THE CYTOPLASM IN HEREDITY

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CHAPTER ONE

Introduction to the Problem

Geneticists have long regarded cell constituents as being divisible into two sharply-defined categories: chromosomal or genic and non-chromosomal or cytoplasmic. Only the chromosomes are considered to carry the basic hereditary units by virtue of the remarkable correspondence between the segregation and recombination of the genes and the behaviour of the chromosomes in reproductive cells. It is the precise pattern of this segregation that forms the basis of mendelism. Non-mendelian patterns of inheritance are often interpreted as demonstrating genetic control by cytoplasmic factors although recently major advances in biochemistry have emphasized the uniqueness of the chromosomal material with the realization that deoxyribonucleic acid (DNA) is the primary genetic substance and that it is located in the chromosomes and generally believed to be excluded from other cell components. These findings began in 1944 when Avery, McLeod and McCarty published their well known results on transformation in Diplococcus and culminated with the pronouncement of Watson and Crick in 1953 on the structure of DNA. A number of experiments have since been performed substantiating the theory that the formation of a new DNA chain takes place in response to directions from a DNA template (Taylor, 1957, Meselson and Stahl, 1958, Kornberg, 1960). It is also well established that sequences of the four kinds of bases in DNA spell out messages specifying protein (Crick *et al* 1961). According to the current hypothesis for the sequence of events leading to the synthesis of protein, chromosomal DNA specifies messenger-RNA (a nucleic acid differing from DNA in one of its bases and in its sugar component) which

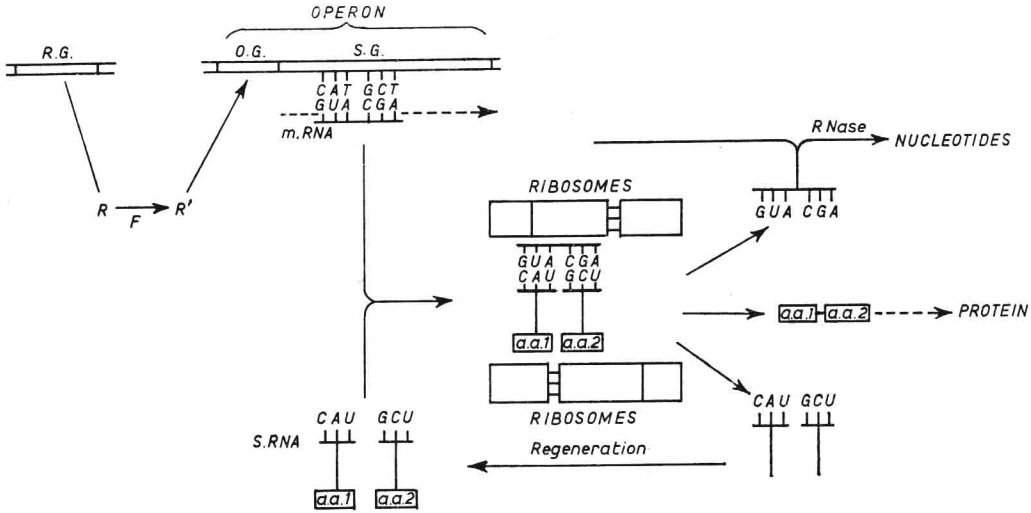

FIG. 1. Model of the regulation of enzyme synthesis (modified from Jacob and Monod, 1962). R.G.: regulator gene; O.G.: operator gene; S.G.: structural gene; R: repressor converted to R′ in presence of effector F (inducing or repressing metabolite); m.RNA: messenger RNA; s.RNA: transfer RNA; a.a.: amino acid; C,G;A,T(U): complementary bases.

moves so as to associate with RNA-containing particles, the ribosomes. Here messenger-RNA functions as the information template for protein synthesis (Fig. 1). How the ribosomes mediate the assembly of amino-acids into specific polypeptide chains is not clearly understood but this problem and others related to it, such as how the messenger-RNA molecule comes off the DNA template, are being tackled with such vigour and ingenuity by a new group of investigators known as molecular biologists, that solutions to these questions are confidently expected in the near future. Mutation is seen as the result of an alteration in one or other of the many possible nucleotide sequences in DNA which would still be compatible with replication. A function for DNA other than specifying protein has yet to be demonstrated. Perhaps this is all that is required, so that given the necessary enzymes the synthesis of all cell components is possible. Put another way, is the code for the entire pattern of every organism contained in the chromosomal DNA or is information from cytoplasmic sources also necessary? The notion that all the structures in the cell can be built up *de novo* by gene action postulates an ideal situation and perhaps this is too much to expect of nuclear DNA although it is clear this substance dominates the general hereditary plan of the cell as a whole. At the same time it must be reiterated that there is no known instance of nuclear genes being directly responsible for the incorporation of macromolecules such as protein into specific cell structures such as ribosomes. During development in higher organisms, the template mechanism insures the precise copying of DNA chains from one cell generation to the next, so that apparently every somatic cell inherits the same complement of nuclear genes. Yet during ontogeny, somatic cells in different tissues show different modifications, the permanence of which has been demonstrated in tissue culture. These inherited differences, or cell heredity, would thus appear to have a basis in the cytoplasm. It may be claimed that cytoplasmic differences are due in the first instance to the action of nuclear genes setting up a cycle of events. Ultimate control of development would then lie with the kind of genes present in the nucleus. Does the nucleus monopolize this control or are there stable hereditary systems in the cytoplasm not subject to modification by the genes which act either directly or in conjunction with gene

