume is turned into superoxide,* due to loss of electrons from the respiratory chain, rather than undergoing four-electron reduction at Complex IV as it is supposed to.[23] (Early experiments[24] suggested that there was negligible leak of electrons during normal metabolism, but this was found[23] to be true only when the substrate for respiration donated electrons mainly via Complex II, which is not the typical situation in vivo.) In view of that, it is

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* *This may even be an underestimate, since it is based on the rate of production of hydrogen peroxide.[23] As noted in Section 3.5, superoxide does not all survive to become hydrogen peroxide, since sometimes it donates its extra electron to ferric iron, thereby turning back into normal molecular oxygen. The one area of the cell where there is likely to be plenty of superoxide but not much superoxide dismutase is the mitochondrial intermembrane space,[25a] and there is an ideal source of ferric iron there: cytochrome c. But of course any electrons donated to cytochrome c are thereby retrieved into the respiratory chain, so that (apart from the pumping of a few fewer protons) the result is as if the electron had never been fumbled in the first place.*

absolutely astounding that our antioxidant systems are able to keep us alive at all. They are just incredibly good already. One reason why it may be very little use to add more antioxidant is that the longer-lived among us are already balancing optimally the levels of the various components of our highly complex and subtle natural system, so that adding more of one of them can only destabilise it. In more concrete biochemical terms, this could derive in large part from the ability of "antioxidants" to become pro-oxidant—that is, to promote rather than inhibit deleterious free radical reactions—if present at high concentrations. Vitamin E has been especially well studied in this regard.[25b,25c]

One can also hypothesize a detailed mechanistic explanation, based on the reactions described in Section 3.7. Superoxide cannot initiate lipid peroxidation directly, but in the presence of iron (including iron bound to proteins, most relevantly cytochrome c) it can cause "branching" of peroxidation chain reactions, which is equivalent to initiation since it turns one chain reaction into two. Furthermore, it was recently established[26] that the initiation-competent superoxide derivative which does nearly all initiation in the mitochondrial inner membrane is almost certainly the perhydroxyl radical, HO_2•, and not the hydroxyl radical (HO•) which most people have usually blamed. (The reason for this, and its rather extensive bioenergetic ramifications, are explored in detail in Chapter 11.) HO_2• is formed directly from superoxide by the addition of a proton, with no involvement of other molecules (even as catalysts), whereas HO• and all the other LECs discussed in Chapter 3 are formed by subsequent reactions, such as the splitting of hydrogen peroxide. We saw in Section 6.5.6 that greater longevity seems to require lowered superoxide production—more assiduous destruction of superoxide seems not to work—so we can infer that more assiduous destruction of downstream products of superoxide will also not work. The relevance of this to the efficacy of dietary antioxidants is that they do not destroy superoxide or HO_2•, only their pro-oxidant derivatives (such as lipid radicals), which, according to the research described above, are of only minor importance in aging.

10.5. Why Hasn't SOS Been Tested in Flies or Worms?

Drosophila (a fruit fly) and *Caenorhabditis* (a nematode worm) might initially appear to be very promising model organisms for testing SOS: not only are they long-standing objects of experimental research, with very extensive genetics, but also their cells are virtually all non-dividing in adulthood. Unfortunately, however, this optimism is misplaced, because SOS almost certainly doesn't happen to them. SOS requires cells to exist for enough mitochondrial generations that a spontaneous mutation can take over the cell. Minimally, this is the logarithm to base 2 of the number of mitochondrial genomes per cell. Since both *Drosophila* and *Caenorhabditis* live only a few weeks, their mitochondria would have to be recycled every day or two at least for SOS to happen. We do not know how fast they do turn over, but a rate of that order seems very unlikely. Indeed, it has been reported that mtDNA in *Drosophila* remains intact during aging:[27] such deletions as are found in adult flies accumulate during development rather than in the adult.[28]

In that case, why do they die so young? A plausible answer is simply that their antioxidant defenses are a great deal less good than ours. For example, flies do not have a gene for glutathione peroxidase,[29] which is one of the central pillars of our antioxidant system (though they do have glutathione reductase).[30] Thus, they die of the basal level of oxidative stress, without needing their mitochondria to amplify it.

10.6. How Can Mutant Mitochondria Survive, Let Alone Out-Compete Working Ones?

The problem implied in this question is that mitochondrial replication requires (as noted earlier) the import of hundreds of nuclear-coded proteins, and the import process

has been shown absolutely to require two things that are normally supplied by OXPHOS. These are (a) a supply of ATP inside the mitochondrion, and (b) a proton gradient across the inner membrane.[31,32] If OXPHOS is not happening, the mitochondrion cannot achieve any further replication (beyond perhaps one or two more divisions using proteins it has already imported) unless both these things are provided in some other way.

ATP is the easier one to obtain without OXPHOS. Recall from Section 9.4 that succinate dehydrogenase is found to be upregulated in anaerobic cells, and that this implies that the entire TCA cycle must be proceeding. But the TCA cycle occurs inside mitochondria, and one step of it, succinyl CoA hydrolysis, generates a molecule of ATP directly.*

The proton gradient is another matter. Since the TCA cycle occurs inside mitochondria, and generates NADH, the only way it can be maintained indefinitely is by reversal of the usual mode of action of the malate/aspartate shuttle (see Section 2.3.2.4). This shuttle normally imports electrons released by glycolysis, which are then fed into the respiratory chain; now, instead, it must export electrons released by the TCA cycle, which are then fed into the PMOR. One of the two carrier molecules that mediate the shuttle is the glutamate/aspartate carrier, which in aerobic cells imports glutamate and exports aspartate; thus, in anaerobic cells it exports glutamate and imports aspartate.

What has that to do with the proton gradient? Glutamate and aspartate are, indeed, irrelevant. But there is one further feature of the glutamate/aspartate carrier which does the trick. Figure 10.1a shows the components of a wild-type mitochondrion which are of most relevance to the proton gradient: they include not only the respiratory chain and the ATP synthase, but also the related metabolite carriers. Every time that the glutamate/aspartate carrier exchanges a molecule of glutamate with one of aspartate, it also transports a proton, in the same direction as (symport with) glutamate. Thus, in its reversed (and up-regulated) mode of action (see Fig. 10.1b), it is exporting protons. One carrier's protons are just as good as another's, for the purpose of making a gradient, so the proton gradient is maintained and protein import is still possible. In theory, if the degree of up-regulation were sufficient, this mode of proton export could suffice to drive ATP synthesis by Complex V (in mutant mitochondria whose mutation was in a respiratory chain gene, so whose Complex V was still intact); but in practice this is very unlikely, since the number of protons exported per pyruvate molecule imported is only about 10% of what the respiratory chain achieves.

It is interesting to note that a system would exist to provide both internal ATP and a proton gradient, even if the TCA cycle did not keep going.[33] Absence of ATP synthesis in the mitochondrion would induce the reversal of the ATP/ADP translocase, which normally imports ADP and exports ATP but would then import ATP generated by glycolysis (and by other, intact mitochondria, while the cell still has some). But intramitochondrial hydrolysis of this ATP (for protein import and other tasks) would release phosphate, so there would also be a reversal of the phosphate carrier. (Neither the phosphate carrier nor the ATP/ADP translocase has any mt-coded components, so this applies whatever the mtDNA mutation.) The phosphate carrier has the same useful property as the glutamate/aspartate carrier: it transports hydroxide ions the opposite way from phosphate, which is electrochemically the same as transporting protons the same way as phosphate (Fig. 10.1c).

It must be acknowledged that both the intramitochondrial ATP supply and the proton gradient are sure to be less in an anaerobic mitochondrion than in a working one, and that its protein import will inevitably be slower as a result. This might be considered fatal to

** Not quite directly, in fact (see Section 2.3.3.2)—bacteria do it directly, but in humans what is generated is GTP, and the extra phosphate bond is then transferred to make ATP. But the point is that OXPHOS is not involved.*

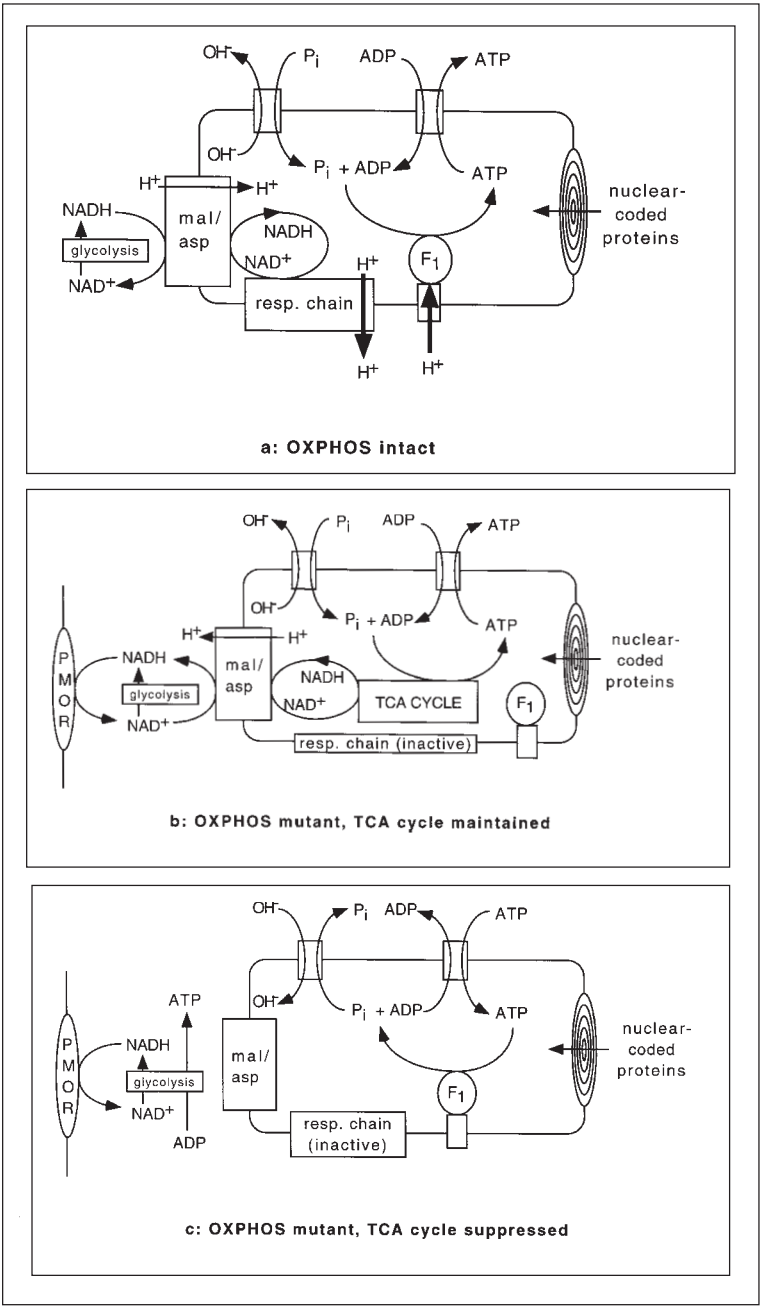

Fig.10.1. Three ways to maintain intramitochondrial ATP and proton gradient.

SOS, since it would surely confer a selective disadvantage. But we must consider with care whether that disadvantage would be big enough to outweigh the advantage conferred by slow degradation. In non-dividing cells, when the mitochondrion has a whole month in which to import the proteins necessary to double its size, an impaired rate of import should not matter, since it could not be rate-limiting. In rapidly dividing cells, however, SOS is short-circuited and this disadvantage may have an important role in eliminating mutant mtDNA[14b] (see Section 10.3.1).

10.7. Why Doesn't the Polyploidy of Mitochondria Protect Them?

Mitochondria do indeed possess more than one copy of the mtDNA. The average in human cells is about five. When a spontaneous mutation occurs, therefore, there is still plenty of backup for the creation of more protein from the other copies. This means that—if, as is reasonable, we presume that the remaining copies can be transcribed and/or translated 25% more often than normal—the affected mitochondrion will not, yet, experience any selective advantage over other, fully wild-type ones. (Note that this is the best-case scenario, because it does not cover the case where the mutation causes the protein to be constructed and incorporated into the enzyme complex as normal, but then to function abnormally. In that case, even one mutant copy may cause a 20% decline in function.)

There is, therefore, still a 50-50 chance that it will be destroyed rather than replicated. If it is replicated, however, there is then a 50-50 chance that the two copies will be segregated into the same daughter mitochondrion. This daughter may then start to feel the effects of a diminished availability of the affected proteins, due to its wild-type mtDNA being depleted by 40% rather than only 20%. The effect may be small, but even a small effect is enough to make a big difference to the probability of going critical and being destroyed before being replicated.

Moreover, the logic of the 50-50 chances mentioned above can be extended to subsequent mitochondrial divisions. A small proportion of spontaneous mutants will therefore inevitably achieve homozygosity in one mitochondrion, even if they have no selective advantage at all until that time, while the majority of spontaneous mutations will be destroyed by random chance before then. (This is called genetic drift, and was discussed in more detail in Section 10.3.1.) Indeed, we already have evidence that that is what happens[34] (see Section 6.6.2).

10.8. What About Mitochondrial Fusion?

This is a phenomenon whose existence in normal mammalian cells has been in dispute for some years, though it is known to be a normal part of spermatogenesis in *Drosophila*[35] and may also occur in mammalian spermatogenesis. Its relevance to MiFRA is with regard to the ability of a mtDNA mutation to out-compete the wild-type mtDNA and take over a cell. The phenomenon of Darwinian selection relies absolutely on the link between genotype and phenotype: that is, on the existence of a population of individuals, of varying genetic content, whose "fitness" is at least partly determined by that genetic content. If mitochondria divide but never (or rarely) fuse, then this is what exists in the cell after a spontaneous mutation. If, on the other hand, mitochondrial fusion is frequent and allows the sharing, by all mitochondria in a cell, of all the proteins that any of them can encode, then the link between genotype and fitness is destroyed and no selective advantage can appear. Therefore, if mitochondrial fusion is indeed frequent in vivo, the apparent clonal amplification of mutations that has been so extensively reported (see Section 6.6) must, somehow, be an artifact. It is thus imperative to establish whether mitochondria fuse in vivo, and if so then how often.

Direct visualization of fused mitochondria ("megamitochondria") has been widely reported in many cell types: in some, such as muscle, they form a reticulated network that appears to span large regions of a fiber,[36] whereas in others, such as kidney, there is an intermediate situation in which mitochondria form filamentous* chains but not more complex networks.[37] However, this is not always present: generally mitochondria appear as separate, small ellipsoids, and the reticulation appears to be induced under conditions of high oxidative stress.[38] The reason for this behaviour is unknown, but it has been suggested that ATP-synthesising proton-motive force can be transmitted along such filaments.[39]

We must be cautious, however, because this visualisation does not tell us that fusion is complete, with the matrix compartments being merged: it is possible that, for example, only the outer membranes fuse while the inner membranes remain separate. Evidence that something like this is indeed the case comes from studies of the mitochondrial proton gradient using the dye JC-1,[40,41] in which different parts of a filamentous mitochondrion exhibit clearly distinct fluorescence characteristics. A single aqueous compartment is always at uniform electrochemical potential throughout (except when there is a continuous current within it, as will be discussed in Section 11.3.7), due to the fantastically rapid conduction of protons in water, so the apparent heterogeneity of proton gradient indicates that the mitochondrion is not fully fused. The phenomenon could, alternatively, be because JC-1 fluorescence is affected by unidentified factors other than the proton gradient, but the face-value interpretation is that the matrix compartments are still separate. Electron-microscope visualisation of such filaments also suggests that their matrix compartment is not continuous,[42] and the electron-microscope evidence with regard to muscle-fiber reticulations is even more unambiguous.[36,43]

A completely different way to assay for mitochondrial fusion is by genetic means. The most compelling experiments showing that it definitely does happen are quite recent.[44] Cell lines were constructed which had mitochondria carrying a particular mutation, and these were fused to other cells whose mitochondria carried a different mutation, such that mitochondria carrying DNA of both types would be able to make all their proteins but the rest would not (or not nearly so rapidly). Initially no mitochondria had both types of mtDNA, because that was how the progenitor cell lines started out; but the fusion cells were rapidly taken over by mitochondria which were capable of OXPHOS, and which thus had both types. This could not have come about other than by complete mitochondrial fusion—not simply fusion of the outer membranes.

Again, however, there is a difficulty in translating these results to the in vivo situation. These experiments applied such enormous selective pressure in favour of the descendants of fusion events that they do not tell us how common the events are: even if they are quite rare, their descendants will rapidly take over the culture. One assay[44] appeared to show that fusion was very common, because it demonstrated the sharing across all mitochondria of a protein that only half the mtDNA present could encode; but this was contradicted by another assay in the same study[44] which indicated that a mutation on one of the mtDNA species, which was known to be recessive, behaved as a dominant mutation. These results could both be explained if a small amount of mtDNA recombination occurs following fusion events. mtDNA recombination certainly happens occasionally, though probably only as an "accidental" side-effect of replication and/or transcription—we known this from the occurrence of mtDNA deletions flanked by direct repeats (see Section 2.4.5), and also because of the detectability of double- and triple-length mtDNA molecules, which has been known

** The filamentous morphology has been known since the earliest days of the study of mitochondria—indeed, it gives them their name, which is derived from the Greek mitos, meaning thread.*

for almost as long as mtDNA's circularity.[45] Conditions that select for fusion will necessarily also select for recombination, as a way of stabilising the hybrid genotype of the mitochondrion and thereby protecting it from genetic drift, as shown in Figure 10.2. And indeed, a more recent study[46] which essentially repeated the above work[44] but using genotypes in which recombination products could be easily detected, found them in large quantity.

In conclusion, therefore, the evidence that incomplete and/or rare fusion of mitochondria occurs is very strong, but the evidence that it occurs fully and frequently enough to obliterate the forces of intermitochondrial Darwinian selection is not at all strong. The evidence does not, therefore, constitute a challenge to the admissibility of SOS as the mechanism of mitochondrial decline during aging.

10.9. Why Doesn't the Body Just Let (or Make) Affected Cells Die?

This question has a simple, short answer: we don't know. In fact, it is not all that easy to establish incontrovertibly that such cells do not indeed die and get replaced. This would give a simple explanation for why we see so few: they struggle on for a little while but then succumb, so the ones we see are those which have gone anaerobic only very recently.

This sounds splendid in principle, but—at least in muscle—it seems to be wrong, probably because of the segmental distribution of anaerobic regions in fibers. The body can repair grossly damaged muscle by proliferation of satellite cells to make new fibers, and this includes fusion of new fibers with the surviving parts of old ones, but the gradual reduction of fiber number during aging[47] suggests that the body may be unable to replace a small segment of one fiber in the middle of a bundle of healthy ones. If so, the only option would be to replace the whole fiber—or, possibly, many fibers—when a segment fails; this scale of fiber turnover would be highly inefficient.

The theory that muscle turnover occurs in response to mitochondrial decline is also challenged by the observed steady accumulation of damaged fiber segments. As noted in Section 5.6, any turnover at all should (if anaerobic cells are indeed the main sources of systemic oxidative stress) lead to an eventual equilibrium situation, where cells are dying and being replaced as rapidly as they are suffering OXPHOS collapse, and not to the steady accumulation of anaerobic cells that is in fact seen.

There is the possibility, however, that whole fibers are destroyed without replacement. This may occur, and would contribute to loss of muscle mass with aging. Moreover, the loss of muscle mass with aging is known to impair many homeostatic mechanisms,[48,49] so can cause increased oxidative stress and accelerate mtDNA damage. This possibility needs further detailed investigation—perhaps also in negligibly senescing species (see Section 12.3).

Cells of some other tissues (such as the liver), however, which can divide on demand but actually do so rather rarely, probably are destroyed fairly quickly when they become anaerobic. If they did not, we would expect to see nearly the same level of anaerobic cells there as in muscle—in fact, probably even more, since the energy utilisation in the liver is very high—but we in fact see only a smaller proportion. (Cells in the liver certainly die for many other reasons, though, so we cannot be sure of this logic.)

A neater—though unmechanistic—explanation is the same as that discussed in Section 6.5.2: we live long enough for our evolutionary niche, so evolution doesn't care. This is certainly not the only example of an "obvious" imperfection (in longevity terms) that evolution has failed to correct: the nonspecificity of macrophages for oxidized LDL, mentioned in Section 5.1, is another. A third is cancer. Malignant tumours can progress beyond a very small size only by the generous co-operation of the body in providing an adequate blood supply, something which one might think it could easily deny. Indeed, some highly promising experimental cancer treatments involve inducing the body not to provide blood supply to tumours.[50a] However, very recent work has shown that older animals have

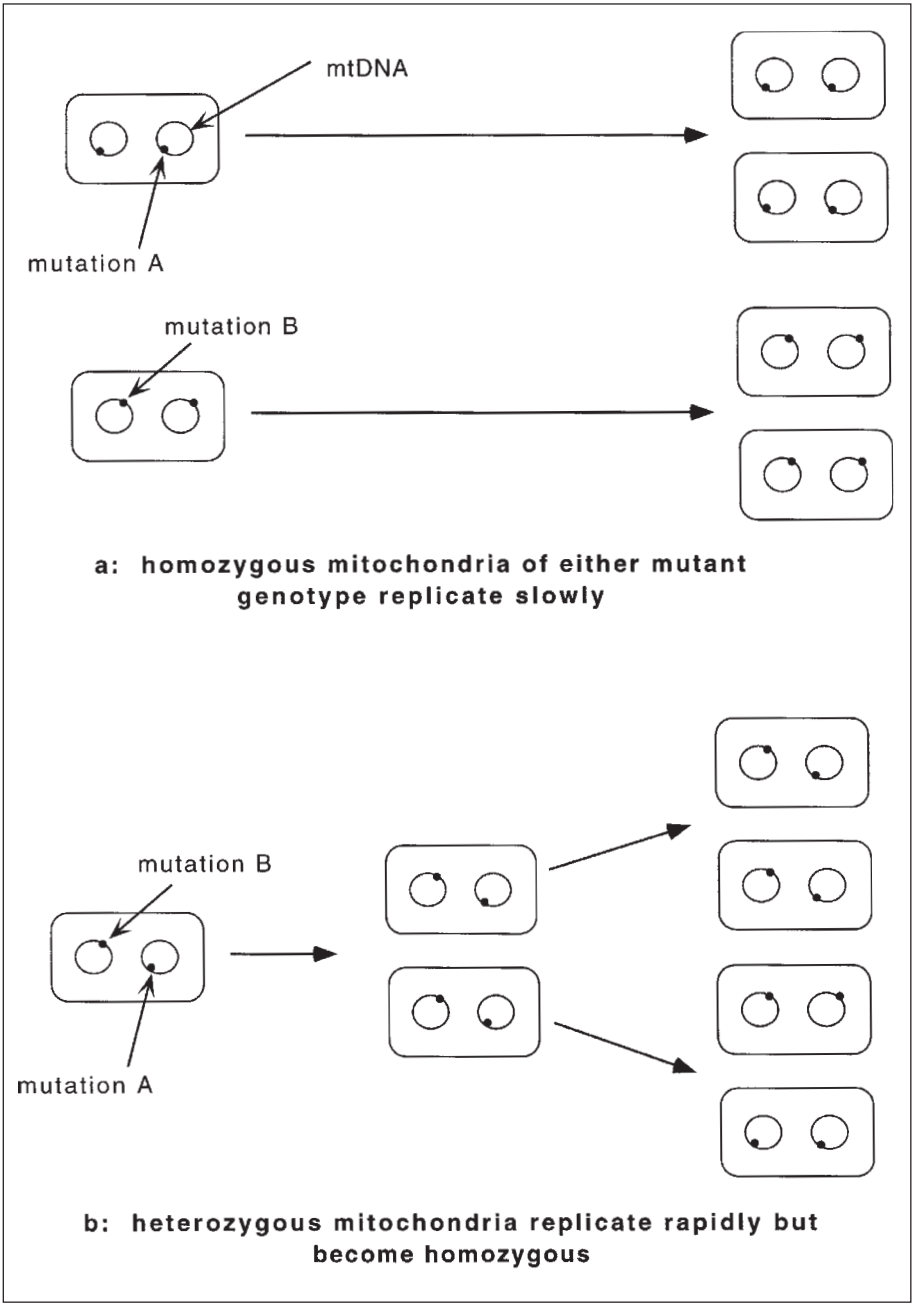

Fig. 10.2. Selection for fusion causes selection for recombination.

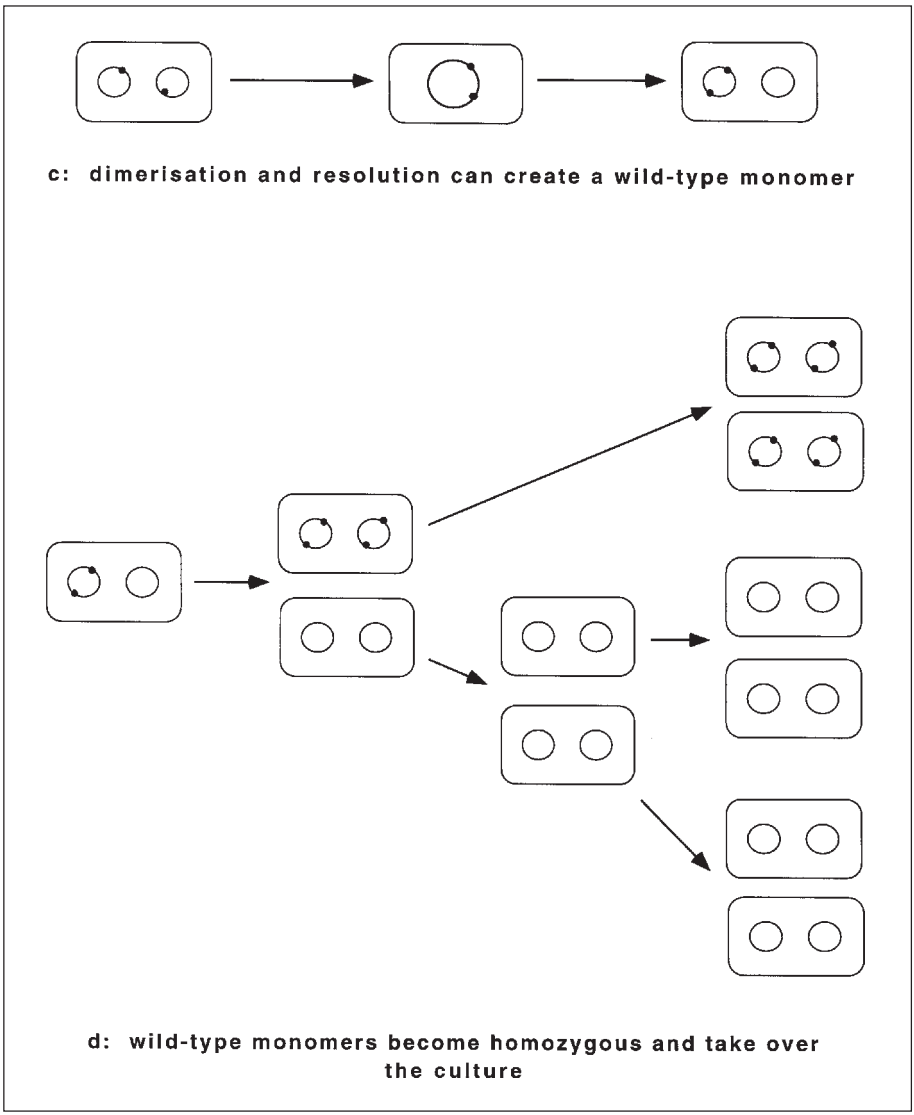

Fig. 10.2., cont. See opposite page

longer anaerobic muscle fiber segments than young ones, suggesting that an affected fiber can last arbitrarily long while the defect slowly propagates along it.[50b,50c]

10.10. What About in Situ Membrane Repair?

In principle, one can imagine enzymatic systems which remove oxidatively damaged molecules from membranes and replace them with pristine ones. In practice, as it happens, such systems do indeed exist. An enzyme which attacks a phospholipid is (naturally) called a phospholipase, and many are known; the one of most interest in this context is called phospholipase A_2. It detaches one of the fatty acid side chains of a phospholipid. (Other

enzymes then remove this chain and replace it with a new one.) But it has the peculiar property that it preferentially detaches side chains that have been oxidized. It is particularly good at this because the chain that it removes, the one attached to the middle of the three carbons that make up the phospholipid's glycerol core, is the one that is most often polyunsaturated (and thus oxidisable). In this way, phospholipase A_2 keeps the level of membrane oxidation down.[51,52]

But it doesn't keep it constant. The property that makes enzymes so very useful in cells is, in this case, a weakness: their specificity. Here, phospholipase A_2 can excise oxidized fatty acid side chains, but only if the oxygen has been incorporated in a place in the molecule that the enzyme can recognise. Lipid peroxidation involves the creation of innumerable varieties of oxidized carbon chain (see Section 3.8), and some of them do not fit the template that phospholipase A_2 recognises. Of course there may be other, parallel systems that do the same job as phospholipase A_2 but on different oxidation products; but still there will be some molecules that none of the systems recognises. If you doubt this, think about lipofuscin. It accumulates in cells during life, and is the indigestible remnant of oxidatively modified lipid and protein; and there we are talking about indigestibility inside a lysosome, where there is a far greater repertoire of destructive enzymes than exists in the cytosol. It is clear, therefore, that a significant subset of the undesirable molecules generated by lipid peroxidation will accumulate in mitochondrial membranes until such time as their cumulative effect causes the mitochondrion to fail, and so to be engulfed by a lysosome, or else to be replicated, diluting the damage.

10.11. How Can SOS Explain mtDNA Depletion?

In Section 6.6 I mentioned that the single-cell analyzes of Müller-Höcker showed that some cells appeared to lose all their mtDNA, not just to become taken over by mutant copies.[53] It is not possible to establish this by looking at enzymatic activity, because the loss of all the mtDNA is functionally equivalent to the mutation of a tRNA—either way, all the 13 proteins cease to be constructed. But several of Müller-Höcker's studies involved staining of the DNA itself, not the protein products.

It is still completely unknown how mtDNA replication is regulated, but we can be sure that the regulation is quite tightly coupled to mitochondrial division. Human mitochondria have about five copies of the mtDNA on average, and this number must be doubled with each mitochondrial division. The copies are partitioned between the two daughter mitochondria. It seems intuitively likely that this partitioning is not always quite accurate, so that perhaps one mitochondrion occasionally ends up with, say, only two copies of the mtDNA. But that should be no problem at all for it, because it can quickly replicate those copies in order to restore its DNA complement to the normal. So mitochondria which undergo this temporary, partial depletion should not be distinguished, during subsequent mitochondrial turnover, from ones which do not.

But there is inevitably a possibility, albeit maybe only a very small one, that a dividing mitochondrion will get the partitioning completely wrong and create a daughter mitochondrion which has no copies of the mtDNA at all. If that happens, then clearly such a daughter cannot haul itself back to normal by mtDNA replication. If it is lucky enough to undergo fusion with another mitochondrion (one which, typically, still has mtDNA) then it will be restored; but, as noted in Section 10.8, fusion seems to be rather rare. And until and unless that happens, it will have exactly the same behaviour—and selective advantage—as a mitochondrion which has (for example) mutated a tRNA in all copies of its mtDNA.

This therefore provides a possible mechanism for the occurrence of cells whose mitochondria are completely lacking in mtDNA. The mispartitioning need only occur less

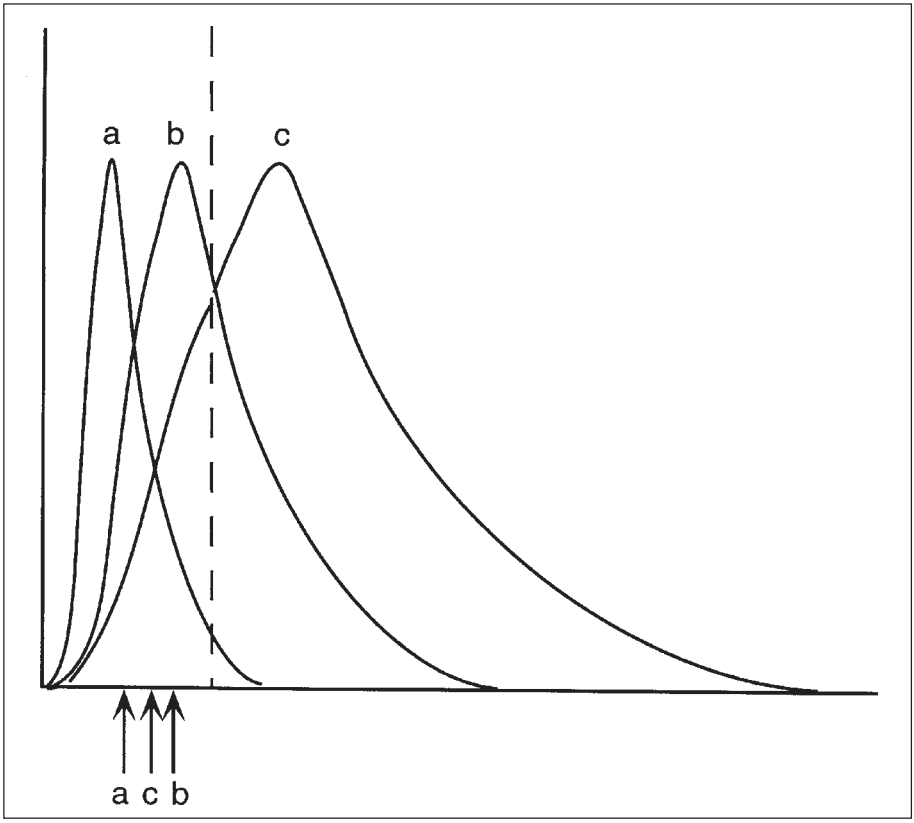

Fig. 10.3. How average subthreshold LDL oxidation (arrows) varies with its overall oxidation.

than once in a billion mitochondrial divisions in order to give rise to the observed frequency of such cells, so I believe that this is a plausible and parsimonious explanation.

10.12. Why Isn't Longevity Mainly Maternally Inherited?

Since mtDNA is almost all maternally inherited, if mtDNA damage drives the rate of aging then the inherited component of longevity might also be expected to be maternally inherited. Three answers are popular; hence they will all be summarized here, even though (as will be seen) only the third really answers the question directly.

Answer 1 is that possibly there is a bias—a greater maternal than paternal contribution to longevity.[54,55] However, no one currently claims that the maternal contribution is, say, 90% of the total.

Answer 2 is that there is no bias because the mitochondrial DNA is not what matters in mitochondrial decline. This view is becoming popular, but it is not really valid to call it the mitochondrial theory of aging. The idea is that the decline in mitochondrial function results from increased oxidative stress, which (by unknown mechanisms) affects the composition of the mitochondrial membrane, and in turn (probably) the efficacy of the respiratory chain. This idea is very strongly supported by recent work.[56a] However, it says nothing about where the oxidative stress comes from in the first place, whereas MiFRA says that damage to

mitochondria is the major source of oxidative stress. It used to be considered satisfactory to invoke the "vicious cycle" as an answer to this—we start off with a little bit of oxidative stress, which has a slight impact on mitochondrial function, which increases oxidative stress, etcetera—but, as discussed in Section 6.6, that logic is very hard to reconcile with the fact that mitochondria in all cells (even non-dividing cells) are constantly recycled. This recycling involves the division of the mitochondrion, which entails the incorporation into it of newly-synthesised, pristine proteins and lipids. That dilutes out the oxidative damage that had accumulated, so there should be an asymptotic, stable level of damage, i.e. no vicious cycle. The only component of mitochondria to which this logic does not apply is its DNA, since the new DNA is synthesised by copying the old DNA so will be as damaged (mutant) as the old copy. So, in summary, if mtDNA damage is not the driving force in aging then nor are mitochondria in general—they can only amplify that force.*

Answer 3, which is the most robust, is that there is no bias because the mtDNA sequence does not significantly affect its somatic mutability. MiFRA is ultimately based on the observation that somatic cells accumulate spontaneously mutant mtDNA. Thus, it says that the rate of aging is determined by the rate at which spontaneous mutations occur. That rate is dependent on, at least:

a. the fidelity of mtDNA replication;
b. the efficacy of repair of mtDNA damage before it becomes stable;
c. the rate at which mutagens (such as free radicals) are generated near the mtDNA; and
d. the rate at which those mutagens are destroyed by antioxidants.

Of these four, only (c) is affected by the mtDNA sequence itself (since free radicals mainly come from accidental loss of electrons from the partly mt-coded respiratory chain). All enzymes responsible for mtDNA replication, mitochondrial biosynthesis in general, repair of mtDNA damage, destruction of free radicals, etc., are nuclear-coded. There is also evidence (from transition/transversion ratio and from the high mutability of the tRNA genes: see Section 2.4.5) that the bulk of spontaneous mtDNA mutation is from replication error rather than from oxidative damage, so (c) may be secondary anyway.

One other way in which the mtDNA sequence can potentially affect its mutability, which has nothing to do with what it encodes, is mentioned in Section 12.1 as a possible test of SOS, because it may be relatively easy to detect.

10.13. Why Don't Plasma Antioxidants Totally Prevent LDL Oxidation?

The problem is as follows: It has long been known that all the cell types present in the arterial wall—endothelial cells, smooth muscle cells, macrophages—can induce LDL oxidation in vitro, but the same studies also found that addition of antioxidants to the culture medium completely prevented LDL oxidation.[57,58] The concentrations of antioxidants which were required for this were well below what exists in plasma.

Far from being a challenge to MiFRA, however, this can be considered as a point in its favour. We cannot doubt that LDL does get oxidized in vivo: this is the conclusion of dozens of studies over the past decade that have explored the etiology of atherosclerosis and LDL's involvement therein. This is therefore a serious paradox, and one that has resisted elucidation for many years. But MiFRA provides a very straightforward explanation. It predicts that the interstitium contains a small—but steadily rising with age—number of anaerobic cells, and that these cells are fairly bristling with lonely electrons all the time. Now, if that same quantity

**A weakness in this logic is that not all the damaged mitochondrial protein and lipid is recycled: a tiny fraction of it accumulates as lipofuscin. Lipofuscin is generally thought to be harmless, but may not be.*[56b,56c]

of electrons were being released into the plasma in an even distribution, we should indeed expect that the antioxidants present in vivo should suffice to absorb them before they can form superoxide or other LECs. But because their release is so focal, it is inevitable that the local concentrations of antioxidants will be saturated. This will result in the annexation of electrons by lower-affinity receptors—particularly by oxygen, which the PMOR does not very readily reduce[59,60] but which is present in much greater concentrations than any antioxidant. The amount of superoxide generated, therefore, will be far greater in the situation described by MiFRA than if the same number of electrons were being released in total but at a uniform rate by all arterial cells.

10.14. Why Doesn't Low Plasma LDL Retard Aging?

The numerous studies of atherogenesis, which have led to a detailed understanding of its mechanisms, were discussed in Section 5.1. A central feature is that macrophages, once attached to the artery wall, express a receptor for LDL particles which is non-specific, whereas the receptor expressed by other cells has an affinity dependent on the particle's degree of oxidation, such that highly oxidized particles are not imported. Atherosclerosis begins when macrophages become engulfed by proliferating smooth muscle cells.

This causative role for LDL oxidation in atherogenesis is now widely accepted,[61] but there is reason to doubt its direct relevance to aging. Atherosclerosis is undoubtedly a major age-related disease, involved in the etiology of both heart attack and stroke. However, its rates of onset and progression are highly dependent on diet, and moreover are far more variable between individuals than are the rates of many other biomarkers of aging. One may therefore wonder whether MiFRA can be held to underlie those other phenotypes of aging. The explanation concerns the **degree** of LDL oxidation.

It was noted above that the standard LDL receptor does not bind oxidized LDL. However, there is a threshold level of oxidation below which LDL is still readily imported by all cells. In a young individual, almost all LDL in plasma is far below this level of oxidation. If the average oxidation of LDL were to double, then the amount that exceeded the threshold for import would rise by a larger factor. But it would still be a small minority of total circulating LDL; the remainder, which was still below the threshold, would nonetheless have an average oxidation level nearly twice the original. Only when average oxidation levels reached a far higher—indeed, unphysiological—value could the average oxidation of sub-threshold LDL slightly diminish, as depicted in Figure 10.3. This means that the blood LDL level does not affect the rate of import of oxidized LDL as it affects atherogenesis: the quantity of LDL imported by a given cell is set purely by its cholesterol requirements, so the average oxidation, not the quantity in transit at one time, determines the amount of oxidized material that is imported. Thus, a role for oxidized LDL in transmission of oxidative stress is consistent with the observation that a diet which promotes low blood LDL levels is a powerful defense against atherosclerosis but does little to retard aging. Rising LDL oxidation will, despite the efforts of arterial macrophages, translate into rising import of oxidized LDL material.

10.15. Isn't This "Reductive Hotspot" Business All Rather Far-Fetched?

Yes. But in my view, to paraphrase Churchill's opinion of democracy, it is the worst theory of aging devised by the wit of man—except for all the others.

This book is not the place to enter into a detailed comparison of the competing claims of the various proposed mechanisms of human aging, so I have restricted such discussion to a summary of my own views at the time I entered biogerontology (see Section 7.4) and this survey of a few items of recent data which I find especially persuasive that MiFRA (in the form presented in Chapters 8 and 9) is on the right track.

A common belief is that experiments designed to accelerate aging are uninformative, because shortening lifespan is easy. This logic is severely flawed: such studies are hugely informative when they "fail"—when the organism exhibits normal lifespan despite the challenge. Two recent examples are especially spectacular, but have received little attention compared to related work that is less relevant to human aging.

The first concerns the possible role of telomere shortening in aging. I mentioned in Section 7.4 that telomeres were shown in 1992 to shorten with age;[62] but in fact, the data presented showed no shortening whatsoever after the age of about 20. This has recently been confirmed in a much bigger study.[63] It makes sense, because the cell type studied was dermal fibroblasts, the ones which replicative senescence studies have most often employed, and these cells must divide during the years when we are growing (since, obviously, our skin is growing in area), but thereafter their turnover is next to nil except when stimulated by tissue damage nearby. But this does not, in itself, tell us whether telomere shortening matters in aging: all it tells us is that telomere shortening of dermal fibroblasts doesn't matter (since it doesn't happen). Therefore, a much more profound conclusion—in short, that telomere shortening doesn't matter in aging at all, at least not in mice—is available from experiments with knockout mice that have no telomerase activity. Their mean and maximum lifespan are absolutely undiminished, even if they are bred together for five generations so that their germ line has progressively shorter telomeres.[64-66] After six generations there are various deleterious effects due to impaired cell division, but that is of no relevance whatever to how normal mice age. Yet, even the researchers who performed this study chose to give more prominence to the phenotype of these sixth-generation mice than to the lack of phenotype of the earlier-generation mice. The result is that much more media attention has been paid to the successful abolition of replicative senesence in vitro by constitutive expression of telomerase[67]—a result which, while hugely important for many biomedical purposes, tells us nothing whatever about aging.

Knockout mice are also the vehicle for the other "failed acceleration of aging" that I find so informative. Knockout mice have been made which lack each of the three isoforms of superoxide dismutase, and only the mitochondrial one causes early (perinatal, in fact) lethality.[68,69] The cytosolic and extracellular ones seem to be wholly dispensable.[70,71] Again, however, the authors of the latter studies[70,71] focused attention not on this aspect of their work but on the finding, much less relevant to aging, that the mice lacking the non-mitochondrial isoforms were less resistant to acute oxidative stress. Partly as a result, much more attention has been paid to the very different (and clearly, again, much less relevant to human aging) results of the corresponding work on fruit flies, whose lifespan is reduced by 80% when they lack cytosolic SOD[72] and increased by 40% when they overexpress it.[73,74] It has been widely overlooked that the survival of the knockout mice is easily the strongest evidence yet for Harman's contention[22] that oxidative damage is especially important in mitochondria. In vitro work has shown a similar pattern for phospholipid glutathione peroxidase (for which knockout mice are not yet available).[75]

I feel that the recent findings that mtDNA mutation levels are actually very high[76-78] must be methodologically flawed, since the phenomenon of clonal amplification of mutant mtDNA must necessarily make highly heteroplasmic cells too short-lived (see Section 9.1). Therefore, if mitochondrial decline really does matter as much as is indicated by the above results, we must either put the blame on lipofuscin (as Brunk has suggested,[56b,56c] but which is not yet supported by in vivo evidence) or else find a way in which a low level of mutant mtDNA can matter. The "reductive hotspot" hypothesis is, as yet, the only way the field has come up with whereby it can matter.

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Chapter 11

A Challenge from Textbook Bioenergetics and Free Radical Chemistry

This chapter might, logically, have been included as a section of the previous chapter, since it addresses a challenge to SOS. A thorough response to this particular challenge, however, is necessarily chapter-sized. The challenge is as follows:

"SOS states that mitochondria which have lost respiratory chain function will do their membranes less LEC-mediated damage. Since such mitochondria increase in number with age, therefore, SOS predicts that the levels of LECs generated by the respiratory chain will fall with age. This is known to be incorrect—superoxide and hydrogen peroxide levels rise with age."[1,2a]

There are two gaps in this challenge. The first is the assumption that the levels of LECs in mitochondrially non-mutant cells—which remain in the great majority throughout life—will stay the same with age. The second is the assumption that the LECs which damage mitochondrial membranes are among those whose levels rise with age. The fragility—indeed, inaccuracy—of these two assumptions is the subject of this chapter.

11.1. The Effect of Oxidative Stress on Non-Mutant Mitochondria

We saw in Chapter 5 that oxidative stress probably impairs everything that cells do. It seems certain, therefore, that oxidative stress makes cells progressively more sluggish in disposing of LECs as they are generated. If so, then an unchanged rate of LEC production (which is what we would predict in mitochondrially healthy cells) will maintain a higher steady-state concentration of LECs.

Thus, superoxide levels may rise with age simply because mitochondrially healthy cells are suffering increasing oxidative stress. Since mitochondrially wild-type cells always outnumber anaerobic ones by a factor of hundreds or more, this change can reasonably be expected to outweigh any reduction in superoxide production by the few mitochondrially mutant cells. The rise in superoxide production with age thus tells us nothing about the **cause** of the rising oxidative stress; in particular it does not eliminate SOS as that cause.

11.2. Perhydroxyl: The Forgotten Radical

The above argument may have appeared to be a clear-cut rebuttal of the challenge with which this chapter began, but it is not. There are both theoretical and experimental reasons to think that, in fact, mutant mitochondria do generate more superoxide than healthy ones. This strikes at the heart of SOS, since without slower peroxidation a mutant mitochondrion will not be amplified. First I shall summarize this theoretical and experimental work, and then I shall explain why higher superoxide production is, after all, compatible with SOS.

The Mitochondrial Free Radical Theory of Aging, by Aubrey D.N.J. de Grey.

11.2.1. Superoxide Stimulation: Theory and Experiment

First the theoretical work.[2b] Coenzyme Q (CoQ), the molecule which transfers electrons from Complexes I and II to Complex III, exists in three forms: ubiquinone (Q), ubisemiquinone ($Q^{\bullet -}$) and ubiquinol (QH_2). The middle of these is a LEC, and is thought to be the respiratory chain component most prone to lose its lonely electron to oxygen, forming superoxide ($O_2^{\bullet -}$). When free in the membrane, CoQ almost never exists as $Q^{\bullet -}$, but it is believed to exist transiently in that state while bound to Complexes I and III,* the proton pumps with which it exchanges electrons.[3-5a] In particular, it probably exists as $Q^{\bullet -}$ at a crucial stage in its interaction with Complex III: the point when it passes from the Rieske protein to cytochrome b. The reason why that point is crucial is that cytochrome b is the only subunit of Complex III that is encoded by the mtDNA. Thus, a knockout of cytochrome b—or, of course, of any tRNAs—would leave CoQ molecules stranded in the radical state (see Fig. 11.1), and thus would cause a rise in superoxide production. This would occur even when Complex I is also failing, because CoQ would still be receiving electrons from the nuclear-coded sources (mainly Complex II). A weakness of this logic is that it assumes that the Rieske protein would assemble into Complex III adequately to be able to accept electrons from ubiquinol, which seems not to be so in yeast;[5b] and, indeed, yeast with no mtDNA seem to make less superoxide than do wild-type.[5c]This may also be so in mammals,[5d] though the opposite has also been reported.[5e] But, importantly, all these studies used dividing cells, in which SOS is not applicable; moreover, it does not help to explain the raised tolerance to hyperoxia of mitochondria whose only defect is in complex IV.[5c]

The experimental evidence that malfunction of the respiratory chain increases the release of superoxide is mainly from in vitro studies of the effects of inhibitors. Many chemicals have been identified which block the respiratory chain; they are often effective antibiotics, since at appropriate doses they work more powerfully on the respiration machinery of bacteria than on mitochondria. Much careful biochemistry, over several decades, has identified the sites at which these various drugs act: not only on which enzyme of the respiratory chain, but whereabouts on that enzyme.[6] These experiments can be coupled with measurements of the rate of formation of superoxide, and can thereby lead to an indication of where in the respiratory chain the superoxide is being made. The conclusion is in accordance with that of the theoretical analysis outlined above: the site of interaction of CoQ with Complex III is a major source,[7-9] as is the site of interaction of CoQ with Complex I, which may operate in a similar way at one or both membrane surfaces.[5a] A recent study[10] presents compelling evidence that the contribution from a functional Complex III is slight when respiration is rapid, but that does not imply that it would be slight when Complex III was dysfunctional.