products in determining cell characteristics? There are obvious difficulties for two genetic systems acting independently in the cell but if their activities were integrated, this might achieve normal development.

Alternatively it may be argued that during development, the nucleus is systematically altered at each cell division so that cellular differentiation may be no more than the expression of nuclear differentiation. Although these ideas of nuclear change have their protagonists (see chapter 6) it is necessary to be more specific as to what is being changed in the nucleus. It is difficult to visualize the DNA itself changing, having accepted the template mechanism of its replication. However it may be that gene action is not entirely located in the DNA and that the protein component of the DNA-protein complex that makes up the chromosome has to be taken into consideration. If this protein were to change during development, and there is evidence that it does, gene action would be altered accordingly. This poses yet another problem as to the mechanism of the change in the protein component.

The problem then may be narrowed down to the finding of a mechanism whereby different genes are called into action in different tissues. Alternatively, and probably on reflexion more accurately, the requirement is for selective repression of genes or groups of genes in certain cell types. It is clear in the fertilized ovum for example, that the full informational content of the organism is held in check so to speak and that most genes are not functioning. That only part of the genetic information is being utilised at any particular time in a cell is indicated by distinct chemically active sites as seen in the chromosomes of insects (Beerman, 1959). These sites which appear under the microscope as swellings or puffs have different locations in different tissues or at different stages of development in the same tissue.

Perhaps the most significant information on repressor systems comes from the work of Jacob and Monod (1961) in bacteria in which they postulate a 'regulator' gene which makes a repressor substance (Fig. 1). This substance is thought to complex with other cell constituents termed 'co-repressors' or 'inducers'. The complex then interacts or fails to interact with an 'operator' gene which functions directly to repress or allow the formation of messenger-RNA by

neighbouring 'structural' genes. The structural genes have the familiar function of specifying enzymes and together with their operator-gene make up units called 'operons'. Ultimate control then would seem to lie with the co-repressors and inducers. In enzyme-forming systems the co-repressor (i.e. the suppressor) is usually the excess product of enzyme action (an amino-acid for example), while the inducer is frequently the substrate of a degradative process mediated by the enzyme (for example, galactose). In other words, regulation of enzyme formation is by feed-back control. Since cells have a limited requirement for many enzymes, the repressed condition must be the most usual one for many operons. Inducer-repressor and co-repressor-repressor complexes must 'recognize' their individual operator-genes implying specificity in these substances. This may reside in a specific amino acid sequence or in a nucleic acid or a combination of both. At the same time the system requires that operator-gene base sequences differ in some fundamental way from those of regulator genes on the one hand and structural genes on the other.

Although extrapolating from bacterial systems to those operating in higher organisms carries certain risks (for example the genetic material of bacteria is not organized in a nucleus surrounded by a membrane), the whole idea of selective control by regulatory genes of genetic information is attractive in problems of differentiation. The need for a system of this kind is all the more pressing when one considers that the cells of higher organisms generally have a great deal more DNA (perhaps a thousandfold in some cases) than bacterial cells, even large ones. Assuming that a limited number of chemical processes can go on in a cell at any one time and roughly to the same extent irrespective of the type of cell, a much more extensive control system would be required in a mammalian cell for example than in a large bacterial cell.

It is now well established that certain diffusible substances are elaborated in differentiating cells which apparently determine developmental patterns. These substances can be extracted and used to induce embryological development along the lines of the donor tissue in cells which would normally proceed along other morphogenetic pathways, (Hillman and Niu, 1963). The indications are that

these substances are RNA-protein and it has been suggested by Platt (1962) that they may act directly on operator genes and by-pass the regulator genes by virtue of their specific base and amino acid sequences which may allow 'recognition' of operator-gene codes. Thus embryological induction molecules would differ from the substrate-inducers of the bacteria. On the other hand, it may be postulated that the RNA-protein inducers carry genetic information directly and are not involved in repressor systems.

Although the Jacob-Monod system of gene regulation is a model of cell economy its merit when applied to higher organisms is to make very plausible the idea of selective gene action in morphogenesis. In the last analysis we are still faced with the problem of defining the properties of the cytoplasmic factors clearly involved in this system but which are now referred back to the regulator- operator level.