11.2.2. Perhydroxyl-Initiated Peroxidation In Vivo

So, what hope is there for SOS? Recall first that superoxide is relatively unreactive, and has long been known not to initiate lipid peroxidation itself[11] (see Section 3.5). Now: in order to maintain SOS as a plausible mechanism, while accepting that mutant mitochondria tend to generate more superoxide, one must argue that the rate of lipid peroxidation is not a function solely of the rate of superoxide production. This could be either because

* *Interestingly, the other enzymes which donate electrons to ubiquinone—Complex II, fatty acyl CoA dehydrogenase and s,n-glycerophosphate dehydrogenase—appear not to contribute to LEC production. This is probably because they do not form $Q^{\bullet -}$ at any stage, but instead transfer two electrons in unison from $FADH_2$ to ubiquinone. The more intricate strategy employed by Complexes I and III is needed in order to couple the electron transport to proton pumping.*

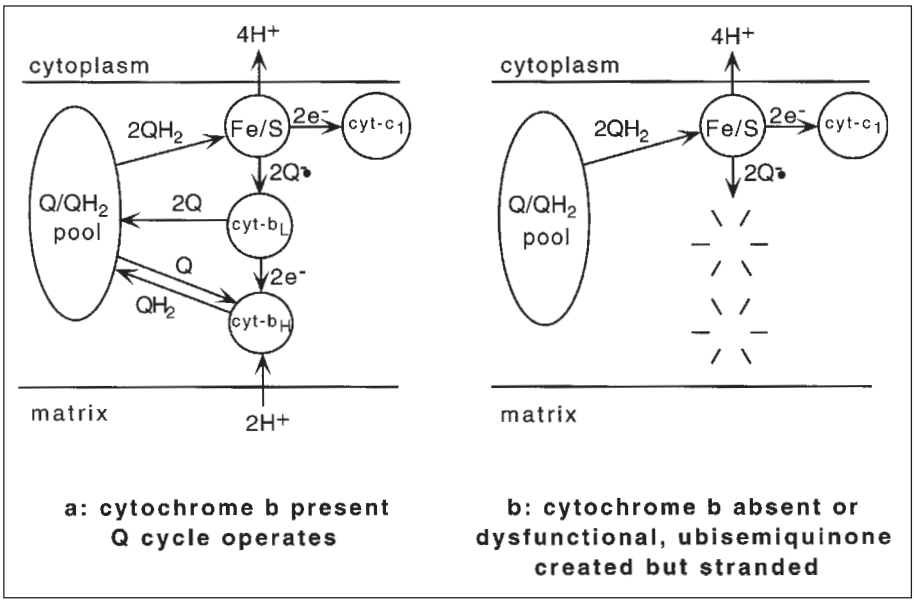

Fig. 11.1. The Q cycle, with and without a functioning cytochrome b.

peroxidation is mainly initiated by some non-derivative of superoxide, whose production does fall as a result of loss of OXPHOS, or else because the main initiator **is** a superoxide derivative but the rate of that derivation falls. I shall argue along the latter lines.

It has been almost universally assumed for many years that mitochondrial lipid peroxidation is mainly initiated by hydroxyl radical, HO•. Superoxide is converted (usually by superoxide dismutase) into hydrogen peroxide, H_2O_2, which can accept an electron from Fe^{2+}• (or Cu^{+}•), and in so doing splits in two to form HO• and water. HO• is vastly more reactive than superoxide and will initiate lipid peroxidation at its first opportunity.

In 1996, however, a very thorough computer analysis was done of the various pathways that can initiate lipid peroxidation in the mitochondrial inner membrane, and it was shown, based on published rate constants, that a completely different pathway is predicted to proceed at a much greater rate than the one just described.[12] Superoxide can, instead, acquire a proton from the aqueous medium—protonation—forming perhydroxyl radical, HO_2•, which is not quite as reactive as HO• but is reactive enough to initiate peroxidation (see Section 3.5). This path had also been known for a long time,[11,13] but it was generally presumed not to be a major source of peroxidation in vivo. The reason its involvement was discounted was that the creation of HO_2• is rapidly reversible—the proton comes off again (deprotonation)—and that that reverse reaction goes much more easily than the original protonation, so that in cells there is predicted to be essentially no HO_2•.[11] There is one caveat, however. The rates of the protonation and deprotonation reactions vary with the pH of the medium, such that if the pH is less than about 4.7 the balance shifts, giving less superoxide than HO_2•.[13] Now, the pH inside cells is much more alkaline than that—between 7 and 7.5, in fact—so theoreticians felt confident in rejecting a role for perhydroxyl. What was overlooked, however, was that the place where the peroxidation of interest occurs, and so in which the pH is relevant, is the surface of the mitochondrial membrane. Recall from Section 4.1 that phospholipids, which are the main constituents of membranes, have a region called the "head group" exposed to

the aqueous medium, and for some phospholipids this group is acidic. Being acidic means that each head group easily loses a proton, leaving it negatively charged so that it attracts free protons into its vicinity. The pH in the water right next to a typical phospholipid membrane, therefore, is considerably lower than in the bulk medium further away from the membrane[14] (Fig. 11.2). The inner mitochondrial membrane has about 20% acidic phospholipids, which gives a pH at the surface about one unit* lower than in the bulk,[17a] so there will indeed be plenty of HO_2• there. Being neutral, HO_2• can also pass through the membrane into the matrix; this may be an important mechanism of proton leak (see Section 8.4),[17b] though unlike straightforward "water wire" transfer of protons it may not be promoted by lipid peroxidation.

11.2.3. Perhydroxyl and SOS

Now for the relevance to SOS. It comes from the fact that pH is, quite simply, concentration of protons in the aqueous medium, and protons are what the respiratory chain pumps. A mtDNA mutation that inhibits the respiratory chain will, therefore, lower the proton concentration—that is, raise the pH—at the outer surface of its host mitochondrion's inner membrane. This will reduce the protonation of superoxide to HO_2• and consequently lower the rate of lipid peroxidation.** It has been argued[18] that all superoxide production must be on the matrix side of the inner membrane, but the evidence cited in support of this[9,19] actually only tells us that some is made inside.

Moreover, there is experimental support for this effect of respiration rate on HO_2• levels. Experiments in 1995 in the laboratory of Joe McCord, the codiscoverer of superoxide dismutase, investigated the effect of respiration on non-enzymatic dismutation of superoxide. Recall from Table 3.3 that non-enzymatic superoxide dismutation does not occur as a reaction between two superoxide anions, but instead either between two perhydroxyls or between one superoxide and one perhydroxyl. (The two-superoxide reaction is not totally absent, in fact, but it is at least six orders of magnitude slower than the others).[20] Therefore, the rate of non-enzymatic dismutation is a measure of pH. And indeed, McCord's laboratory established that non-enzymatic dismutation of externally generated $O_2^{\bullet -}$ in the presence of isolated mitochondria was much faster when their respiration was rapid than when it was inhibited.[21]

On top of all this, a perusal of Table 3.3 shows us that even the levels of HO• may fall when respiration slows! This is worth explaining, because the simulation noted above[12] is currently the only work showing that HO_2• initiates most lipid peroxidation, and many biologists (with history on their side, I fully acknowledge) are very wary of simulations. The reason is that reaction 8 in Table 3.3, which is essentially the only way that HO• is made in vivo, requires not only superoxide but also H_2O_2, which is created by superoxide dismutation. Now, the intermembrane space is one place in the cell where superoxide may be plentiful but superoxide dismutase is absent,*** because the cytosolic version of the enzyme is too

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** The literature is strewn with statements of this pH difference, varying from 2.5[15] to only 0.3;[16] but these have discussed either unrealistic membrane composition or the pH not quite at the surface. The calculation cited here[17a] is based firmly on well-supported measurements of the relevant biological parameters.*

*** The same argument implies that loss of respiratory capacity will lower the pH at the inner surface of the inner membrane, so increasing the levels of HO_2• there. This increase will be much less, however, than the decrease in HO_2• levels at the outer surface, since the pH inside will always remain above the value of about 6.5 generated by the head groups. Thus the net HO_2• concentration on either side of the membrane will be reduced by an inhibition of respiration.*

**** SOD was originally reported[22] to be present in the intermembrane space, but more recent studies[23,24a] have refuted this. The existence of a compartment lacking SOD may explain why birds have paradoxically low antioxidant enzyme levels (see Section 6.5.6).[24b]*

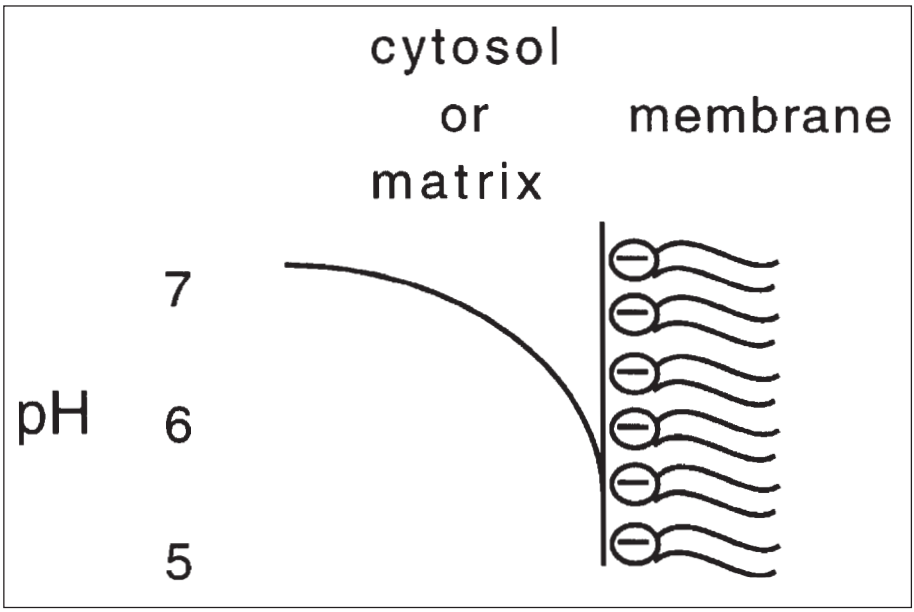

Fig. 11.2. Variation of pH near a phospholipid membrane.

big (as are nearly all proteins) to fit through the pores in the outer membrane and the mitochondrial version is similarly trapped by the inner membrane. Therefore, the H_2O_2 formed in the intermembrane space is that formed by non-enzymatic dismutation, which (as noted above) slows down when respiration slows down. This has been further confirmed by the histochemical detection of singlet oxygen in the intermembrane space;[24c] recall from Table 3.3 that singlet oxygen is generated by the non-enzymatic, but not the enzymatic, dismutation reaction.[24d] Most analyzes have ignored non-enzymatic dismutation and therefore presumed that the H_2O_2 in the intermembrane space gets there by diffusion from either the cytosol or the matrix (where it was formed by superoxide dismutase); but if that is wrong, then the overall level of H_2O_2 may well fall when respiration slows, even if the level of superoxide rises. Yet another reason why this is so derives from the presence in the intermembrane space of cytochrome c, which (when in the ferric state) assiduously detoxifies superoxide back to oxygen by accepting its lonely electron,[25] but which also (when in the ferrous state) just as readily[26] donates an electron to HO_2•, forming—yes—H_2O_2. This too would, therefore, happen at a slower rate if there were less HO_2• present.

Again it may seem as though I have comprehensively rebutted the challenge with which this chapter began. Again, the truth is very different.

11.3. Mitchell's Oversimplification

I may as well warn the reader honestly, in advance, that this section is the most arcane in the book. Bioenergetics is a challenging discipline at the best of times, and the particular topic to be discussed here is one regarding which specialists have been at loggerheads for over thirty years. I include it because MiFRA is incomplete without it, but it may be skipped without loss of continuity.

11.3.1. The Spectacular "Diffusion" of Protons

When I was a schoolboy, my classmates and I were walked through an experiment that taught the concept of pH, and in particular the fact that it is a logarithmic scale. The fact that neutral pH is set at 7 is because of a property of water mentioned in Section 2.3.1.1: in pure water at standard temperature and pressure, almost exactly one H_2O molecule in 10^7 is dissociated, with one proton being separated from the rest of the molecule. This means that the molecule which remains is a hydroxide anion, OH^-, and the proton attaches to a different water molecule forming a hydronium cation, H_3O^+. Thus, the proportion of hydroniums is also one in 10^7, so the product of the proportions of hydroxide and hydronium ions to neutral water is 10^{-14}. The reason the pH scale is possible is that if alkaline chemicals are now added to the pure water, to increase the number of hydroxides, they neutralise exactly enough of the hydroniums so that the product of the proportion of hydroxide to neutral water and that of hydronium remains 10^{-14}. The same is true if the chemical that is added is an acid, so increases the hydroniums instead. Thus, for example, a solution of acid that is at pH 3 has one in 10^3 of its water molecules existing as hydronium and one in 10^{11} as hydroxide.

The fact that neutral water has a pH of 7, i.e. that only one in ten million water molecules is dissociated, means that adding a tiny amount of a strong acid (that is, an aggressive donor of protons to water making hydronium) to a large amount of pure water can change its pH by a detectable amount. The school experiment that I mentioned above was designed to impress this upon us: we would first add a pH-sensitive dye, phenolphthalein, to a large flask of pure water, and then add concentrated acid one drop at a time. The dye stayed the same colour for a while, and then the whole flask changed colour with the addition of just one more drop.

But that, to quote Arlo Guthrie,[27] is not what I came to tell you about. I came to talk about a feature of that experiment which is every bit as striking as the one I just described—indeed, I can visualise it to this day—but which was not pointed out to us and which none of us noticed at the time. When the crucial drop is added, the whole flask changes colour at once—so quickly that one cannot see it spreading out. Think now what happens when one adds a drop of a strong dye to pure water. It spreads out at a highly dignified pace. The behaviour of pH change bears no comparison.* (We will cover the mechanism behind this in some detail below.) And, potentially, this knocks a huge hole in the logic of Section 11.2.

The flaw (or not, as I will argue here) in the logic of Section 11.2 concerns my claim that the rate of a particular mitochondrion's proton-pumping significantly determines the pH—proton (strictly, hydronium) concentration—at its inner membrane's surface. This would be uncontroversial if it referred to anything other than protons, but protons, as explained, diffuse in water at a phenomenal speed, orders of magnitude faster than anything else does. So fast, in fact, that the effect of a given mitochondrion's proton-pumping on its own surface pH can be calculated to be infinitesimal; what matters is the average proton-pumping efficacy of all mitochondria in the cell. The pH would vary somewhat, at least temporarily, if all the mitochondria in a cell stopped pumping protons simultaneously; but that is not the situation which SOS proposes.

I shall present in this section my reasons for believing that the above "flaw" is itself flawed, because the proton concentration right next to the membrane is held some way from equilibrium with the concentration further away—and is held there by OXPHOS, such that, as SOS requires (and as is indicated by the dismutation results noted in Section 11.2.3),[21] the rate of OXPHOS determines the distance from equilibrium. This is a severe deviation from

* *In fact, this can partly be put down to the fact that small molecules, such as hydronium, diffuse faster than large ones such as dyes. But not totally: the observed rate of diffusion is even faster than would be predicted on that basis.*[28,29]

standard bioenergetics, but most of my arguments are not new. The final link is due to me, however.[30]

11.3.2. Is the Bulk-to-Bulk Proton Gradient Adequate?

Mitchell's chemiosmotic theory, described in Section 2.3.4.2, was first published[31] at a time when the mechanism of oxidative ATP synthesis had been the object of a huge amount of research worldwide for some years, and everyone engaged in this research had (as it turned out) been frustrated as a result of a shared false assumption—that there existed a chemical intermediate which transfers energy between the respiratory chain and the ATP synthase. Because the field was so fixated on the existence of such an intermediate, it took many years for Mitchell to win people over, even though in hindsight there can be no doubt that his theory—even in the rather preliminary form in which he first presented it[31]—had all that a theory ought to need in order to be compelling: elegance, simplicity, and absolute accordance with experimental data.

This is well known. What is much less well known is that a second battle has raged over the chemiosmotic theory, beginning in 1969, which is still unresolved. In this case it is the other way around: a challenge to, rather than a defense of, one aspect of Mitchell's model. Perhaps by way of atonement for their unjustifiable reluctance to accept the original chemiosmotic theory without a great fight, bioenergeticists have been, if anything, even more adamant in their subsequent reluctance to entertain variations to it. This particular variation not only has extensive experimental support: it is also very relevant to SOS. Thus it will be explored in detail here.

The chemiosmotic theory as originally set out in 1961[31] stressed that the proton pumping of the respiratory chain generates a proton gradient across the mitochondrial membrane, which forces the weaker proton pump (the ATPase) into reverse, constructing rather than hydrolysing ATP. This concise statement of the chemiosmotic hypothesis can usefully be broken down into four testable postulates:

1. The respiratory chain transfers protons across the membrane.
2. The ATP synthase also transfers protons across the membrane.
3. Protons do not generally pass across the membrane by other means.
4. In general, ions also do not pass across it freely, only when actively transported.

In that original paper, Mitchell noted that the ATP synthase would only synthesise ATP if there were a substantial asymmetry in the distribution of protons on either side of the enzyme; with no asymmetry it would catalyse the reverse reaction, the hydrolysis of ATP to ADP and phosphate. He mentioned that this asymmetry could be manifest as a difference in pH or as an electrical potential difference, or as a combination of the two; he did not, however, speculate on what combination was present in actual mitochondria in vivo. But in 1966 he published a much more detailed restatement of the theory,[32] in which he laid great stress on the concept of proton-motive force as the combination of these two components; furthermore, he defined them more precisely than before. One, usually denoted ΔpH, is the difference in pH between the bulk aqueous phases on either side of the membrane. The other is called $\Delta\psi$, and it derives from the fact that the membrane is generally impermeable not only to protons but to other ions. It is the difference in electrical potential, caused by a tiny imbalance in the concentrations of all ions added together, between the sides of the membrane. Mitchell found it necessary to invoke $\Delta\psi$ in order to reconcile the chemiosmotic theory with the (by then) known fact that the mitochondrial ΔpH in vivo is nowhere near enough to force ATPase into reverse, i.e. to drive ATP synthesis.

It is important to note that Mitchell's invention of the concept of the bulk-to-bulk proton-motive force as the driving force of OXPHOS was not merely a clearer exposition of what he had written in 1961. It incorporated a new assertion, over and above the four

postulates he had originally discussed (and all of which had, even by 1966, already been quite thoroughly confirmed by experiment). This new assertion is that the bulk aqueous phases inside and outside the mitochondrial inner membrane are each electrochemically homogeneous: that the combination of the electrical potential and the pH is the same everywhere in the matrix and also the same everywhere in the cytosol, so that the difference between the two—the proton-motive force, as Mitchell had defined it—would be the same when measured between any point in the cytosol and any point in the matrix as it was across any of the OXPHOS enzymes. This model is known as the delocalized chemiosmotic theory.

This may seem, at first hearing, to be an altogether uncontroversial assertion. After all, the pH and electrical potential are formed by protons and other ions, all of which can surely move freely throughout the aqueous space that they occupy (the matrix or the cytosol), so it seems hard to question the assertion, i.e. to propose a proton-motive force between two points within the same aqueous compartment. Indeed, no one really did question it—until they were forced to.

Researchers of course sought to measure the proton-motive force between the cytosol and the matrix in order to test this refined chemiosmotic hypothesis. This was not very easy, though, because mitochondria are so small. One cannot get an electrode inside a mitochondrion, for example. Therefore, measurements were made by indirect means: by measuring the rate at which detectable (usually fluorescent) ions passed through the membrane in appropriate conditions, and calculating how strongly they were being pushed.[33,34] These experiments confirmed that the proton-motive force was about what it needed to be to drive ATP synthesis.

But in 1969, Henry Tedeschi and colleagues reported[35] a much lower—indeed, negligible—value. This would have been unexciting if their method of measuring it had been similar to what others had used; it is, after all, not uncommon in science for experiments whose design initially seems valid to be found at fault in later years. But they had used a method which was not the same at all, and which, most importantly, was far more direct. They had succeeded in doing what I just said was impossible—getting an electrode inside a mitochondrion, thus allowing them to measure the potential difference between the matrix and the cytosol purely electrically, avoiding any inferences based on the behaviour of chemicals not present in vivo. They did this initially by using mitochondria that are a great deal larger than normal, and in later experiments by making normal mitochondria swell.

This result was not well received. For the next decade and more, bioenergeticists raised challenge after challenge to the validity of Tedeschi's techniques and/or results. Each time, he and his coworkers responded by improving the experimental design so as to confirm that the result was real. In the late 1970s, they succeeded in showing that the mitochondria were generating ATP at the usual rate, even while they were impaled by an electrode and their $\Delta\psi$ was being measured (and found to be about zero).[36] They also showed, by ingenious use of a mitochondrion impaled by two electrodes, that the impalement was real—that the electrode was not just encased in an invagination of the (unpunctured) membrane.[37] Finally, in 1984 they eliminated the possibility that the swelling of the mitochondrion had somehow lowered its internal pH, allowing ΔpH to drive ATP synthesis unaided.[38]

Most specialists, however, remain sure to this day that, robust though the evidence appears to be that these measurements are reliable, the chemiosmotic theory is simply too well confirmed to be rejected on this basis. (No discussion whatever has appeared regarding the challenge to Mitchell's model posed by the superoxide dismutation results discussed in Section 11.2.3,[21] doubtless because they were not presented as such.) They have decided that there must be something wrong with the experiments that report inadequate $\Delta\psi$, even though exactly what is wrong has not been established. But, as stressed above, Tedeschi's

results do not challenge the chemiosmotic theory sensu 1961, only its later elaboration. Conversely, verifications of the chemiosmotic theory have involved the way in which mitochondria function, and in how this function can be inhibited or stimulated. They have not, in particular, addressed directly the question of whether the immediate environment of the mitochondrial membrane is (for these purposes) faithfully represented by measurements of the medium some way away from the membrane.

11.3.3. Macroscopic Restriction of Ion Movement

One important factor that may well have caused Tedeschi's work to be so poorly received was that he never proposed an alternative hypothesis to Mitchell's. In fairness, though, it is indeed hard to see how an aqueous compartment can be anything other than electrochemically homogeneous, because ions—especially protons—diffuse so fantastically fast in water.

One candidate for a barrier to homogeneity[39] is the shape of the inner membrane. The intricate cristae into which it is folded will inevitably hinder the flow of protons, and it is reasonable to suppose that occasionally the membrane will come together and separate a scrap of cytosol in the intermembrane space from the main bulk. Topologically, that scrap of cytosol would then resemble the inside of a chloroplast's thylakoid membrane: since it was no longer in electrochemical contact with the cytosol, proton-pumping would be able to acidify it sufficiently that the pH difference across the membrane could drive ATP synthesis unaided. But this will probably not happen often enough, or anyway not for long enough at a stretch, to affect the pH to a chemically significant degree. Moreover, we must recall that some of Tedeschi's experiments were performed with "giant" mitochondria, whose outer membrane had been removed and whose inner membrane had then been swollen by osmosis to its maximum possible size—in other words, ironing out all the cristae. And those mitochondria still phosphorylated properly, with apparently not nearly as much bulk-to-bulk proton gradient as should be needed according to Mitchell. So this idea—which can be termed the "pseudothylakoid hypothesis"—is not the answer.

11.3.4. Totally Localized Coupling

So far, we have seen that in order to reconcile Tedeschi's results with Mitchell's 1961 theory, protons must somehow be restrained from rapid movement in all three dimensions within the cytosol and the matrix. There are two basic alternatives to three-dimensional proton movement, and both have been explored: namely, two-dimensional and one-dimensional. The one-dimensional version has been tested experimentally, and appears not to be true. What it says is that the primary proton pumps (the enzymes of the respiratory chain) and the secondary ones (the ATP synthases) are arranged in one-to-one juxtaposition, either all the time or periodically, such that they pass protons between them by direct contact. If this were so, protons would by and large never escape into the bulk aqueous phase, so we would indeed not see a $\Delta\psi$.

A rather elegant test of this involves noting that it predicts not only an inhibition of proton movement into the bulk, but also inhibition of proton leak across the membrane. As noted in Section 8.4, leak exists in an easily measurable amount; it varies with the proton-motive force, as one would expect. Now, the proton gradient can be reduced by adding an ionophore (a chemical that simply makes the membrane leakier to ions), such as carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (mercifully abbreviated FCCP); and it can also be reduced by adding ADP and phosphate, because that will stimulate the ATP synthase to work faster and dissipate the proton gradient. So, if the ATP synthase receives its protons only by a direct transfer from a respiratory chain enzyme, then adding ADP and phosphate will cause a rapid loss of proton gradient and rise in respiration rate, whereas the

addition of FCCP will cause a slower response, dependent on the rather few protons that leak out of the localized coupling system. In fact, the kinetics in the two cases are the same.[40]

11.3.5. Proton Conduction Very Close to a Phospholipid Membrane

So much for the idea of one-dimensional proton movement; what about two dimensions? The definition of the two-dimensional model is that protons involved in OXPHOS can move freely within the vicinity of the mitochondrial membrane, but (for some reason) cannot move away from the membrane. The challenge, therefore, was to identify such a reason.

First of all we must be clear about the relationship between Mitchell's model and the surface effects described in Section 11.2.2. Mitchell asserted that the water throughout the cytosol, including right up to the membrane, was always electrochemically homogeneous (due to the very fast conduction of protons): the combination of pH and electrical potential was uniform. This is not to say that the pH is uniform right up to the membrane—there is no doubt that the pH is lower near the membrane, as explained in Section 11.2.2—but rather that the gradient in pH (see Fig. 11.2) is exactly cancelled out by a gradient in electrical potential (Fig. 11.3). In particular, Mitchell's model did not allow for any non-uniformity deriving from proton pumping.

The theoretical breakthrough (or so it briefly seemed) that Tedeschi had failed to provide came in 1979, when Douglas Kell proposed a reason why the pH right at the membrane may indeed be influenced by proton pumping, without a counteracting change in electrical potential there.[41] This can only be so if there is some barrier to the movement of protons in the direction perpendicular to the mitochondrial membrane, so that they cannot move equally freely in all three dimensions. He named his model the "electrodic" view, for reasons which will become apparent.

The idea relies on the details of how water conducts protons, which is very different from how metals do it. We saw in Section 11.3.1 that this conduction is fantastically rapid; the way this works at the molecular level is called the Grotthuss* mechanism,[42,29] which makes use of two properties of water:

1. That each hydrogen atom is linked by a covalent bond to one oxygen atom and by a hydrogen bond to another, and
2. That excess protons are always bound to water molecules, making hydronium, H_3O^+.

Conduction is a two-step "hop and turn" process, whereby in step one hydrogen bonds become covalent and covalent bonds become hydrogen bonds, and in step two some of the water molecules rotate to allow another hop (Fig. 11.4). The rotation part is proposed[43-45] as an explanation of why ice conducts much better than water, despite being composed of molecules whose freedom to rotate is clearly less than in liquid water: in ice the tendency of water molecules to dissociate is much less, so that "neutral pH" is about 10.5, and this reduction in the number of protons present at any one time means that protons can usually hop by quantum tunneling, for which the rotation step is not necessary.

There is one more feature of the pH near a phospholipid membrane that we must bring into play before Kell's idea can be presented. The acidity of the membrane head groups must create not only an acidic environment but a steeply graded one—that is, an electric field, similar to that surrounding an electrode immersed in water. Now, some molecules

** The paper in which de Grotthuss suggested this mechanism[42] is the oldest publication referenced in this book, dating from 1806. It is amusing to note that he suggested it as a general mechanism for the transmission of charge in ionic solutions, a hypothesis which was rapidly shown to be false; thus, by the attachment of his name to the mechanism of proton conduction he has been honored for an idea which was almost, but not quite, completely wrong.*

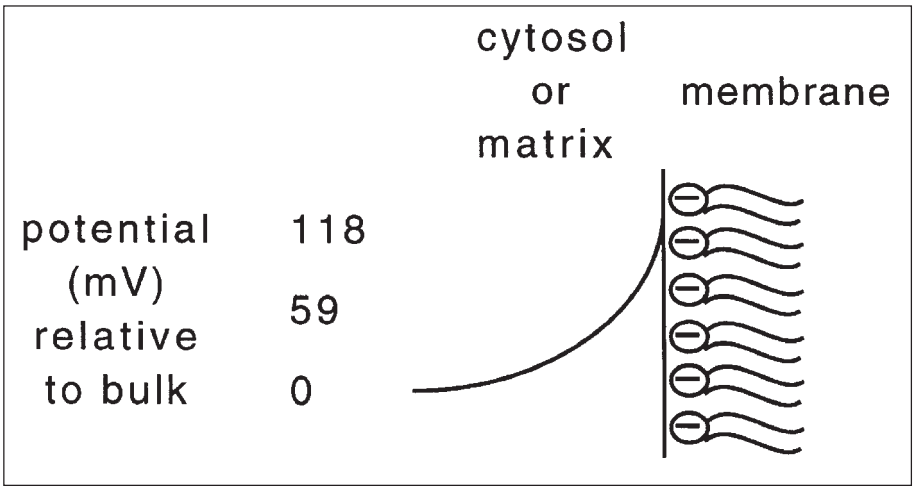

Fig 11.3. Variation of potential near a membrane (independent of proton pumping).

have an intrinsic variation in the density of charge within themselves, due to their molecular structure: one end of the molecule is more positively charged, the other end more negative. Such molecules are called polar. Some polar molecules have one charged end and one neutral end, and others have opposite charges at either end; the latter are called dipolar. Any dipolar molecule which is placed in an electric field will have a tendency to orient itself in such a way that its own positive charge is nearest to the negative side of the field, and conversely its negative charge is nearest to the positive side.

The reason why all this is relevant is that water is a dipolar molecule. Its two hydrogen atoms are attached on either side of its oxygen atom, but not in a straight line—they make an angle of about 130°. Thus, water molecules in an electric field will tend to "point" (if you think of that angle as an arrow) towards the positive side of the field. There is also random movement and reorientation going on all the time—that is what keeps water liquid—but calculations show that the field next to the inner mitochondrial membrane will be strong enough to inhibit that natural random orientation of water molecules, and make them mostly line up with their oxygen atoms away from the membrane and their hydrogen atoms towards it[28] (Fig. 11.5). Hydronium is also a dipole—the three hydrogens arrange themselves to make a pyramid with the oxygen[46]—so it behaves the same way. Finally we come to Kell's insight: that this bias of orientation produces, effectively, a series of one-molecule-thick layers of ice.* There will be a two-dimensional hydrogen-bonded network of water molecules coating the membrane, oriented to allow ease of proton transfer, thus facilitating conduction of protons across the face of the membrane. Not only that: the hydrogen-bond connections between the layers will predominantly not be favorable to the relevant proton transfer, thus impeding conduction perpendicular to the membrane. Therefore, proton conduction would be predominantly in two dimensions. Kell proposed[41] that this semi-permeable "insulation" of the surface water from the bulk would allow the respiratory chain to generate a greater

** Ice-like in regard to freedom of rotation, but not in regard to conduction, since the pH is around 6.5 whereas the pH of pure ice is about 10.5.[43-45] The inferences with regard to conduction thus derive only from the restriction on rotation caused by the electric field.*

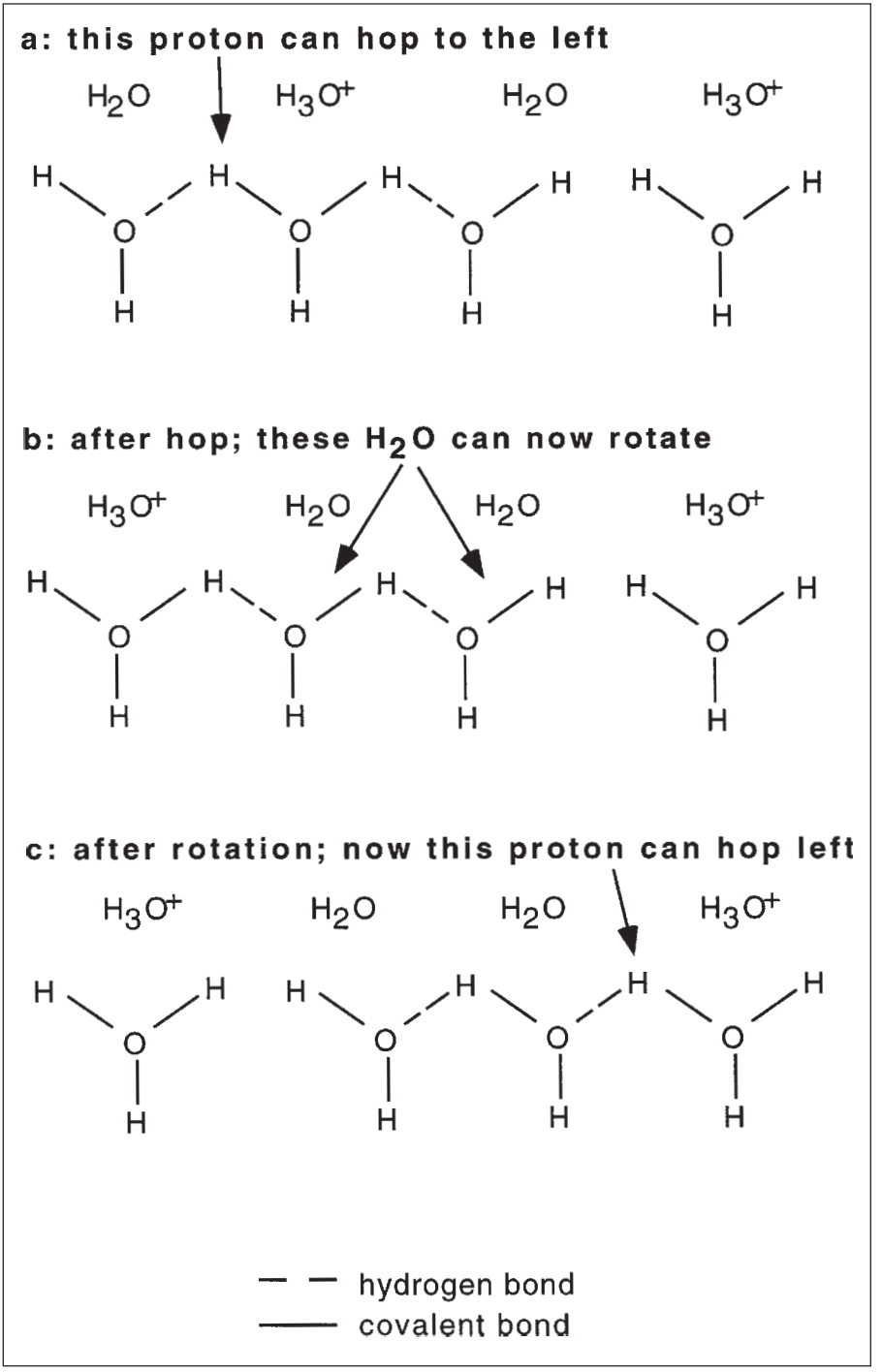

Fig. 11.4. The Grotthuss mechanism of proton conduction in water.

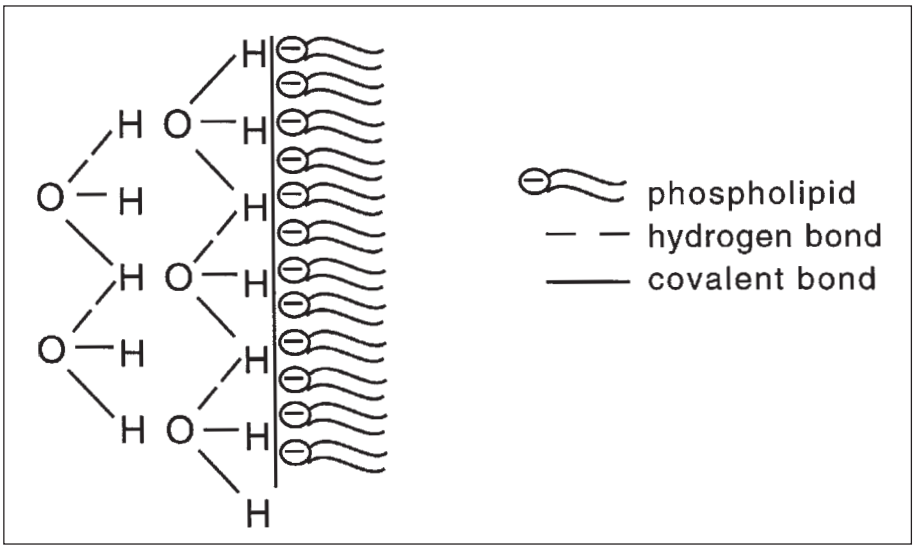

Fig. 11.5. Orientation of water and hydronium layers near the membrane.

density of protons at the inner membrane's outer surface than in the bulk cytosol, and similarly a lower density at the inner surface than in the bulk matrix. The surface-to-surface difference in proton density (i.e. in pH) would be what was felt by the ATP synthase, so the idea was that a $\Delta\psi$ was not needed after all, because the surface-to-surface pH difference was enough to drive ATP synthesis unaided. The proposed variations of proton-motive force are shown in Figure 11.6, with notation that will be used hereafter for the forces between various locations.

The proposal that proton conduction is not isotropic in three dimensions has been confirmed experimentally. Experiments in the laboratory of Teissié have shown that protons move along the surface of a membrane much faster than they move away from or towards it.[47] Other work has, conversely, demonstrated the existence of a barrier to proton movement perpendicular to the membrane.[48-50]

A particular attraction of this hypothesis was that it did not simply give theoretical credence to Tedeschi's findings: it also reconciled them with the confirmation of Mitchell's "delocalized" model that had repeatedly been achieved using ion-distribution methods (see Section 11.3.2). This was because the ionic dyes that were used for this purpose were, necessarily, ones which were relatively soluble both in water and in lipid, so that they could diffuse across the membrane. And such molecules are exactly the sort that will act chaotropically on the water at the membrane surface: that is, disrupt its organisation, allowing protons to move in the third dimension, perpendicularly to the membrane, at a much higher rate than usual. That easier proton flow will rapidly dissipate δp_c and δp_m. Kell proposed that the mitochondrion responds to this collapse—which necessarily equates to a collapse of ATP synthesis—by cation transport that generates a real $\Delta\psi$; that new, non-physiological $\Delta\psi$ then causes diffusion of the ionic dyes, whose interpretation as a real $\Delta\psi$ is thus correct.

This potentially saves SOS from the challenge described in Section 11.3.1, that proton conduction is too rapid to allow an individual mitochondrion's respiration rate significantly to affect the pH near its inner membrane. The faster the mitochondrion is respiring, the more of a proton-motive force it will make between the surface and the bulk (on both sides

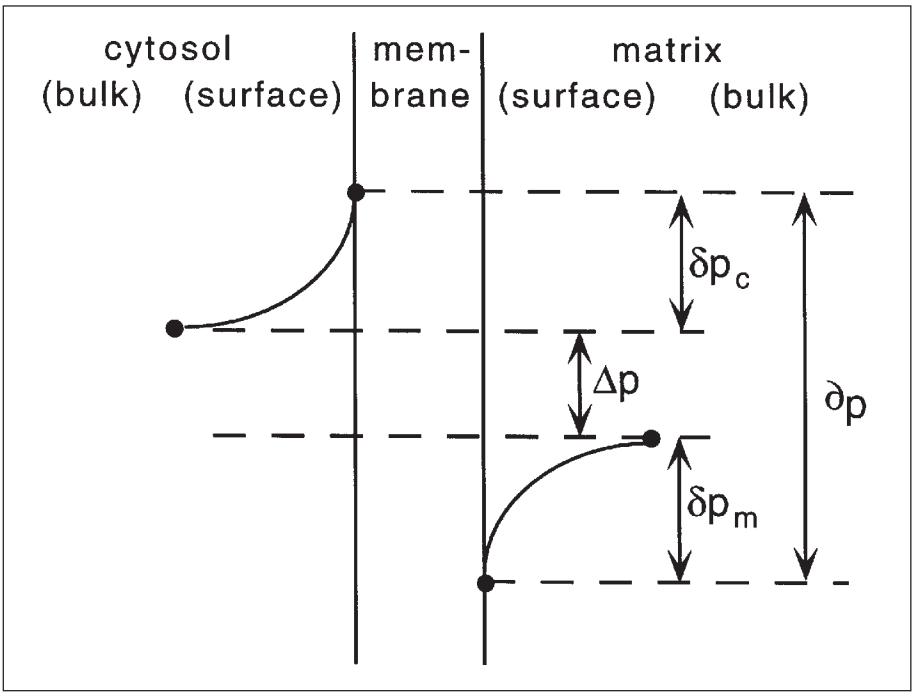

Fig. 11.6. Variation in proton-motive force ("p") according to the electrodic hypothesis.

of the membrane). This force will be composed entirely of pH difference, since all ions other than protons are unaffected by the organisation of water and will therefore move to equilibrate any difference of electrical potential between surface and bulk. Therefore, the pH at the outer surface of the inner membrane will be lowered by respiration, and conversely raised by the inhibition of respiration that arises when the mitochondrion becomes genetically unable to build the respiratory chain. The effects on the rate of lipid peroxidation then follow as discussed in Section 11.2.

11.3.6. The Gap in the Electrodic Hypothesis

Nonetheless, this revision of the chemiosmotic theory has not been accepted. There is a major gap in its logic, which was in fact identified in 1981 by Mitchell himself.[51] It is so conclusive that Kell also rapidly discarded the electrodic model in favour of a much more complex scheme[52] which invokes additional, still unidentified proteinaceous components of the proton circuit. That model also has difficulties; it will not be explored further here.

The problem with the electrodic model becomes apparent when one bears in mind that it is proposed to apply to mitochondria at steady state. That is, not non-respiring ones, but ones whose rate of respiration (and ATP synthesis) is constant. The organised orientation of the surface water will impede proton movement perpendicular to the membrane, but—this is the crucial point—it will not abolish it. Thus, if there is a proton-motive force between the surface and the adjacent bulk on one or both sides of the membrane (δp_c or δp_m)—as there must be if ∂p is to exceed Δp (Fig. 11.6)—then there will be a net flow of protons down that proton gradient, which, however slow, will inexorably

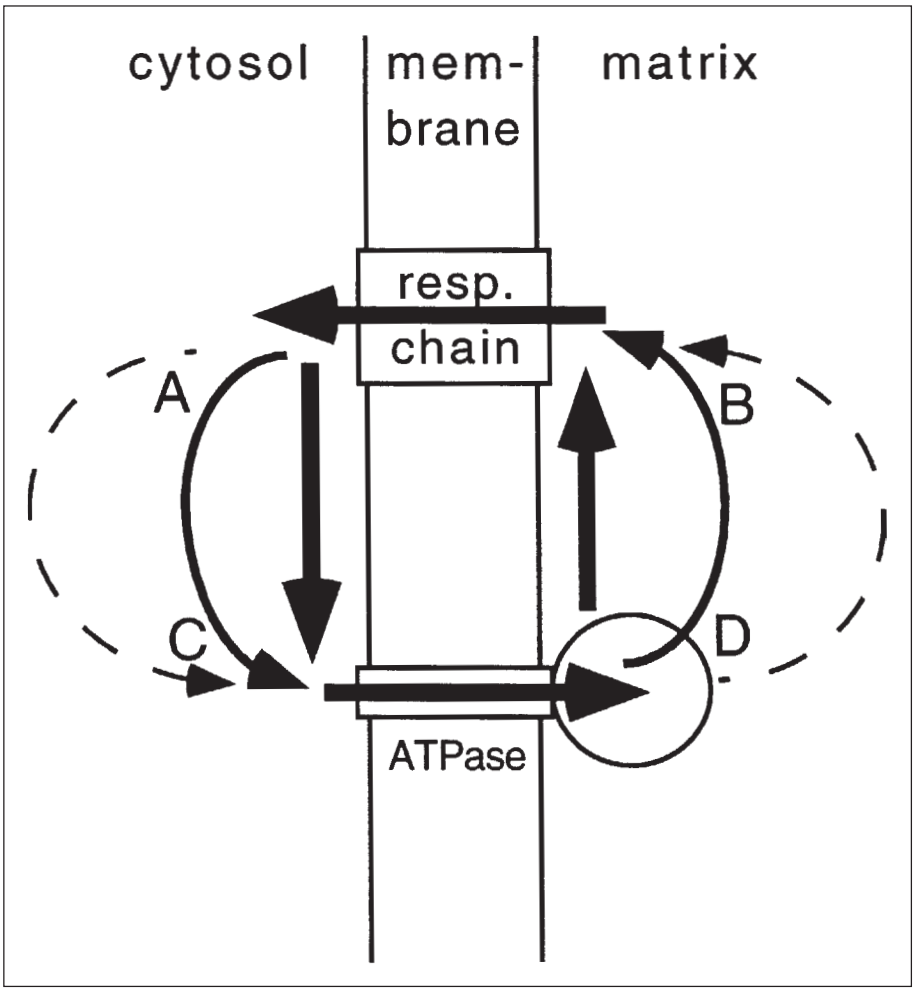

Fig. 11.7. Routes of proton flow according to the electrodic hypothesis.

continue until δp_c and δp_m have been completely dissipated. This can be depicted another way (Fig. 11.7): the currents at C and D are required to equal those at A and B, but if there is a δp_c or δp_m they cannot.

11.3.7. Resuscitation of the Electrodic Hypothesis

This story (so far) closes with my very recent contribution,[30] which proposes to resolve this difficulty and thereby render the electrodic model admissible again. The dissipation of δp_c and δp_m can be avoided if the proton current that they drive is somehow compensated by a separate current the other way. I realized that it was an oversimplification to reduce the system to just the mitochondrial membrane, the respiratory chain and the ATPase, because steady-state OXPHOS also requires the steady-state operation of several metabolite carriers. The one of most interest is the phosphate carrier, which has a high rate of turnover (once per ATP molecule generated in the matrix) and also, crucially, transports phosphate in

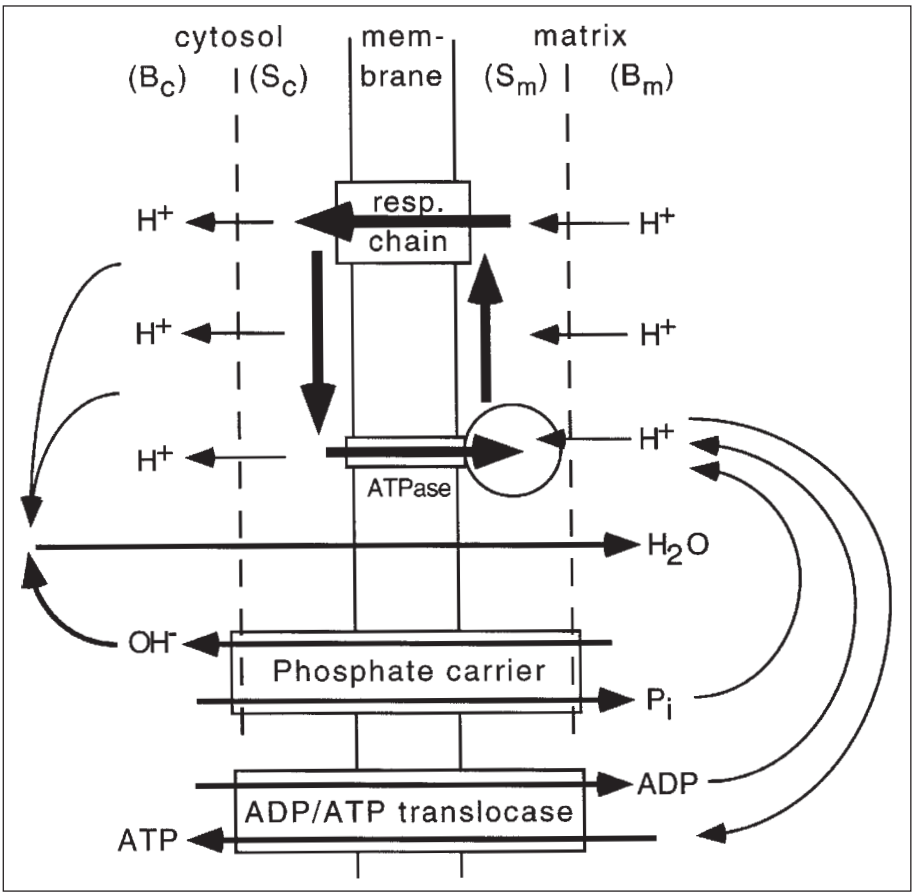

Fig. 11.8. Routes of proton and hydroxide flow incorporating the phosphate carrier.

antiport with hydroxide.[53,54] The relevance of this is that the permeability of the inner membrane to water[55] renders hydroxide transport functionally equivalent to reverse proton transport, so that if the phosphate carrier were trapping hydroxide ions in the matrix and releasing them in the cytosol at some distance from the membrane—perhaps a nanometer or so further away from it than the distance at which the respiratory chain and ATPase were trapping and releasing protons—then the inevitable (see Section 11.3.6) steady-state current in the aqueous medium, perpendicular to the membrane, would be sustained indefinitely (Fig. 11.8). It is plausible that hydroxide trapping and release should indeed occur some way from the membrane, because they are in more plentiful supply there (on account of the pH gradient, Fig. 11.2) so the carrier can achieve faster turnover. Another circumstantial point in favour of this idea is that the one energy-transducing biological system which is not associated with hydroxide transport, namely the thylakoid of chloroplasts, does make a sufficient ΔpH (bulk-to-bulk) to drive ATP synthesis unaided. A third one is the coordinate loss of inner membrane cardiolipin and OXPHOS performance with age: cardiolipin is the only charged phospholipid present in significant amounts in that membrane (see Section 4.1), so cardiolipin depletion reduces the organisation of surface water and allows more protons

to cross between surface and bulk, thus reducing the surface-to-surface proton-motive force (∂p) and impairing OXPHOS. It has indeed been shown[49] that the degree to which proton conduction is preferentially lateral varies with the density of negatively charged head groups.

This idea is too new to have undergone the detailed analysis by the bioenergetics community that will be needed before it can be accepted as a valid refinement of the chemiosmotic theory. I anticipate that such scrutiny will be intensive, because the ramifications of this model for our understanding of mitochondrial function are very profound. For example, one vital role of mitochondria in vivo is cellular calcium homeostasis: they are able to take up and store calcium when there is an excess of it in the cytosol, so stabilising its cytosolic concentration. The textbook model for how they achieve this relies on the presence of a large bulk-to-bulk $\Delta\psi$, which causes cations (of which calcium in solution is of course one) to leak, slowly, through the mitochondrial membrane. If there is in fact no $\Delta\psi$, that mechanism must be radically revised.

Presuming that this model survives such scrutiny, however, it finally shows—after a whole chapter of twists and turns—that SOS is, after all, compatible with the rise in superoxide levels that is caused by certain types of respiratory chain inhibition.

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Chapter 12

Some Testable Predictions of MiFRA

It has often been observed that gerontology is heavy on data but light on theory. A consequence of this is that, when a theory is propounded which fits the data already available, it may be unusually difficult to identify experiments to test it: the problem is that so many of the experiments have already been done in the attempt to inspire a theory in the first place. Nonetheless, several tests of MiFRA are available. Before introducing them, it may be worth stressing that each really only tests a component of MiFRA. In a sense this is inevitable for a theory with so much structure and detail; furthermore, it inspires confidence that, if MiFRA is broadly correct but some details are wrong, the falsification of those details will lead more rapidly to a corrected hypothesis than is typical when an entire theory has to be abandoned.

12.1. Some Predictions of SOS

The hypothesis that SOS is the mechanism of mtDNA decline with aging makes a specific prediction with regard to which mutations will and will not accumulate in affected cells. As noted earlier, SOS is proposed to occur when the mutation has the effect of reducing the proton gradient at the inner membrane of its host mitochondrion. Any mutation that inhibits the respiratory chain will have this effect, but a mutation that inhibits the ATP synthase, without at the same time inhibiting the respiratory chain, will not. Therefore, point mutations in the two ATPase subunits should not accumulate in postmitotic cells.

I noted in Section 8.5.4 that there is already preliminary evidence in support of this. Müller-Höcker has reported seeing cells which lack ATPase activity, but that all such cells had also lost cytochrome c oxidase activity.[1] However, this result must be considered preliminary since the antibody used stains the wholly nuclear-coded F_1-ATPase as well as the F_o. Similarly, Schon has reported a failure of accumulation of point mutations in the region 8991-8995 within ATPase 6, determined by an assay which did show slight accumulation at a tRNA site[2] (see Section 6.6.4). Again, though, the fact that this study only assayed for mutations at five of the 844 base pairs in the ATPase genes weakens the confidence with which one can cite it in support of SOS. What is needed is a systematic survey of point mutations throughout the mitochondrial genome, to establish which do and do not accumulate.

It must be stressed that such a survey should, if at all possible, look at mutations that are known (or seem very likely) seriously or totally to disrupt the relevant gene's function. The preference of most studies to date has been to pursue point mutations which have been shown to cause inherited diseases, but the fact that such mutations are inherited strongly suggests that they have only a rather slight deleterious effect on gene function: if they were more serious, they would cause embryonic lethality. This is clear from an examination of the range of mutations so far identified as causing inherited diseases: all are missense mutations, which change one amino acid into another, as opposed to nonsense mutations which turn an amino acid codon into a stop codon and thereby truncate the protein[3] (see Section 6.6.5).

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It is also conceivable that certain phenotypically silent mtDNA mutations may affect the rate of SOS, by varying the tendency of the mtDNA to undergo spontaneous mutation (see Section 10.12). We do not as yet know what proportion of amplified mutations are deletions, but we do know that the most commonly seen deletions are flanked by direct repeats, and in particular that the longest relevant (see Section 2.4.5) direct repeat in the mtDNA, 13 base pairs,[4] flanks a deletion which is seen much more commonly than any other.[5] The 26 base pairs which form this repeat are not all "fixed": some of them are third bases in protein-coding genes, which could be changed without affecting the encoded amino acid sequence. What they would be expected to affect, though, is the frequency with which the common deletion occurs, since the direct repeat would no longer be perfect. Thus, if it turns out that deletions are much commoner than point mutations in anaerobic cells, there would be value in assessing whether silent polymorphisms in these 26 base pairs correlate with longevity. Since such polymorphisms would be maternally heritable, such a study can make use of databases of historical records, such as that maintained by the Mormons in Salt Lake City. (That database has been used for such studies in the past).[6]

One characteristic of mitochondria that is superficially promising for testing SOS, but less so on closer analysis, is the rate of mitochondrial turnover. Turnover rate was first measured over thirty years ago,[7-9] so the experimental aspect is not particularly problematic; what is difficult is to identify predictions of SOS that such measurements might test. An illustrative example comes from a recent comparison of turnover rates (or of their presumed effects) in the central nervous system versus the peripheral nervous system.[10] The CNS has higher energy utilisation and shows higher levels of mtDNA damage with aging than the PNS, but CNS mitochondria appear to be recycled more slowly than PNS ones. This might be thought incompatible with SOS, since SOS predicts that the rate of turnover is increased by more rapid membrane damage (which should correlate with mtDNA damage). But a simple resolution derives from the observation that the CNS must generate a lot of heat purely to maintain brain temperature, whereas PNS neurons do not need to do so since their surrounding muscle does it for them. This suggests that CNS mitochondria are probably maintained in a leakier state than PNS ones, which is most easily done by recycling them more slowly.

The description of SOS given in Section 8.5.2 made no attempt to suggest a detailed biochemical pathway for the targeting of lysosomes to unacceptably leaky mitochondria. This is another area in which specialists in the relevant cellular components (lysosomes, in this case) may be able to lend weight for or against SOS by identification of such a pathway.

Finally, it should be possible to test SOS in vitro using human cells. The experiments of Chambers and Gingold[11] (see Section 8.3) cannot be copied in every detail, because human cells are not so obliging as yeast in their cell cycle; but culture conditions should be obtainable in which the rate of cell division is substantially less than that of mitochondria, and so in which lysosomal degradation of mitochondria is occurring. The recent work of King's laboratory[12] on cybrid muscle fibres (see Section 6.6.3) is a highly promising step in this direction.

12.2. Some Predictions of the Reductive Hotspot Hypothesis

This component of MiFRA is rather less comprehensively supported by existing data, so is correspondingly more amenable to testing.

One prediction is that the PMOR system itself should be much more active in cells which have lost OXPHOS function. Antibodies to some of the respiratory chain enzymes, notably cytochrome c oxidase (Complex IV) are widely available. Histochemical assays for the PMOR are likewise already established.[13] Studies of muscle tissue may be able, therefore, to establish whether PMOR hyperactivity colocalises with inactivity of Complex IV, as has

already been shown to be the case for succinate dehydrogenase.[14-16] Conversely, if the PMOR is not up-regulated then we would expect to see an up-regulation of lactate dehydrogenase (LDH), which is the enzyme that turns pyruvate into lactate for export from the cell if the TCA cycle is not maintained. Assays for lactate dehydrogenase activity are routine.[17]

The maintenance of the TCA cycle in anaerobic cells is not a vital component of the mechanism described in Chapter 9, but it would certainly make that mechanism's effects much more severe, so it should be tested. Histochemical demonstration that the enzyme is present is strong evidence that it is active, but falls short of proof. However, there are simple direct tests of whether ρ^0 cells do this: the simplest are to ask whether they generate CO_2, and whether they can grow on non-fermentable carbon sources (such as, most simply, the necessary exogenous pyruvate).

Likewise, there are well-developed assays for superoxide production;[18,19] but the rate of single-electron reduction of extracellular oxygen by ρ^0 cells has, to my knowledge, been established only under conditions where other electron acceptors are plentiful.[20] (One early study[21] may point the way here: it measured reduction of oxygen but did not identify the product.) Alternatively, the extracellular superoxide dismutase is predominantly bound to endothelial cell surfaces. rather than free in plasma,[22a] so any preferential colocalisation with cytochrome c oxidase inactivity in tissue may well be directly visualisable. A new SOD assay using cerium appears to allow accurate histochemical visualisation of all three SOD activities and may be particularly useful in this regard.[22b]

A third prediction is that chemicals which inhibit the PMOR should reduce the oxidative modification of LDL in blood plasma. One chemical is known which inhibits the PMOR: pCMBS, or p-chloromercuriphenylsulfonic acid.[23] This chemical is toxic, so it clearly cannot be used as an anti-aging drug; but it may well be possible to assay its effect on LDL oxidation in rodents before its toxic effects mask that. Alternatively, this may be studyable in vitro. The oxidation of LDL by cultured aerobic cells is totally inhibited by physiological levels of vitamin E or other antioxidants.[24] Incubation of LDL with ρ^0 cell lines in a physiologically realistic medium should allow measurement of its rate of oxidation, which should be non-zero if they are using the PMOR heavily.

A fourth prediction is that individuals with dysfunction in the machinery of LDL import into cells will incur oxidative stress somewhat more slowly, and thence age more slowly in general. This may be harder to test, however, because the import of cholesterol into cells is a very definite requirement, so that a deficiency in the normal uptake pathway is likely to be compensated for by a stimulation of some secondary pathway, or else by a rise in the overall LDL level in the blood. (The latter is what is seen in sufferers from familial hypercholesterolaemia, a genetic defect in the LDL receptor).[25] Either of these would negate the retardation of oxidative stress that is predicted above. Again, however, in vitro experiments may be more straightforward. There are numerous ways to quantify the level of oxidative stress in cells: these include the concentrations of hydrogen peroxide,[26] of lipid peroxidation products,[27] and of oxidatively damaged proteins.[28] The level of oxidation of LDL can also be quite accurately controlled in vitro. Thus, an approach to testing the influence of LDL oxidation on intracellular oxidative stress would be to incubate cells for an extended period in conditions where they were induced to import LDL at physiological rates, and measure the dependence of one or more of the above indicators on LDL oxidation levels.

12.3. Negligible Senescence: Predictions of MiFRA

As noted in Section 6.5.5, numerous species of cold-blooded vertebrate grow throughout their lives, and some of these live exceptionally long—so long, in fact, that it is possible that they do not senesce (their future life expectancy does not diminish with age) at all. It is,

however, essentially impossible to distinguish between extremely slow senescence and non-senescence. Perhaps the strongest evidence that these species really do not senesce comes from their maintenance of fecundity,[29,30] since it has long been a central tenet of evolutionary theory that reproductive senescence tends to precede other aspects of aging; however, this has yet to be tested in a representative variety of species.

If mtDNA decline is indeed so important in aging as is claimed here, then we can infer that any vertebrate which essentially avoids senescence must also avoid mtDNA decline. A technically straightforward experiment, therefore, is to measure the levels of any class of mtDNA lesion (either deletions or point mutations) in animals of a given negligibly senescing species spanning a wide range of ages.

Similarly, downstream effects of oxidative stress are just as straightforward to measure in negligibly senescing species as in others. Accumulation of lipofuscin, of oxidatively damaged proteins and of cross-linking in collagen have all been the subject of preliminary studies,[30,31] but much more extensive analysis is clearly required before we can form a detailed picture.

A third aspect of negligibly senescing species which is strongly predicted, though not absolutely required, by MiFRA is that they should have virtually no cell types which become unrenewable in adulthood. This does not mean they have no cells which are postmitotic—that would indeed be remarkable, since, for example, the syncytial character of muscle fibres is a fundamental consequence of their construction, and syncytial cells are constitutionally unable to undergo further mitosis. But, unless the cells continue to grow indefinitely without division—something that should be readily detectable in dissected muscle as increased length and/or cross-sectional area of fibres—it does mean that all postmitotic cells must be associated with precursor cells which can proliferate to replace them as and when they die, and also that such replacement actually occurs (as opposed to the situation in mammalian muscle, for example, where it occurs in response to larger-scale cell death such as a wound or toxin-induced necrosis[32] but does not maintain fibre number during aging).[33] Biogenesis of muscle fibres and nerves throughout life has indeed been reported in amphibians (though not specifically in negligibly senescing species),[34] but such work clearly needs to be conducted on a larger scale.

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Chapter 13

Prospects for Intervention

The retardation or reversal of aging has been a desire of mankind for as long as we can trace, but biologists have not worked particularly hard to achieve it. One reason for this—a very poor one, in my view—is that it is clearly a very hard problem, and therefore one on which progress is likely to be slow and patchy. A big problem with patchy progress, for the researcher, is that one's ability to attract funds for subsequent work is always dependent upon one's record to date, with the result that both success and failure in science tend to be self-perpetuating. NB: I call this a poor reason, but of course (a) I speak with the luxury of a theoretician, whose budget is negligible compared to those of the experimentalists whose work I study, and (b) it goes without saying that the funding regime I describe is not the fault of scientists. Therefore I certainly have sympathy with the widespread disinclination to work on very hard problems. I just don't like it.

The other main reason why manipulation of the aging process has not been a popular research field is rather better. It is that the problem is not only very hard, it is also so diffuse that people have not had much idea where to begin. If a scientist needs one thing more than any other, it is self-motivation. This is hard to sustain if one has no real confidence that success in the project immediately underway will constitute real progress towards one's ultimate goal. And such, unfortunately, has been the situation in gerontology. We have mountains of data on what makes for a long, healthy life, but we have lacked sufficient interpretation of that data to be able to formulate a realistic plan for its exploitation to engineer an even longer one.

Now, however, that psychological barrier is crumbling. In the foregoing chapters of this book, I have set out a description of how we age which, though still only hypothetical and in need of rigorous testing, is a great deal more detailed that any that have preceded it. Detail is the keystone of scientific confidence: if one describes a process only in broad terms, however plausible they may sound, others will harbour doubts that some problems have been overlooked, but if those broad terms can be broken down into lower-level parts, while retaining overall plausibility, those doubts are correspondingly assuaged. This is not a peculiarity of science—it is very much the same as the description of a mathematical proof, or the design of a large computer program. Detail is explanation, and explanation is reassurance.

Moreover, as noted in the Introduction, it is a matter of opinion just how strongly confirmed by experiment a hypothesis should be before one invests significant resources in an enterprise which would be valueless if the hypothesis were wrong. The main criterion which modulates that cutoff is one's perception of how valuable the enterprise would be if the hypothesis were right. In the case of MiFRA, many people—including, without doubt, people with the necessary skills and resources—consider that the enterprise of retarding aging is quite valuable enough to motivate investment of those resources on the basis of MiFRA's present degree of confirmation, prior even to the undertaking of the tests discussed in Chapter 12. The only factor that has held back such work hitherto has been the presence

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of unsettlingly large gaps in the theoretical framework: something which, as previous chapters have discussed, is broadly a thing of the past.

Consequently I shall devote this chapter to an examination of how, if the theory set out in previous chapters is indeed correct, we may in principle be able to retard human aging. The two approaches which I consider most promising will then be analysed in detail in the following chapters.

13.1. Some Probably Futile Approaches

In principle, if there is indeed a single chain of events which dominates the rate at which we age, the progress of aging could be greatly retarded by breaking any link in that chain. Some such treatments might not be able to reverse aging that has already occurred, but a clean break of any link in the causal chain should put a brake on further progress. Similarly, a treatment that only weakens, rather than breaks, one of the links would still retard aging, albeit to a lesser extent. The first question one should consider, therefore, is: "Supposing (for sake of argument) that MiFRA is correct, which links in it are the most amenable to disruption?"

Here are the possibilities which seem to be available. They each seek to subvert some link in the chain of events leading from mtDNA mutations to systemic oxidative stress, and are listed in causal order with respect to that chain. In theory one might extend the list to include treatments of the effects of oxidative stress, but I have avoided this because, as discussed in Section 6.5, the strong evidence from inter-species comparisons is that such "late-acting" interventions (by which is meant causally late, as opposed to late in the lifespan) are ineffective if the tide of early events is allowed to continue unabated.

a. Stop the spontaneous mutation of mtDNA
b. Repair spontaneous mutations of mtDNA
c. Introduce extra, wild-type mtDNA into mutant mitochondria
d. Stop OXPHOS from fumbling electrons and making LECs
e. Stop LECs from damaging mitochondrial membranes
f. Destroy mutant mtDNA before it takes over the cell
g. Reverse SOS—give mutant mtDNA a selective disadvantage
h. Abolish cells' reliance on wild-type mtDNA for OXPHOS
i. Abolish cells' reliance on OXPHOS for autonomous ATP synthesis
j. Kill cells that have lost OXPHOS function (have become anaerobic)
k. Prevent anaerobic cells from causing the peroxidation of plasma lipids
l. Prevent mitochondrially healthy cells from importing peroxidised lipids

A reasonable first step in deciding which of these is most realistically addressable is to consider what the body already does. Options a, b, d, e, k and l can, I feel, be excluded from further consideration on the grounds that the human body already works very hard to achieve them, by means that have been described earlier in this book, and this work is done by genetically determined machinery that has been developed by natural selection. Humans are among the longest-lived species for our metabolic rate, so there is unlikely to be any grossly suboptimal feature of this machinery. It is possible that, by studying species which do even better than us (as has been eloquently urged by Austad)[1] we could identify some of the slightly suboptimal ones, manifest as refinements that these species have achieved, but mimicking those refinements might be impractically laborious even with the development of reliable gene therapy (which is discussed in Section 13.4). Moreover, even the most exceptional birds achieve mortality rate doubling times (see Section 17.1) only about 50% greater than ours,[1] so this approach could only retard aging by that factor; the deleterious effects of introducing such genetic changes into a genome that has evolved without them are virtually certain to outweigh that and result in no net slowdown of aging. The only way

to do better than that would be to identify refinements that *no* species has yet found; that would require an astronomical improvement in our understanding of the relationship between a protein's sequence and its function.

Option i is the next against the wall. OXPHOS appears to have evolved only once in history; it is an astoundingly subtle process. Our chances of coming up with something equivalent (which lacks OXPHOS's side-effects) are vanishingly small. This is not least because the laws of thermodynamics dictate that energy conversion can be done efficiently only by extracting it in a series of small steps. It is no use getting energy out of chemical bonds (in glucose, etc.) if it mostly goes into heat—it has to be channelled into phosphorylation—and any such system must, in order to achieve the same energetic efficiency, be about as complex as OXPHOS itself is. Biology we might hope to tinker with; physics is another matter.

Now let us consider option g. I believe that it can be discarded even without extending it to a specific proposal for implementing it (which, in the first place, is not by any means easy to think of). If mitochondria with reduced ability to generate a proton gradient were somehow selected against, it follows that mitochondria with an unusually large proton gradient would be selected for. And, as noted in Section 8.5.4, this is exactly the predicted property of mitochondria that have suffered a point mutation in an ATP synthase subunit. Such mitochondria are clearly no more useful to the cell than those which SOS amplifies. In fact, the reversal of SOS may be even worse than that: it may be rapidly lethal to all cells, irrespective of their mitochondrial genotype(s). If it were implemented in the most direct way, by reversing the preference of lysosomes to digest mitochondria that have become dysfunctionally leaky to protons, then in one mitochondrial generation all cells would become populated mainly with mitochondria that are burning oxygen for all they are worth and making no ATP.

That leaves four lines of exploration: c, f, h and j. All have been touted as realistic ways forward. However, I believe that c and f are likely to fail. The problem they both face is that the restoration of normal mtDNA in the cell cannot be instantaneous: it can only act to increase the number of functioning mitochondria, steadily, until the cell is restored to health. But, as we have seen, a cell some of whose mitochondria are working and some not exerts a huge selective pressure to amplify the mutant ones and to destroy the wild-type ones. I therefore predict that either option c, reintroduction of wildtype mtDNA[2-4] or option f, destruction of mutant mtDNA (either explicitly or by suppressing its ability to be replicated) so as to promote amplification, by default, of the residual wildtype mtDNA[5-7] will be overpowered by selective pressure the other way. Destruction of mutant mtDNA has an additional risk: it may in fact accelerate the loss of OXPHOS. The fact that we can make mtDNA-less (ρ^0) cells in vitro by inhibiting mtDNA replication tells us that mitochondria can, if forced, divide even when they have failed to replicate their DNA. Genetic drift ensures that nearly all mitochondria in a heteroplasmic cell are homozygous; thus, many cells may harbour some mitochondria homozygous for a mild, hypomorphic mutation which reduces OXPHOS but not so much as to give a selective advantage. (This is particularly the case in the inherited diseases discussed in Section 6.6.5.) If this hypomorphic mtDNA were destroyed, mitochondria would be formed which had no mtDNA and were thus completely OXPHOS-less; these, unlike the hypomorphs, would be clonally amplified and take over their host cell (see Fig. 13.1).

This leaves us with options h and j, which are the subject of the rest of this chapter and the following two. Some may feel that the process of elimination I have conducted above is unjustifiably cavalier. I certainly see nothing wrong with trying to influence the aging process by any of the means I have rejected above. For example, option a might be attempted by targeting a SOD to the intermembrane space, as has recently been done in yeast;[8] option k

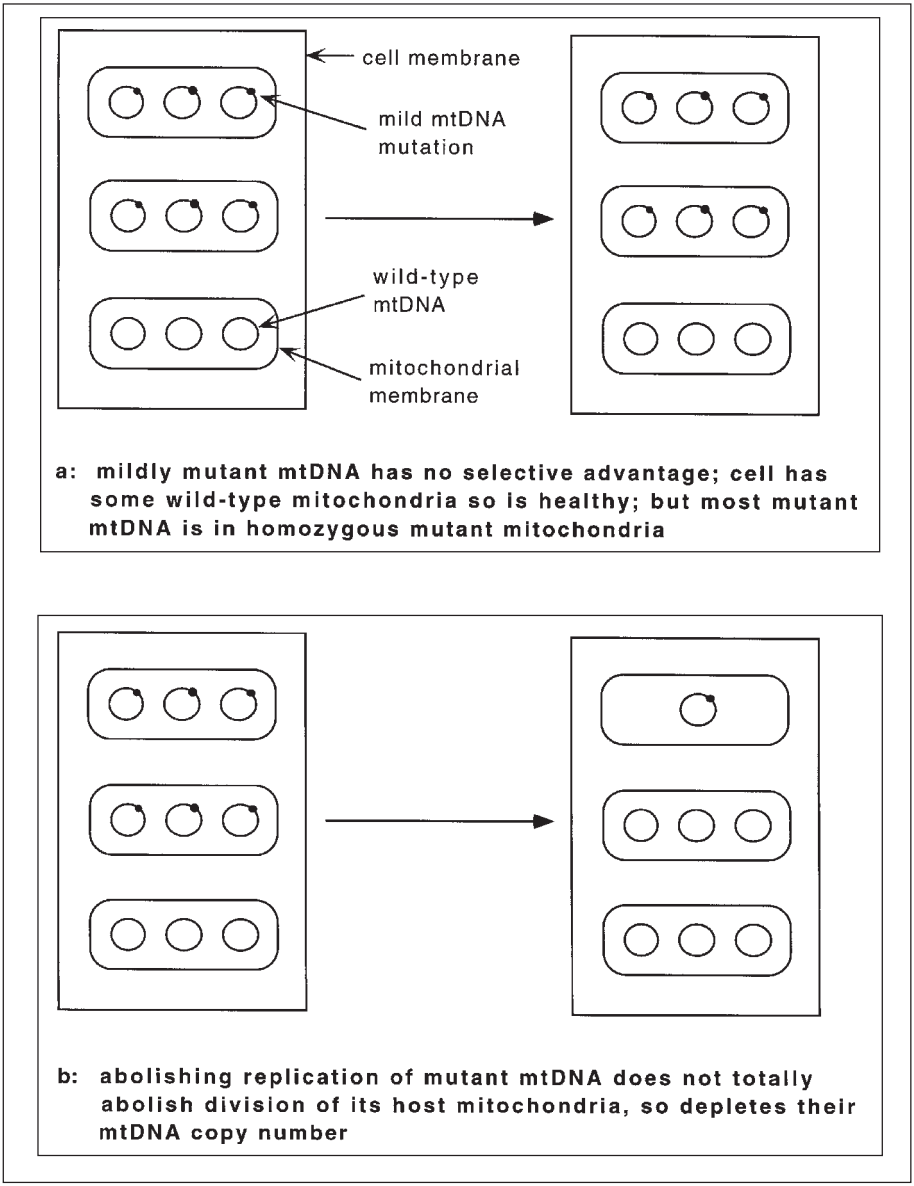

Fig. 13.1. An undesirable potential result of selectively inhibiting replication of mutant mtDNA.

could be tried by inhibiting the PMOR without killing overexpressing cells. My only purpose is to draw attention to their potential difficulties, so that such efforts do not proceed in a vacuum, oblivious to the obstacles ahead.

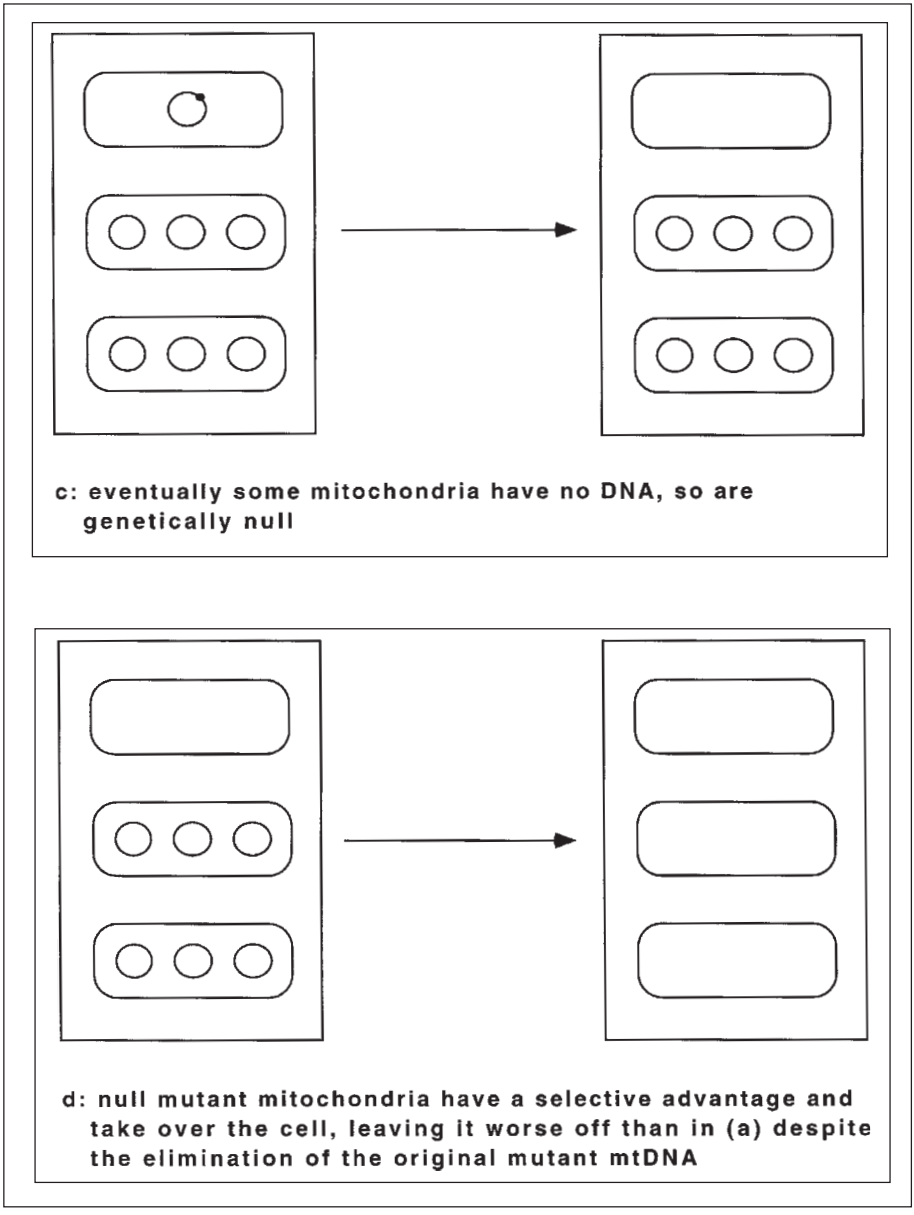

Fig. 13.1. See opposite page.

13.2. A Way to Obviate mtDNA

The proteins encoded in the mtDNA are vital, integral components of the OXPHOS machinery. In rejecting option i, I have thus already accepted that these genes are indispensable. What is not so clear, however, is that they need to be carried in mitochondria. Could we not make copies of those genes and incorporate them into cells' nuclear DNA?

These copies would be vastly less prone to spontaneous mutation than their mitochondrially-located counterparts, since they would be

1. far from mitochondria and the LECs they produce,
2. safe from replication error, since the nuclear DNA of non-dividing cells (which are the ones that matter) does not replicate, and
3. safe from the huge selective pressure for mutations that affects mitochondrially-located DNA.

When a mtDNA mutation occurs, it will then not matter whether or not it is amplified, because the protein products of the mutant gene will not be necessary to the host mitochondrion. (And anyway, SOS predicts that it will actually not be amplified, because the host mitochondrion will be generating a normal proton gradient using the nuclear-coded protein.) In genetic terminology, the nuclear copy will complement the mutant mitochondrial one. This technique is termed allotopic expression of the mtDNA genes.

There are numerous difficulties with this treatment. In focusing on it, I am in no way belittling these difficulties; all I claim is that none of them is so great as those which confront the alternative approaches rejected above. They will be discussed in detail in Chapter 15.

13.3. A Way to Destroy Anaerobic Cells

Next, consider option j. Its logic is that OXPHOS collapse of only 1% of cells would be perfectly tolerable if it did not affect all the rest of our cells so badly. If those cells were removed, therefore, we would not suffer noticeably from their absence and we would benefit greatly from the loss of their toxic electrons. Accordingly, I think their removal is a plausible avenue for intervention, and I will examine it in detail in Chapter 14.

This option for retarding aging has one clear advantage relative to complementation of mtDNA and one clear disadvantage. The disadvantage is that, unless and until ways are found to stimulate replacement of the ablated cells, there would be a steady—albeit slow—decline in the functionality of non-dividing tissues. This will be addressed further in Section 14.4.

The advantage is that such a treatment may be developable—to a degree of reliability that makes it useful—more quickly than the complementation of mtDNA. This is because complementation of mtDNA by nuclear genes will definitely not be possible without gene therapy, whereas ablation of anaerobic cells may well be achievable by simpler technology. A discussion of what gene therapy is (and aims to become) is therefore appropriate.

13.4. Gene Therapy

Gene therapy is the treatment of a medical condition by adding DNA to a patient's cells. The DNA that one would add depends, naturally, on the condition which is being treated; in principle one can add whatever DNA one wants. In general one would want to add new, intact copies of genes whose existing copies were mutant, thus relieving the symptoms of the mutant genes.

Development of treatments involving gene therapy really breaks into two parts: design and delivery. That is:

1. figure out what DNA to insert into the nucleus in order to achieve the effect;
2. figure out how to actually get that DNA into the nucleus of our cells.

13.4.1. How Close Is It?

Delivery of DNA to cells is very complex. Luckily, however, it is the same for any DNA, and there are plenty of ailments other than aging which are caused by mutations and which could be treated by inserting new DNA. Thus, there is already a huge research effort worldwide seeking ways to do this. As with any technological advance, no one really knows how long it

will be before this research succeeds in perfecting a safe, reliable treatment. All that can be said is that there are many labs around the world trying to achieve it, that they are employing a wide variety of techniques, and that they are constantly reporting encouraging progress in trials. This has led, importantly, to increasing public optimism about timescales on the part of specialists.[9] If forced to guess, I would say that truly general-purpose techniques for DNA delivery to somatic cells will probably achieve a level of reliability sufficient to gain governmental authorisation within 20 years, though almost certainly not within 10 years. Consequently, I believe that safe gene therapy may have become widely available by the time all the other problems with these proposed interventions are solved.

13.4.2. What Could It Achieve in Regard to Aging?

Again, no one really knows. By the time it is available, however, we may have a more accurate idea. This is because there is a far simpler technology, already routine in many laboratories worldwide, with which we can simulate the effects of perfect gene therapy in mice. Mice are not men, so the effect achieved in mice might not be an absolutely reliable indicator of what could be done for humans, but it would certainly be a broad hint.

This simpler technology is called germ-line transformation. Functionally, it is the same as gene therapy except for the target cells. Gene therapy targets somatic cells, which make up almost all of our body but are not passed on to our offspring. Germ-line transformation targets egg cells, which (when fertilised) give rise to all the cells that form the embryo. This means that germ-line transformation is far more dangerous for humans, and also is of no benefit to those of us who are already alive. But it can be used in mice, and since mice have such a short lifespan it can give us hugely valuable information quite quickly.

Therefore, the likely scenario for option h (obviation of mtDNA) is that we will be able to "prototype" the whole treatment, and therefore test the whole theory laid out in this book, by generating mice with appropriately modified genes of the mtDNA in the nuclei of all their cells and, well, just sitting back and watching them age—or not, as the case may be. If their lifespan is indeed increased significantly, efforts to apply the same treatment to humans using gene therapy will become motivated.

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Chapter 14

Ablation of Anaerobic Cells: Techniques and Hurdles

14.1. Apoptosis: A Ready-Made Cell Killer

The death of cells is not always something that the body seeks to avoid. There are two major pathways involved in cell death, called necrosis and apoptosis; apoptosis is the one that will be highlighted here, because it has features which appear quite readily amenable to recruitment for the purpose under discussion. One is that it is a highly orderly, regulated process, so that once initiated by means that we engineer it is likely to proceed "hygienically"—that is, without toxic consequences for healthy cells nearby. (Necrosis should not be discounted, however—see Section 14.5.)

The other feature of apoptosis which may be of particular use is that it occurs during embryonic development: it is used in the construction of large-scale features such as the capillaries of the kidney. This means that the body is somehow able to generate a signal that induces certain cells to enter the apoptotic program while other cells very nearby do not. The cells cannot be acting purely independently of each other, since in that case the structure that resulted would not have the necessary organisation. And that is why the signal we engineer, which causes anaerobic cells to enter apoptosis, need not necessarily be a genetic signal, delivered by as yet unperfected gene therapy. In fact it may not need to enter the cell at all, since (as noted in Section 9.4) these cells should have easily detectable surface features such as a highly up-regulated PMOR.

14.2. Specificity and Frequency of Treatment

Any medical treatment that is designed to induce changes—in this case, death—in some cells but not others has an inherent risk of inaccurate targeting: both of inducing the changes in cells where they are not wanted, and of overlooking cells where the changes **are** wanted. Since this treatment has not even begun to be developed, we of course can have no idea how prone it will turn out to be to either class of error. All we can say is what scale of error rate is likely to be acceptable for the purpose.

In particular, there is a trade-off between how accurate the treatment is in targeting the correct cells and how often it will need to be administered. If the ablation of anaerobic cells could be done perfectly, MiFRA predicts that the rate at which cells will enter SOS thereafter would revert to that of a young individual, and would not return to the level it had before that first treatment until the individual was perhaps one and a half times as old as they were then. (Not twice as old, because MiFRA only asserts that mtDNA decline is the dominant determinant of lifespan, not that it is the sole one.) By this reckoning, treatment would only need to occur every few decades. In practice, it is unrealistic to expect every single anaerobic cell to be removed each time, so treatment would have to be more frequent than that.

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This begins to look like a serious problem once one considers the course that aging takes. The rate at which cells enter SOS evidently accelerates rather rapidly during life.[1,2] Thus, a 10% false negative rate (that is, a removal of only 90% of the anaerobic cells) seems inadequate; we would want to aim for at most 1% in order to restore the number of such cells to levels obtaining many years previously.

However, this should be easier to achieve if we can live with erring considerably the other way, and we probably can. Since so few cells are affected by SOS, it seems likely that even a 100% false positive rate (that is, a removal of as many healthy cells as anaerobic ones) would have no detectable deleterious effect. A further encouraging point with respect to frequency of treatment is that it should not need to become progressively **more** frequent as an individual gets older (presuming we solve the other problems).

Another reason for optimism is based on the observation that gene therapy may not need to be involved (see Section 13.3). It is not particularly fanciful to imagine a chemical capable of selectively triggering apoptosis in cells with a hyperactive PMOR, which could be administered without medical supervision—perhaps even orally. Such an option would of course allow the possibility of very frequent, even daily, treatment; this would in turn allow a very high false negative rate for each individual treatment.

14.3. Disposal of Dead Cells

This is very unlikely to be a problem. The apoptotic program is just that: a program. Once the initiating events are in progress, there is a very precise and well-controlled series of changes of expression of various proteins, many of them already well characterised, which control not only the cell's cessation of function but also its destruction by macrophages. It is reasonable to suppose that induction of apoptosis of anaerobic cells will involve the stimulation of an early event in normal apoptosis; thus, the strong likelihood is that all the usual downstream events will follow without further intervention. That includes disposal of the "carcass."

14.4. Replacement of Dead Cells

This is much more likely to be a major obstacle. Many cell types in mammals certainly become depleted during life, as noted in Sections 5.3 and 5.5.[3-5] This has been proposed to be a trade-off mechanism, since the organism would benefit from the replacement of the dead cell only if that replacement is accurate, and there is always the risk of DNA replication error possibly leading to cancer. Thus, if a cell of a particular type becomes inviable quite frequently, such as in the liver, then it must be replaced regularly in order that the tissue will last a lifetime. But if the tissue in question is less severely exposed to toxins, its cells may die only very rarely; in that case, enough cells may remain by old age even if no replacement whatsoever is done. In practice, many cell types appear to adopt this latter strategy most of the time, though they can be stimulated to proliferate in exceptional circumstances such as wounding.

What, then, is likely to be the consequence of removing anaerobic cells, in terms of their replacement? My hunch, and it is only that, is that the affected tissues will respond very much as now, by not replacing them, and that this will have minimal impact on the tissue because so few cells are involved. Moreover, as noted elsewhere we must clearly address the general issue of replacement of dead cells (irrespective of why they died) in tissues that are not usually inclined to replace them; it is reasonable to hope that such treatment, once it is developed for seriously affected cell types such as thymocytes and fibroblasts, will be adaptable to muscles and possibly even nerves too.[6]

14.5. Segmental Cell Death

The last obstacle to this treatment may well be the most severe. We saw in Section 9.6 that muscle, though not being the tissue type most affected by mitochondrial decline in terms of performance, is probably the most important in terms of its toxicity because we have so much of it. Thus, unless we can apply this treatment to muscle it is probably not going to do us much good. This means that we must address a feature of SOS in muscle that was discussed in Section 6.6.1: its segmentality.

Recall that the proportion of muscle fibers that have lost OXPHOS activity by old age, as observed in transverse sections (where the muscle is cut perpendicular to the fibers, so that each fiber appears as a disc) is under 1%, but the length of an anaerobic segment as revealed in longitudinal staining (where a single fiber is separated from the sample and stained along its length) is almost always under 1 mm. Since fibers can be many inches long, this means that a hefty proportion of fibers are anaerobic somewhere or other. Apoptosis is inducible in muscle fibers, but is unlikely to stop at the same boundaries that mitochondria do: if it kills anything then it will probably kill the whole fiber. Thus, the damage done to tissue by this treatment may, after all, outweigh its benefits.

The main reason not to regard this as a complete show-stopper for the whole idea is that we still do not understand apoptosis all that well, especially not in tissues (such as muscle) where it plays a very minor role—if any—in normal tissue development and turnover. I consider it quite possible that, as our understanding increases—and it is increasing extremely quickly as I write, with the elucidation of the roles of caspases, cytochrome c, Bcl-2 and several other proteins—we will learn how the apoptotic program can be modulated to restrict itself only to a small segment of a muscle fiber. That would not be the end of the story, since the complete absence of this segment might have effects on the rest of the fiber, such as the failure to transmit the action potential when a motor neuron fires. Again, however, such problems seem to me to be potentially remediable if satellite cells can be recruited to replace the deleted segments; the new fibers formed after a wound fuse with the surviving portion of existing fibers, so this is not particularly unrealistic.

Another plausible way out of this difficulty would be to stimulate **necrosis**, rather than apoptosis, in the affected cells. Necrosis can be induced in small regions of muscle tissue by bupivacaine hydrochloride, and if the concentration is appropriate the fibers are killed but the neighbouring satellite cells survive and regenerate the tissue.[7,8] This has been used recently as a way of reducing the levels of mutant mtDNA in muscle of a sufferer from an inherited mtDNA mutation.[9] The difficulty with necrosis is that, unlike apoptosis, we do not yet understand how to trigger it selectively in individual cells: it is therefore a treatment which, at present, is potentially useful only for reducing the extremely high tissue-wide levels of mutant mtDNA seen in these strange inherited diseases.

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CHAPTER 15

Transgenic Copies of mtDNA: Techniques and Hurdles

The conclusion of the previous chapter is somewhat ambivalent: I believe that selective ablation of affected cells is a concept worthy of consideration and research, but may in the end prove unable to retard systemic mtDNA decline all that much. Therefore, I feel that only one approach to subverting mtDNA decline is really likely to work well: complementation of mtDNA mutations with transgenic copies introduced into the chromosomal DNA.

This approach has been discussed for some years, starting with the first demonstration of its feasibility in 1986[1] and with suggestions for its use in treating diseases[2] and aging.[3] Consequently there are many aspects of it which merit detailed description here. The first nine sections of this chapter deal with potential obstacles to mitochondrial gene therapy by protein import; the first eight will be argued to be either spurious or currently hypothetical, and therefore not meriting concern at this point, but the ninth—the mt-coded proteins' hydrophobicity—is acknowledged to be very real. The remaining sections of the chapter focus on ways to overcome or sidestep that obstacle.

15.1. Changes to the Coding Sequences

A gene that is encoded in the mitochondrion is translated using mitochondrial tRNAs; a gene encoded in the nucleus is translated using cytoplasmic tRNAs. It was explained in Sections 2.4.4 and 10.2 that the amino acid sequence resulting from translation of a given nucleotide sequence will be different in the two cases—the genetic codes are different. Therefore, if we take a gene from the mtDNA and insert it into the nucleus, the protein resulting from its translation will almost certainly not work, because it will include numerous incorrect amino acids. Worse, it may be prematurely truncated by virtue of a codon that should mean an amino acid being, instead, seen as a "stop" codon; this will be the fate of UGA codons, as explained in Section 10.2.

The way to avoid this difficulty is to change the DNA sequence, before inserting it into the nucleus, to mirror the differences of genetic code. That way, the correct amino acid sequence will result from cytoplasmic translation. Of course that will mean that the DNA would now generate the wrong sequence if it were translated in mitochondria; but we will not need intramitochondrial translation, so that is no problem.

The introduction of base-pair changes in this way is well within the scope of existing molecular biology. In fact it was first achieved (in yeast) in 1986,[1] and a highly efficient technique for making multiple such changes simultaneously was developed more recently.[4]

15.2. Targeting to Mitochondria

It is not going to be any use, however, to restore to cells the ability to make their mt-coded proteins, if those proteins are not then incorporated into the mitochondrial OXPHOS

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machinery. In Section 2.4.3 it was explained that, since nearly all mitochondrial proteins are already encoded in the nucleus, a system exists to transport them into mitochondria. This system is now fairly well understood; in particular, we know that the addition of a particular type of sequence at the beginning of such a protein causes it to be imported all the way into the matrix.[5] Therefore, if we simply prepend the nucleotide sequence of such a signal onto each of the inserted genes, there is a chance that the encoded proteins will thereby be transported to mitochondria. Once inside, the presequence is removed and the protein will be indistinguishable (in theory) from what it would have been if it had been constructed in the mitochondrion in the first place. Thus it should be incorporated correctly into the OXPHOS machinery.

This sounds straightforward in principle, but would it work? We certainly have reason for hope, since the yeast gene that had been recoded in 1986[1] was properly imported and incorporated, so that it really worked, as long ago as 1988.[6] Other proteins have so far proved harder (see Section 15.9), but there are after all only 13 proteins, 13 problems.

15.3. Regulation of Expression; Copy Number

It may have caught the reader's notice that the proposal discussed above is to incorporate "copies" of the mt-coded genes into the nucleus, but that no mention has yet been made of how many copies. This is indeed an issue of central importance to the proposed treatment, because the mt-coded proteins are all subunits of enzyme complexes, of which other subunits are already nuclear-coded. If the mitochondrion has too much of one subunit relative to another, it may fail to assemble any complexes correctly. This obstacle has been recognised ever since protein import was first proposed as a treatment for aging.[3]

In fact, however, we can turn the above observation to our advantage. Since all 13 mt-coded proteins do their work in direct physical combination with nuclear-coded ones, they must normally be generated in exactly the same stoichiometry, relative to those nuclear-coded ones, as they exist in the complete complex—usually 1:1. So, for example, in order to get the right amount of cytochrome b, we could embed our nuclear copy in the same regulatory DNA that naturally surrounds the Rieske protein. In this way we should ensure that exactly two copies of the inserted gene will do the job, since that is the number of copies that cells have of their nuclear-coded genes.

How can this be correct, since cells have so many mitochondria, each of which has its own DNA (and in several copies, at that)? Surely that means that we must insert thousands of copies into the nucleus in order to achieve correct levels of expression? Well, we cannot escape the fact that cells manage with two copies of the gene for cytochrome c, etc., compared to thousands of copies of that for cytochrome b etc. Therefore, for some reason the mitochondrially-encoded genes must be transcribed and/or translated thousands of times more slowly than the nuclear-coded ones. The question is, why? Conceptually, there are two possible answers: either there is an intrinsic feature of the genes' DNA sequence that makes the nuclear ones much easier to translate, or else the transcription and/or translation machinery in the mitochondrion is simply much less efficient than that in the cell. The former alternative is biologically implausible, since the mtDNA is made up of the same chemical constituents as the nuclear DNA. The latter must therefore be correct. But then, if we insert a gene into the nucleus, it will promptly benefit from the more efficient machinery, so it need only be present in the same copy number—two—as the naturally nuclear-coded genes.

15.4. Stoichiometry: Interference by Endogenous Wild-Type mtDNA

Germ-line transformation gives us no choice about which cells include the inserted DNA: they all do. Gene therapy may in time be more selective, but probably not at first. This

means that cells whose mtDNA is perfectly intact, which means nearly all cells, will nevertheless have nuclear (transgenic) copies of it. Thus, there will be a copy number problem after all, since these mitochondrially healthy cells will be expressing twice as much of the mtDNA-encoded proteins as of the solely nuclear-coded ones. A factor of two may not sound like much of a burden, but the assembly of enzyme complexes is a very subtle process which is still poorly understood, so there may be very deleterious effects. Indeed, this may be the reason for the failure, a few years ago, of an in vitro attempt to make a working nuclear-coded ATPase subunit 6.[7] This is discussed further in Section 15.8.

If this does turn out to be a barrier, however, there are various way that we might get around it. One is to disable some of the nuclear-coded genes that are responsible for transcribing and/or translating the mitochondrial copies. This would leave the inserted copies as the only functional ones, so the correct stoichiometry would be restored. Also, it would then not matter whether the mitochondrial copies were mutant or not; they would already be functionless.

However, this approach has the disadvantage that the disruption of mitochondrial transcription or translation must absolutely be effected only in cells whose inserted mt-coded genes are all working. It is quite possible that gene therapy will (at least at first) be unable to incorporate the engineered DNA into every single cell for which it is intended. If copies of the mitochondrially-coded genes reach, say, 90% of cells, and independently the DNA that disrupts mitochondrial transcription or translation also reaches 90%, then there will on average be 9% of cells which are no longer able to use their mtDNA but which also have no functioning replacement. Since under 1% of cells exhibit OXPHOS failure naturally, this would be worse than doing nothing at all! It would thus be necessary to arrange a very tight linkage between the two treatments, so as to allow mtDNA still to be used in cells where the replacement DNA was ineffective. This may be more easily said than done. A second problem is that all known techniques for disrupting a particular nuclear gene have an error rate, so that they occasionally disrupt miscellaneous other genes too. Such disruption might easily kill the cell, or even induce a tumour.

15.5. Interference by Mutant mt-Coded Proteins

An alternative approach to the stoichiometry problem would be to incorporate into the inserted DNA a mechanism that stopped it from being transcribed until such time as the cell got into OXPHOS difficulties. This would have the same effect: until and unless SOS destroys the cell's OXPHOS capacity, only the mitochondrially-coded copies are used, and after that only the transgenic copies are used (since the mutant copies are useless). It should also be quite easy to arrange, since there will be some fairly unsubtle intracellular signals (such as the up-regulation of the PMOR) that can be used to trigger the switching-on of the inserted genes. The switch itself can be constructed by numerous methods that are already standard molecular biological tools, such as FLP recombination.[8] It may be necessary to make the switch reversible, since cells may transiently get into a state where, for example, the PMOR is briefly asked to work very hard but not because of mtDNA failure; if so, then the detection mechanism would also need to distinguish between OXPHOS recovery due to the action of the transgenic DNA and recovery that would have occurred anyway, since in the former case the switch should clearly not be reversed when OXPHOS recovers.

The main shortcoming of this approach to the stoichiometry problem comes from the mutant mtDNA. Some mutations (e.g. in tRNAs) will simply cause no mt-coded protein to be constructed; cells taken over by mutations of that sort should respond properly to the mechanism just outlined. But other mutations may only change the amino acid sequence a little: enough to stop the protein from working, but not enough to stop it from being incorporated into an enzyme complex. It will thus compete with the correct copies being

imported from the nucleus, with the result that (as well as the stoichiometry still being wrong) half the copies of that enzyme complex will be non-functioning.

Again, there are potential ways round this. One possibility is to insert into the nucleus not only the genes for the mt-coded proteins, but also a gene whose product would enter mitochondria and inhibit mtDNA transcription or translation. This is equivalent to disrupting a nuclear-coded gene for mtDNA transcription or translation, but without the drawback of potential damage to other genes (see Section 15.4), since it would act not on the nuclear gene itself but on its protein product's (mitochondrial) site of action.

15.6. The Inconvenience, but Irrelevance, of Mitochondrial RNAs

Some observers have suggested[7,9,10] that rescue of mtDNA mutations by importing normally mt-coded proteins will not be any use in retarding aging, because many of the mutations that are found to have occurred in anaerobic cells affect the mitochondrial RNA-encoding genes, in particular the mt-coded tRNAs, and we have no idea how to get cytoplasmic RNAs into mitochondria. This is a logically flawed objection, however. The only thing a mitochondrial RNA does is to participate in the construction of mt-coded proteins. Conversely, the only reason that a mutation in a mitochondrial tRNA gene is harmful to OXPHOS is because it causes the absence of those proteins, which are integral components of the OXPHOS machinery. Thus, successful import of all such proteins from the cytosol will rescue not only mutations in their mt-coded genes but also mutations in tRNA (or rRNA) genes—or, for that matter, any other mtDNA mutation such as a deletion.

However, it is a trifle inconvenient that tRNAs are such frequent victims of—"hotspots" for—spontaneous mutations. What it means is that, even if the theory of aging presented in this book is the truth, the whole truth and nothing but the truth about the aging process, we are still rather unlikely to bring about more than a slight slowdown in aging until we get all 13 genes to work like clockwork from nuclear copies. If 12 of them are working, and one not, and a tRNA gene goes down, that thirteenth protein will be lost and the cell will become just as anaerobic as if the 12 were not working. This means that, initially, studies of the efficacy of particular genes in their inserted copies will probably have to be done using carefully predefined mutations in the mitochondrial copy of the relevant gene, in vitro.

Or will it? One highly ingenious way of studying each protein's import in isolation from the others has been developed by Claros and coworkers.[11,12] Rather than seeking restoration of OXPHOS, they constructed "tribrid" proteins: they took the sequence of the OXPHOS enzyme subunit, but then, as well as attaching a presequence to induce import they also attached a "postsequence," encoding a polypeptide that would thereby be attached to the C-terminal end of the imported protein. This C-terminal polypeptide's activity required it to be imported into the matrix, but was not related to OXPHOS. Since the C-terminal end of the protein is the last part to be imported, if this activity was seen then it indicated that the whole protein must have been successfully imported. And since the function was autonomous, not requiring cooperation from any mt-coded proteins, a mutation in a mt-coded RNA would have no effect on the assay. Claros et al did this work in yeast, with a protein that mammals do not actually have (because it is used to splice introns of some mitochondrial genes, and mammalian mtDNA has no introns), but the principle can be applied just as well to any nuclear-coded mitochondrial protein whose endogenous chromosomal copy has been mutated.

15.7. Unfolding, Refolding and Prevention of Folding

It must also be borne in mind that the successful import of a nuclear-coded protein into the mitochondrion is not the end of the incorporation process. Proteins derive their specific properties from their stable three-dimensional structure, which is defined by their

amino-acid sequence. Import involves the unfolding of this structure,[13] so that a protein which has just been imported is in fact completely useless until it has resumed its proper configuration. The refolding process seems to differ greatly from one protein to another: some seem to refold all on their own, while others need help from "chaperone" molecules. Mitochondrially encoded proteins must also, somehow, fold into the correct configuration as they come into existence during translation, but we have no idea whether the machinery (if any) that does this will work on a protein that is emerging from the membrane rather than from a ribosome. We'll just have to try it.

15.8. Fallacious "Existence Proofs" That This Cannot Work

As if all the obstacles discussed in this chapter were not enough, the plausibility of this proposed treatment has also been challenged on the basis that we have no idea what other, as yet unknown vital function mtDNA may have, that has caused its retention during evolution. It is certainly fair to ask why mtDNA still exists, and if no satisfactory answer can be found then one would be justified in worrying that its replacement by nuclear transgenes might have unknown deleterious effects. That is why I laid out in so much detail, in Section 10.2, the best guess as to why mtDNA still exists. Given such a robust explanation, I claim that there is no reason to anticipate such problems. If they arise, of course, we will have to tackle them; but we can cross that bridge when we come to it. Two possibilities should be mentioned, however, since they may be testable.

One suggestion[14] was based on a similarity between one mt-coded gene, ATPase subunit 8, and a bacterial toxin called *hok*. Why is this potentially relevant? Well, the role of a protein in the cell comprises not only what it does, but also what it does not do; many mutants in model organisms cause their phenotype by expressing a perfectly correct protein in an incorrect place, where it happens to be toxic. We have no information* regarding how the various mitochondrially-encoded proteins might interact with cytosolic ones, as they would be in danger of doing if they were constructed by cytosolic ribosomes. It would be highly valuable to explore this possibility at an early stage in the attempt to develop the proposed transgenes; this can of course be done before import has been perfected, since it is the behaviour of the protein prior to import which is of interest. If any toxicity is discovered, techniques to circumvent it may include use of a presequence or chaperone that prevents the offending protein from adopting its usual three-dimensional configuration (and hence activity) until after import, as will be discussed in another context in Section 15.10.[15]

Another proposed reason "why mitochondria need a genome"[16a] is that the mt-coded proteins have sequence characteristics that are preferentially recognised as targets for export from the cell, so that if they were nuclear-coded they would end up on the outside of the cell before they could be targeted to mitochondria. This is an intriguing idea, but is based only on sequence similarities, not on observation of such export. And again, even if it turns out to be true it is something that we might hope to subvert by suitable presequences and/or chaperones.

Finally, as noted in Section 15.4, there has been one failed attempt at allotopic expression—with ATPase subunit 6. (Actually there have surely been many other such failures, but this one has actually been reported—albeit only in a meeting abstract.)[16b] Not only did the transgenic copy fail to rescue a mutation in the endogenous gene: it actually impaired OXPHOS in mitochondrially wild-type cells. This was interpreted by some as implying cytosolic toxicity. However, the study involved expressing the protein at very high levels. If its import was failing, due for example to its hydrophobicity (see Section 15.9), then the

* *However, this is another possible explanation for the non-function of a transgenic ATPase subunit 6 (see Section 15.4)*

mitochondrial protein import machinery may have become saturated by "stuck" proteins and so unable to import other, normally nuclear-coded proteins. This would certainly cause the observed OXPHOS inhibition.

15.9. Import of Very Hydrophobic Proteins

This section discusses the only problem facing the protein import project which is, honestly, going to have to be attacked by trial and error all the way, and which is therefore, arguably, the hardest. On the other hand, initial stabs at it have met with quite significant success, so it may turn out to yield quite quickly after all.

In the description (Sections 2.1 and 10.2) of how we came to have and keep mitochondrial DNA, and in particular the genes for exactly these 13 proteins, I noted that there is a particular characteristic, hydrophobicity, which is shared by all these proteins (because it helps them to become embedded in the inner membrane, where they function) and which seems to make them difficult to import. In fact there are measures of hydrophobicity that discriminate almost all mt-coded proteins from those that are imported.[11] Thus, if we take the signal presequence from some random imported protein and attach it to a mitochondrially-encoded protein, we cannot by any means be sure that import will occur—the protein may get stuck part-way through.

One can learn much about why this may be from examination of the signal presequences of imported proteins. These proteins, of course, have varying degrees of hydrophobicity, albeit less than the mt-coded ones. Interestingly, the more hydrophobic imported ones tend to have a longer signal presequence.[17] On the basis of this observation, researchers have examined the effect on importability of attaching longer-than-normal presequences. These were made in the most simplistic way imaginable: just by attaching two copies of a standard presequence end to end. And indeed, it was found that several proteins which could only be imported very inefficiently were thereby imported more easily.[18,11]

Unfortunately, the successfully imported proteins were not quite so hydrophobic as most of the 13 of interest, and when ones with that greater degree of hydrophobicity were tried, no import occurred even with the duplicated presequences. Thus, unlike any of the difficulties discussed in previous sections, this obstacle to mitochondrial gene therapy by protein import is still, unarguably real.

15.10. Hints from Other Organisms

It is all very well to say that the presequence design technique tried so far[18,11] is simplistic, but that does not tell us what sophistication to try that might be more effective. I think we may find it very difficult to guess a design strategy which will enable import of proteins that are completely unimportable at the moment. We will need help. The source of such help is, as usual, nature: organisms that already encode any of the relevant 13 on nuclear genes will provide a far better example than extrapolation from the presequences of more easily importable ones.

A further reason for optimism that other organisms can be of use is that the mt-coded 13 are all very highly conserved across taxa. It is of course likely that an example from another animal will be more applicable to the human gene than one from a more distantly related organism, but even very primitive eukaryotes have sufficiently similar homologues of these genes that we should not ignore them.

At the time of writing, however, little effort has gone into this line of attack. Two genes—subunits 2 and 3 of cytochrome c oxidase—have been found to be nuclear-coded in certain plants (*Vigna radiata*[19] and *Selaginella elegans*,[20] respectively), but I know of no attempt to import their human homologues using their presequences. A blue-green alga,

Chlamydomonas reinhardtii, offers even more opportunity: its mtDNA encodes only seven of the 13.[21] But, currently, there is no information regarding the missing six. Much further work is clearly needed in identifying and experimenting with nuclear-coded homologues of our mt-coded genes.

A second highly relevant aspect of import is chaperones. The (nuclear-coded) beta subunit of the yeast ATPase has an anomalously long presequence; it was recently shown[15] that this sequence acts as a **cis**-chaperone, keeping the business end of the protein in a semi-unfolded state while it is still in the cytosol, so that import is easier than if it had folded up into its final configuration. An even more encouraging finding of this study[15] was that this property of the presequence was not specific to that one protein: it also accelerated the import of other proteins to which it was attached. It has not yet been tried on the mt-coded proteins; the results will be of great interest.

15.11. Do We Really Need Complex I?

I mentioned in Section 2.4.4 that the *Saccharomyces cerevisiae* (yeast) mtDNA encodes only six of the 13 proteins that ours does, but that this was not helpful in the same way as the plants discussed above, because the seven others are not present in the *S. cerevisiae* nucleus either. The huge, 40-odd-subunit Complex I that accepts electrons from intramitochondrial NADH and passes them to ubiquinone has been completely discarded, and in its place there is an enzyme which is composed of only one (nuclear-coded) polypeptide. This enzyme does exactly the same job ... except for one thing: it does no proton-pumping.

This led most people, myself included, to give no further thought to the relevance of yeast to development of this technology. Seo et al[22] were not so unimaginative. A number of diseases are known which stem from mutations in Complex I subunits, so it is clear that we need it; but, they thought, do we actually need its proton-pumping? Could the toxicity of these mutations be primarily due, instead, to the production of more LECs, or to the impaired recycling of NADH back to NAD^+? To test this possibility, they introduced a copy of the yeast gene into mammalian cells: this was easy, because (being nuclear-coded) it already uses the same genetic code, and furthermore the mammalian mitochondrial processing peptidase (the protein which removes the presequence of imported proteins after they are imported into mitochondria) recognises the same cleavage sequence as the yeast one, so no re-engineering whatsoever was needed. At the time of writing only very preliminary results are available, but cells mutant in Complex I but expressing this transgenic yeast gene appear to grow faster than controls, and also to use less glucose, indicating that they are performing OXPHOS with the yeast protein. A further indication of OXPHOS function is that these cells can grow well on galactose, which is a much poorer substrate for glycolysis than glucose.[23] Furthermore, they appear to generate fewer LECs: this may be because Complex I is probably the major site of LEC production in the respiratory chain, whereas the yeast enzyme may work by direct two-electron transfer from NADH to ubiquinone, i.e., not generate the risky ubisemiquinone intermediate. (Recall that this is thought to be how the mammalian FAD-dependent electron transporters, such as Complex II, avoid LEC production—see Section 11.2.1.) If this idea really works, the impact on development of protein import would be dramatic, since, of the six mt-coded proteins which have so far not been found nuclear-coded in any species, four are subunits of Complex I.

15.12. Cotranslational Import

The idea that a protein's hydrophobicity hinders its importability into the mitochondrion is based on the assumption that the complete protein exists in the cytosol at some point. This is not necessarily so. Many extracellular proteins are secreted from the cell while they are being built, so that only a very short stretch of the protein is ever in the cytoplasm. There

is compelling evidence that some mitochondrial protein import is similar: in yeast, some ribosomes engaged in translation are found bound to mitochondria.[24,25] This is actually no great surprise, since the signal that targets most proteins to mitochondria is at their N-terminus, which is synthesised first. Thus, this signal becomes "visible" to the targeting machinery before the protein synthesis is complete. In other words, contranslational import may not be obligatory; it may just happen by chance some of the time.

This interpretation is supported by a number of other points. Firstly, it seems that only a minority of import is cotranslational: no transcripts have been found exclusively (or even predominantly) associated with mitochondria-bound ribosomes rather than free ones.[25] Secondly, the acceleration of import that is sometimes achieved by duplicating a protein's leader sequence[18,11] (see Section 15.9) appears hard to explain on the basis of "more strenuous" import, since (based on our current understanding) the import machinery would not be expected to bind both sequences simultaneously; but it is easy to explain on the basis of more rapid targeting to mitochondria leading to more cotranslational (hence successful) import, since the targeting machinery will see a bigger signal more quickly on average.

These considerations might suggest that it would be difficult to exploit cotranslational import for the present purpose. Since no protein is known which is predominantly imported cotranslationally, we must presume that there is nothing about the signal sequence (other than its size) which promotes such import, and we know (see Section 15.9) that bigger is better but not good enough. If contranslational import is a matter of chance, therefore, we would need to increase that chance. Pessimism may be premature, however. One approach that might possibly achieve this would be to exploit the nuclear genome's codon bias.* Codon bias is thought to be self-sustaining, by virtue of rare codons being represented by small numbers of tRNA genes or by tRNAs with low efficiency. Thus, the idea is to give the transgenes deliberately terrible codon bias—to construct them with a large number of codons which are rare in human nuclear DNA, on the basis that they will typically be more slowly recognised and translated than normal, giving more time for import to begin (and, once begun, to keep up with translation). In bacteria, codon choice can alter translation rate by as much as sixfold,[26a] so this approach has potential. Promotion of cotranslational import is a possible reason why overexpressing a protein involved in nucleocytoplasmic transport improves import of moderately hydrophic proteins.[26b]

The consideration of cotranslational import suggests another possible obstacle to mitochondrial gene therapy, however: it is quite conceivable that most mt-coded proteins are cotranslationally exported into the inner membrane. If they are, then the problem of folding (discussed in Section 15.7) becomes altogether more likely: it may very well be much easier for a protein to go straight into the membrane as it comes off a ribosome than as it comes through the Tim machinery, since the ribosome can face the membrane whereas the Tim machinery is facing the wrong way.

** Codon bias is a numerical property of a collection of sequences of protein-coding genes—typically, of the set of all sequenced genes of a given species. Amino acids are encoded by triplets of nucleotides, so there are 64 possible triplets (codons), but there are only 20 amino acids. Thus, most amino acids are encoded by more than one codon—sometimes as many as six. One can therefore compare two synonymous codons (ones that translate to the same amino acid) with regard to how often they each appear in the collection of sequences. One will appear more often than the other: sometimes, it turns out, much more often. The difference between these pairs of numbers is the set's codon bias.*

Table 15.1. mtDNA gene complement in various species, relative to humans

Species	Nuclear in humans, mitochondrial here	Mitochondrial in humans, absent here	Mitochondrial in humans, nuclear here
Most animals	none	none	none
Nematodes, *Mytilus*	none	ATP8?	ATP8?
Most fungi	ATP9	none	none
S. cerevisiae, *S. pombe*	ATP9	NAD1-6, NAD4L	none
Most plants	RPs, SDH, etc.	none	none
Beans e.g., *V. radiata*	RPs, SDH, etc.	none	COX2
Selaginella elegans	RPs, SDH, etc.	none	COX3
Chlamydomonas reinhardtii	none	none?	COX2, 3, ATP6, 8 NAD3, 4L ???

15.13. Semi-Import

This is an approach to complementation of mtDNA mutations that has so far not been explored at all. It is inspired by the observation that the 13 mt-coded proteins are components of transmembrane enzymes, so might theoretically be able to sink into the membrane from the outside, rather than taking a detour into the matrix. They would still have to be transported across the outer membrane, so this could be called "semi-import". Recall from Section 2.4.3 that this is exactly the route taken by some inner membrane proteins, particularly the anion carriers;[27,28] thus, there is no requirement for novel mechanisms to divert the proposed transgene-encoded proteins away from the Tim23-Tim17 complex (the system that transports proteins into the matrix). The signal sequences that direct these naturally semi-imported proteins to the mitochondrion are not N-terminal and are never removed, so incorporation of such a signal into the proposed transgenes faces the problem of potentially rendering the protein non-functional; but we do not yet know nearly enough about these signals to assess the true scale of that obstacle.

Realistically, however, we probably cannot expect this approach to work in the general case. The OXPHOS enzymes are composed of so many subunits that their correct assembly is bound to be dependent on the arrival of each protein from the "expected" side of the membrane. Nevertheless, a few of the 13 may be able to find their correct juxtaposition despite arriving from the "wrong" side; for all we know, those may be the few whose import into the matrix turns out to be the hardest. Thus we should continue to bear this alternative seriously in mind.

15.14 Inteins: A More Coercive Approach

Inteins[30] are self-splicing protein introns: they post-translationally excise from the protein in whose gene they are encoded, ligating the "exteins" on either side with a bona fide peptide bond. Most have been found in Eubacteria and Archaea, but six are known in eukaryotes, including two in the nuclear genome of yeasts. "Split inteins" have also been discovered,[31] in which the two exteins are initially encoded on separate mRNAs but nevertheless ligate to give the same end result.

The relevance of these remarkable entities to mitochondrial gene therapy is that they can be inserted into the middle of a hydrophobic domain, so as to make it much less resistant to unfolding; such a protein should be much easier to import into mitochondria. Inteins must be located immediately N-terminal of a cysteine, a serine or a threonine, but other than that they seem to be able to excise from any extein context.

The major requirement that must be met before inteins can be applied to allotopic expression is that the inteins must be prevented from excising while still in the cytosol. Natural inteins require no cofactors or chaperones for excision, so this requires modification. A simple, but uncertain, approach is to develop conditional inteins which excise in the mitochondrial matrix due to its distinctive chemistry (for example, its pH, which is half a unit or so higher than in the cytosol). Alternatively, one might exploit split inteins, which may be inactivated in the cytosol by the attachment of the N-terminal leader sequence that must be provided in order to direct the preprotein to the mitochondria in the first place. This idea is very new and promising, especially because (unlike the other techniques discussed above) it has a realistic chance of working even on the very hardest of the "dirty baker's dozen".[32]

15.15. More Exotic Technologies

In closing this chapter I think it is worth stressing that, though the technology involved in mitochondrial gene therapy may appear daunting today, other technologies with which we are now completely familiar appeared equally daunting not very long ago. Conversely, methods that are even more firmly in the realms of science fiction in 1998 may come within reach in years to come—and may do so with very little warning. One current idea for targeting exceptionally hydrophobic proteins into mitochondria comes under this heading, so I will briefly sketch it.

It is to exploit the methods used by purely destructive cellular machinery. Most damaged proteins are degraded by a machine called the proteasome, a rather large multi-subunit structure which chews polypeptides into small segments of one or a few amino acids. The overall shape of the proteasome is a tube, and the catalytic core lies right in the centre of the tube; this means that the target protein must be unfolded on its way in. The particular relevance of this is that some target proteins may be extremely hard to unfold (partly, though not only, for reasons of hydrophobicity). Recent high-magnification imaging[29] appears to show that the proteasome achieves this unfolding by rapid motion of the ends of the tube: that is, by shaking the target protein into an unfolded state. Theoretically, this machinery at the end of the tube (though not, of course, the proteolytic core!) could be attached to a mitochondrial import complex and act as a precursor to, and facilitator of, transmembrane protein import.

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Chapter 16

Prospective Impact on the Healthy Human Lifespan

The message of the previous two chapters is that we have a realistic chance of achieving, in only a few decades, a degree of control over the rate of human aging which far exceeds anything that has hitherto seemed feasible. Since that conclusion is rather dramatic, I will use this penultimate chapter to stand back from the technical details and critique it on various other grounds, as well as briefly analysing some of its scientific predictions. The closing chapter then considers a few implications of these predictions, for society at large and (more especially) for the gerontologist; that discussion has the brevity and limited scope which is appropriate in a book principally focused on the scientific issues, but it cannot be omitted entirely.

16.1. Plausibility of Engineered Negligible Senescence

A number of eloquent and senior gerontologists have dismissed the possibility of great extensions of healthy lifespan on purely biological grounds, by examining what would be necessary in order to achieve massively improved maintenance and repair of tissue. All such arguments of which I am aware are drastically flawed. Most of the examples I critique in this section come from a very clear and information-packed book published only four years ago by a very prominent gerontologist;[1] they are suitable for analysis not only for that reason but also because that book, like this, was written so as to be accessible to a wide audience.

16.1.1. Plausibility of Indefinite Maintenance

One of the commonest arguments that indefinite maintenance is impossible appeals to an analogy between the human body and man-made machines. An example is that the heart is a pump, with many similarities to man-made pumps, albeit with somewhat better efficiency, durability and reliability than any which man has built so far. From this is inferred that, once it does finally begin to lose functionality, the heart can only be kept "on the road" by being overhauled in the same sort of way as a man-made pump. In particular, it would have to be stopped for the duration of that overhaul, and a backup pump substituted. In the specific case of the heart it is unclear how this can be considered a remotely conclusive argument against the feasibility of such maintenance, since exactly that substitution is already done during heart transplants. But there is a much more fundamental flaw in it, which applies equally to any organ, irrespective of our ability to overhaul it by invasive surgery: namely, that it is over-anthropomorphic with regard to scale. It is indeed clear that an engineer cannot overhaul a heart-sized, man-made pump, with a spanner and screwdriver, without first stopping it. But if the pump were the size of St. Paul's Cathedral, while nevertheless built entirely from independently replaceable components that were all the size of bolts and screws, then the only problem that the engineer would have in performing the overhaul while the pump remained in operation would be with the G-forces. And that—in fact, a

The Mitochondrial Free Radical Theory of Aging, by Aubrey D.N.J. de Grey.

much larger difference in scale than that—is the correct analogy to consider when assessing the plausibility of maintenance by biotechnology.

16.1.2. Plausibility of Indefinite Repair and Regeneration

Repair is not easy to distinguish qualitatively from maintenance; if one accepts a quantitative distinction then perhaps it can be defined as maintenance on a larger scale. Further along this spectrum are processes which are even more dramatic, and which may more accurately be termed regeneration.

Certain parts of the human body are never repaired at all, even if the damage they suffer is very small. Examples are the enamel of teeth and the sensory hair cells of the inner ear. In order to retain function of such parts indefinitely, we must either develop gene therapy treatments that revive the regenerative capacity which exists in other vertebrate species but which we have lost, or else rely on artificial replacements. Enamel is a fantastically durable material, but some man-made ceramics certainly now equal it. Loss of hearing, due to death of sensory hair cells, would be very difficult to repair artificially on a cell-by-cell basis, but the replacement of the entire auditory apparatus has been very effectively achieved with the development of cochlear implants, whose insertion is now a routine operation in the treatment of profoundly deaf individuals whose auditory nerve is known to be functional.[2]

Another cell type which is purportedly incapable of regeneration in humans is the neuron. However, this is a shortcoming which does not extend even to all homeotherms, since some birds are known to renew parts of their brain every year. Moreover, there have been reports during the past few years of the discovery of neuronal precursor cells in the brains of both adult mice and adult humans.[3,4] The desirability of a turnover of neurons has sometimes been questioned on the basis that it would erase parts of our memory; but the nature of memory—though certainly still very poorly understood—is probably not a case of individual facts or skills being stored in individual cells or connections: it is likely to be a great deal more "holographic" than that. Thus there is no reason to think that a very slow turnover of neurons—which is all that is necessary—would have any effect at all on memories that are being used enough to be retained in normal life. Explorations of such treatment are already being considered.[5,6a,6b]

The final class of regeneration that must be considered is that of highly complex structures. This includes large-scale reconstruction, such as of limbs, and also small-scale but highly intricate reconstruction, such as of the connections between the individual neurons of a severed optic nerve. Both are classic cases of repair which is far beyond our natural capacity, and is also quite impossible with present surgical techniques; the reconstruction of a complex three-dimensional structure such as a limb is a technology which, in many people's eyes, is permanently in the realms of science fiction. Again, however, there is a remarkable capacity for such regeneration in certain other vertebrates—the best-studied examples are amphibians, such as *Xenopus* for the optic nerve[7] and axolotls for the limb.[8] I suspect that surgical techniques will in fact improve rapidly enough to make artificial repair possible within not many decades, so that genetic approaches will not be required. However, I also feel that it is unwise to describe as "surely unreasonable" the prospect of restoring to humans a biological repair capacity that exists in other vertebrates. Vertebrate genomes vary quite considerably at the sequence level, but hardly at all at the level of the gene; thus it is possible to study almost any genetic interaction in one vertebrate and apply the resulting knowledge to another. The cascade of genetic events involved in regenerating a complex structure is obviously very intricate, but it is no less studyable for that—indeed, it is virtually the same as studying embryonic development, which is of course a flourishing field of biology. A recent review[9] noted that, though we certainly do not yet know how to stimulate such regrowth in mammals, there appear to be no showstopping barriers to the development of such therapy. There is even a residual ability of some mammals to regenerate somewhat

smaller structures: mice, for example, can fully and perfectly regrow the tip of a toe so long as they retain the stem cells at the base of the claw,[10] and a comparable effect has been reported in young children.[11]

16.2. Is MiFRA a Theory of Aging?

One can also seek to challenge a theory of aging that proposes a specific mechanism, as noted in Section 7.1, on the grounds of insufficient verification of the mechanism's dominant role. I believe that, while more testing of MiFRA is undoubtedly warranted, it is too well supported by the evidence for its conclusions to be legitimately ignored—especially since, as they relate to extension of healthy lifespan, those conclusions are so dramatic. Taken down to its bare bones, MiFRA can be summed up in four statements:

1. SOS is the main cause of the accumulation of non-dividing cells with no OXPHOS function.
2. That is the main cause of the progressive increase of oxidative stress throughout the body.
3. That is the main cause of the decline of cellular and extracellular maintenance.
4. That is the main determinant of the rates of all currently immutable aspects of human aging.

If all these four statements are true, then abolition of step 1 (by the treatment described in Chapter 15, or in some other way), or of step 2 (e.g. by the treatment described in Chapter 14) would slow down all aspects of human aging by a large factor. How sure can we be that they are true? Let us consider them in turn.

Statement 1 is very strongly supported by current evidence, as discussed in Chapter 8. Furthermore, the details of the SOS mechanism are sufficiently precise that, I believe, any experiment which showed SOS to be incorrect would almost certainly also show how it could be corrected—in other words, it would at once give us a replacement theory which was closer to being correct. More importantly, however, it does not matter exactly how OXPHOS fails in these cells: what matters is that this failure may realistically be avertable by gene therapy, as discussed in Chapter 15.

Statement 3 is also very strongly supported. All maintenance processes in the body are mediated by proteins, which can be generated "at will" by all cells. The only requirements for indefinite retention of those maintenance processes, therefore, are:

a. The supply of sufficient nutrients and oxygen to make the ATP to drive maintenance. This is not limited—at least, not by our biology.
b. The genetic ability to generate this ATP. As observed earlier, this remains intact in at least 99% of cells—and would be so in virtually 100% of cells if the therapy described in Chapter 15 were achieved.
c. The retention of wild-type, and properly regulated, genes encoding the proteins involved in maintenance (which are all nuclear-coded). The rate at which genes become mutant has been computed in various ways, such as from the rate of incidence of cancer, and is thus known to be far too low to account for more than a tiny fraction of the observed rate of decline of maintenance.[12] There is likewise, as yet, no evidence to support the theory that misregulation (for example, dysdifferentiation)[13] occurs in vivo to an extent that could significantly influence the rate of aging.
d. The absence of an increase in the average rate of occurrence of damage requiring repair. Such an increase necessarily impacts the efficacy of maintenance. But damage can only arise (if we exclude macroscopic physical injury or exogenous toxins) from endogenous chemical damage; and the only endogenous toxins—accumulating sources of this damage—are pro-oxidants. Thus, accelerating damage from internal causes can result only from increasing oxidative stress.

Statement 4 is essentially a one-sentence summary of Chapter 5. The major classes of deleterious, late-onset macroscopic change in the human body, which were enumerated and described there, are all maintenance failures. At first glance this is virtually a truism, since maintenance is simply the avoidance of degradative changes; after maturity (or, in women, menopause), all changes are degradative and are reasonably classified as aspects of aging. In fact it is not quite so simple, because the abolition of a decline in the quality of maintenance is not equivalent to abolition of damage, only to abolition of acceleration of that damage. Thus, with permanently youthful maintenance processes we would still age, but at a constant, rather than accelerating, rate. That is quite sufficient, however, to justify statement 4, since the degree of acceleration of aging over a lifetime is very substantial.

Only statement 2 is somewhat less well supported, as yet. It seems highly likely that the process described in Section 9.6 (haemin-driven oxidation of LDL components, which promote further chain reactions after import) occurs to some extent, since all the component steps are chemically favoured. But the only evidence that this process (together with parallel ones also initiated by the PMOR of anaerobic cells) is the main source of oxidative stress is negative: that there seems to be no other process to account for it. Inside a mitochondrially healthy cell, the only macroscopic irreversible change (in the sense defined in Section 5.6) that occurs with time is the accumulation in lysosomes of lipofuscin, a fluorescent concoction of protein, lipids and iron atoms which is popularly known as "age pigment." But it is unclear how lipofuscin can be doing cells any harm at all (except in the extreme case of the aged retina: see Section 5.4), since it is packaged up in lysosomes. It is thus very hard to blame lipofuscin for oxidative stress (though not completely unreasonable: Brunk has suggested[14,15] that it causes problems passively, by attracting the futile attentions of hydrolytic enzymes, which are thereby in shorter supply to digest newly-phagocytosed material). Similarly, no other extracellular mediator of oxidative stress has been convincingly proposed. Some antioxidant hormones decline in activity with age, but their supplementation seems to confer no great benefit, suggesting that they are only peripherally involved. Finally, insofar as there remains doubt that anaerobic cells are the main source of oxidative stress, one must recall that there is no shortage of available tests, as was discussed in Section 12.2.

In summary, then, there appears to be a significant possibility that the theory presented here is correct in the strong sense defined in Section 7.1, namely that complete abolition of the effects of somatic mtDNA mutations would slow all other aspects of aging by at least a factor of two.

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Chapter 17

Conclusion: The Role of the Gerontologist Today

Why did I write this book?

Firstly: not just because I believe that what I have written is true—that would not have been sufficient. This is certainly not the first book ever published which suggests the significant possibility of great increases in healthy human lifespan in the foreseeable future, and the tradition in which it follows is not all that enviable. Its predecessors have usually been swiftly and roundly dismissed by professional biologists as being scientifically unfounded. In general, moreover, that criticism has been absolutely valid. If primary literature has been cited at all, its conclusions have generally been greatly exaggerated or mis-stated in being interpreted as supporting the book's message: perhaps deliberately, but more often through simple lack of understanding on the part of the author. I contend that the theory set out here cannot be challenged on such grounds; but that is not, in itself, a motivation for making an assertion which is sure to be met with widespread skepticism.

Nor have I been motivated by any perception that retardation of aging is or is not desirable—a matter on which there is sure to be profound disagreement. The explanations noted in Section 1.4 for why many gerontologists play down the medium-term likelihood of greatly retarding aging are predicated on the belief that it will not happen. A rather different reason, which may affect some gerontologists' public statements, is that it may indeed happen but would be socially (or morally) a bad thing, so should be played down in an effort to diminish the worldwide efforts to achieve it. I do not know whether it would be socially bad, and I can of course only speak for myself with regard to its morality; I am quite certain, however, that the pace of biotechnological advance can be only minimally slowed by such talk, and therefore that this argument for or against discussion of the prospect is ill-conceived.

My actual reason is a different sociological one. Many gerontologists indeed believe—firmly—that healthy human lifespan will not be greatly increased in the lifetime of anyone alive today. I absolutely accept that if indeed it will not, then a belief to the contrary on the part of the general public would certainly be a bad thing. My difficulty, as presented in the rest of the book, is that I do not agree with the premise. In the previous chapter I addressed various arguments that some distinguished gerontologists have presented for dismissing this possibility, and why I think those arguments are flawed. In this final chapter I shall explain why I believe that, if they actually are flawed, the perpetuation of the erroneous conclusion that lifespan is broadly immutable for the foreseeable future is extremely dangerous for society, and therefore that an open and well-informed debate of the question is of great urgency.

The Mitochondrial Free Radical Theory of Aging, by Aubrey D.N.J. de Grey.

17.1. Demographic Challenges

Demographics is, without doubt, one of the most valuable areas of gerontology today. Policy-makers throughout the world must plan many years ahead in order to ensure that economic stability is maintained, and they can only do this if they have a reasonably accurate idea of, among other things, how much money will be required to support those who are unable to support themselves. The largest sector of society to which this applies is the elderly. It is by no means easy to predict, even roughly, how many old-age pensioners there will be in a given country ten or thirty years from now, and guesses based on intuition have tended to be wrong. Therefore, policy-makers rely heavily on the calculations of specialists who have developed sophisticated statistical techniques for giving reliable predictions.

One of the earliest students of demographics was Benjamin Gompertz, a 19th century actuary. His most lasting contribution to the field[1] was his observation that there was, in the human societies that he studied, an exponential relationship between age and mortality rate. (The mortality rate of a group of individuals is simply the proportion of that group which will die within the next year.) Thus, if one identifies a large group of people who were 55 seventy years ago, and their age at death is plotted, it is found that (say) 5% died within eight years, then 10% of the remainder within the next eight years, then 20% of the remainder within the next eight years, and so on; a hypothetical, exact Gompertz distribution is shown in Figure 17.1. (The "of the remainder" aspect is important—it is why the graph in Figure 17.1c has a "tail" while that in Figure 17.1b does not.) The magic number eight, above, is called the mortality rate doubling time, or MRDT. This geometric progression obviously reaches 100% at a finite age, which implies that humans have a maximum lifespan potential that is exceeded only by a very small number of "outliers."* The accuracy of the Gompertz relation is impressive and has not diminished since his time; attempts are still being made to explain it in mechanistic terms.[2] Moreover, the MRDT of human societies appears not to have increased measurably during the past century, despite the unarguably immense advances in health care. For these reasons, many demographers base extensive and detailed predictions on the assumption that the MRDT will also not increase significantly in the foreseeable future.

All predictions of this sort are, by definition, based on extrapolation. I have been challenged by distinguished demographers in the past on the basis that a dramatic increase in life expectancy within a few decades is "nearly mathematically impossible," by which is meant that the required rate of increase of lifespan would be, say, an order of magnitude greater than it has ever been in our history. The underlying presumption is that, intuitively, this is fantastically unlikely.

However, it is easy to think of technological advances that have transcended this sort of logic. One clear example is the history of intercontinental travel. In 1900, one could have looked at the rate at which ocean-going liners were getting faster and made reasonable predictions for how long it would take in 1950 to travel from London to New York. Those predictions would not have been in the region of a few hours, because they would not have taken into account the advent of aeroplanes. Similarly, any argument about longevity that is based on extrapolation from past trends does not take into account the likely advent of gene therapy as a routine treatment and the possibility that it may have a similarly dramatic impact on the shape of demographers' graphs.

** Study of why these outliers exist, in the face of the Gompertz distribution's remarkable accuracy for the rest of the population, is rightly a flourishing research topic.[3] They are not the "tail" of Figure 17.1c, which depicts a hypothetical, precise Gompertz distribution. But here I want to focus on the population in general, so rare exceptions are of relatively minor relevance.*

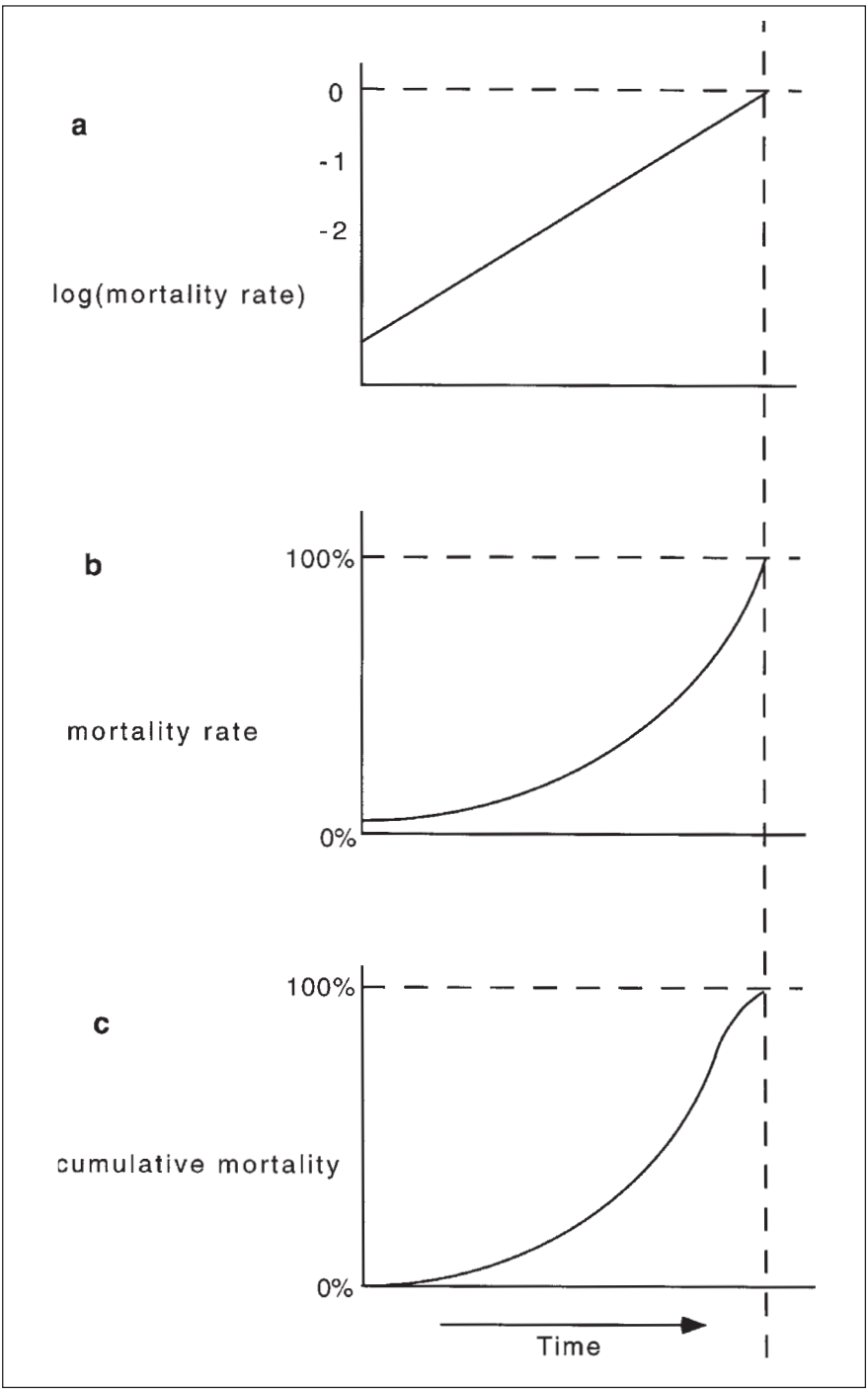

Fig. 17.1. A Gompertz distribution, plotted in three different ways. Note that the tail in panel c is the same length whatever the population size.

The demographer may retort, in turn, that even very great medical advances will have only a small impact on maximum life span. The basis for such a belief is that, as simple mathematics easily reveals, the only way that we can substantially increase maximum lifespan is by increasing the MRDT. If the MRDT remains at around eight years, then a halving of overall mortality rates (that is, achievement of a situation in which people survive half of the diseases, accidents etc. of which they die today) would shift the graph of mortality to the right by only one MRDT—eight years. But, again, this logic rests on extrapolation: in this case, on the assertion that the MRDT will be as impervious to future medical advances as it has been to past ones. The mutually synergistic interactions between various degradative processes, described in Chapters 5 and 7, suggest exactly the reverse—that substantial retardation of any one of them, but particularly of mtDNA decline, would indeed stretch the MRDT.

17.2. Accidents, Diseases and Lifestyle

Another reason why a great increase in lifespan may arrive sooner than is usually supposed is that people may hasten it by caution in everyday life. A number of studies have calculated a supposed upper bound on average human lifespan, by analysing how many people die of accidents and avoidable diseases and how many of "old age" (variously defined) and calculating an average lifespan if all death from the latter were removed.[4] I have never seen, however, a calculation of that sort which takes into account the prospect of changes in lifestyle that people might be expected to undertake in order to reduce their chances of dying of causes other than old age. It seems highly probable to me that a 50-year-old man (who wanted to live a long time) considering a parachute jump would be more circumspect about it if he were risking indefinitely many years of life than if, as now, he were only risking 30 or so. Activities that endanger the lives of others, such as driving, might very easily be outlawed. Once we begin to incorporate such factors, the numbers that come out of these calculations may be remarkably different.

17.3. An Operational Definition of the "Defeat" of Aging

"Immortality" is a word so indescribably loaded with implications having nothing to do with my message that its use in this sentence is the only one you will find in this book. It is nevertheless necessary to consider, for sociological reasons if no others, where the practical boundary comes between increased lifespan and indefinite lifespan. It has been said that an indefinite lifespan is a theoretical impossibility, because there will always be some biological process, however slow, that will catch up with us in the end. This is logically incorrect, because "all" that is in fact needed is a steady stream of medical advances which, cumulatively, increases lifespan (for people of all ages) by a rate faster than the passage of time. If one doubts the plausibility that such a stream will happen, once begun, one should reflect that our understanding of the human body is rapidly—and not asymptotically—improving while the body itself is not getting more complex.

I believe that in practice, therefore, what matters most from a sociological point of view is not whether (or when) large numbers of people are actually living much longer than now but when such a thing becomes perceived to be attainable. This perception will not necessarily even await the advent of effective human life-extension therapy. A single well-documented case of a treatment that doubled the longevity of mice, and which most specialists declare likely to work comparably well in humans too (as would probably be the case for both the approaches discussed here, in Chapters 14 and 15) would be quite sufficient, I believe, to trigger a drastic alteration of the public mood with regard to all life-threatening aspects of everyday life. This alteration would be hardly less great than if the announcement

had been of a treatment that completely abolished all aspects of human senescence, since the latter announcement (or something not far short of it) would at once be generally seen as likely in relatively few years. For sociological purposes, therefore, I contend that it is pointless—and therefore unwise, since it is a distraction—to distinguish between degrees of life extension that exceed a factor of two.

17.4. Possibilities Versus Probabilities

The predictability of scientific progress is not good. A classic case is the history of the search for a polio vaccine,[5] which reminds us that it is not always the optimists who get it wrong:

1. In 1912, one of the leading experts was asked how far away the field was from developing a polio vaccine. He said they were only a few years away.
2. In 1949, a vaccine still did not exist; another expert was asked the same question. He opined that a vaccine was still decades away.
3. Later in 1949, a vaccine was created.

Bearing this in mind, let us briefly set aside the question of whether the scenario I have outlined will actually occur; all I have claimed, after all, is that it is a realistic possibility. Let us simply consider the consequences for society if it were to occur, and our potential to influence those consequences by our prior actions. The innumerable changes to society that would ultimately result are daunting enough; what is even scarier is the transition to that state, since there would inevitably be a period during which the availability of the necessary medical treatment fell short of universal. In view of this, it is very hard to be sanguine that a poorly anticipated transition to a society in which life expectancy is much higher would be a smooth one. A better planned transition, on the other hand, might be less traumatic: not because the public would be any less energetic in their efforts to acquire the treatment at once—that is surely unrealistic—but because the period of limited availability might be greatly shortened by forward planning.

I feel, therefore, that gerontologists have a critical responsibility to remain alert to the medium-term medical prospects of such an eventuality, so as to ensure that policy-makers have as much warning of it as possible. This book has only touched very briefly on the social consequences of a greatly increased lifespan potential, and not at all on the specific difficulties that would face us during the transition to it; for a treatment which expertly blends the social and scientific issues, the reader is recommended to reference 6, and especially to its closing chapter. I have, rather, simply tried to make the case that a wide-ranging discussion of those issues should not be put off. I do not think that inhibition of that discussion, by persistent and vocal overestimation of the time before such an advance could possibly occur, can be described as erring on the side of caution.

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