All the foregoing arguments are based on the assumption that the sole repository of DNA is the nucleus or rather, the chromosomes. The idea of cytoplasmic inheritance would become much more feasible if DNA were found in association with cytoplasmic structures and attention will be given here to experiments purporting to demonstrate this to be the case. Further evidence might come from the demonstration that the stable RNA of cytoplasmic components is genetically active. A template mode of bio-synthesis of RNA using polyribonucleotides as primers has already been described (Nakamoto and Weiss, 1962) so one of the requirements for genetic continuity, namely self-replication, now seems available for RNA. That protein can undergo self-replication by a template method seems to be a remote possibility. This is mainly because of the 3-dimensional complexity of protein molecules compared to nucleic acid chains, the replication of which is essentially a two-dimensional concept.

Genetic analysis, that is the analysis of offspring in a cross in terms of segregation and recombination patterns is basically an abstraction. It was through cytological investigation that tangibility was given to mendelian concepts and similarly, speculation on the genetic functions of cytoplasmic structures may be put on a firmer basis by investigations with the electron microscope in an attempt to

correlate heritable changes with alteration or loss of cytoplasmic constituents. Already electron microscopy has revealed a morphological framework of great complexity inside the cell (Fig. 2, *after p.* 8).

Not only has the ultrastructure of distinct cytoplasmic organelles such as mitochondria and plastids been revealed by the submicroscopic ground substance in which these elements are located is seen to present a picture of a complex, polyphasic mixture at electron microscope resolutions. Indeed, electron micrographs of this material are quite unintelligible to the uninitiated. The experienced observer however can readily detect various membranous elements or lamellae which delimit whole areas into canals, tubules and vesicles collectively called the endoplasmic reticulum by Porter and Kallman (1952). Profiles of these membranes seen in sections of cells may be smooth-surfaced or rough-surfaced the latter being due to the presence of numerous small particles believed to be ribosomes attached to the outer surfaces of the membrane. Palade (1956) regards the endoplasmic reticulum as 'a continuous network of membrane-bound cavities which may be involved in the import, export and circulation of various substances' thereby emphasizing the physiological importance of these structures. Rigorous control of the movements of metabolites, substrates and enzymes is necessary in the cell and a membrane-enclosed phase in the cytoplasm would provide this by setting up electrical membrane potentials which seem to be necessary in life processes. As well as supporting a system of membranes, and organelles, the continuous phase or matrix of the cytoplasm contains varying amounts of resolvable dense particles of dimensions around 200 Å. The matrix itself is apparently structureless but may contain aggregates of macromolecules as yet undetected by present methods. Marked variation is seen in the morphology of the endoplasmic reticulum in different cells and it is possible to identify cells with tissues on the basis of the particular form of the endoplasmic reticulum which they display. Differentiation thus involves the elaboration of distinct patterns of organization of cytoplasmic elements and the elucidation of the mechanism of this process is the formidable task confronting the student of morphogenesis. That some of these cytoplasmic elements may have genetic continuity and carry informa-

tion vital to the process is one aspect of the problem under discussion. Another aspect that will be considered is the part played by cytoplasmic, non-genetic factors in regulating the flow of genetic information. In view of the extreme complexity of the cytoplasm taken as a whole a good starting point would be to consider individual organelles from the point of view of structure, function and genetic variability and to extend the investigation to other cytoplasmic constituents. In this way it may be possible to arrive at some conclusion as to the nature of their inheritance, and to obtain clues as to the mechanism of other systems of cytoplasmic control.

CHAPTER TWO

Organelle Inheritance: Mitochondria

Structure and function

The presence of mitochondria in respiring cells of nearly all organisms is an indication of the fundamental nature of the part they play in cell function. In the light microscope this organelle is usually rod-like of about one micron in width and over three or four microns long. In some cells the shape may be more spherical in which case a distinction from other granules is possible by staining methods (see review by Baker, 1958). Although considerable variation in size and shape may be seen in mitochondria of different tissues, there is a remarkable constancy of form in tissues of similar function indicating a correlation between mitochondrial morphology and cellular activity. Electron microscope studies of mitochondrial sections give a consistent picture of the architectural framework as being comprised of a double membrane the outer smooth, the inner involuted, with the infoldings or cristae usually protruding some way into the central fluid or matrix (Fig. 3). Both membranes are about 185 Ångstroms thick and are connected by septae. They have a high lipid and protein content and a recent model of the molecular organization of the membrane based on results from the electron microscope and x-ray diffraction studies, shows a double layer of lipid molecules with each surface covered by a layer of protein molecules (Lehninger 1961). This protein-lipid arrangement may be regarded as the basic structure of the two membranes but since they appear to differ considerably in function, it is likely there are dissimilarities at the molecular level. The outer membrane for example appears to function in connection with

osmotic phenomena and is able to shrink or swell in response to external stimuli. The inner membrane on the other hand is more stable to alterations in the medium and is now generally believed to carry the enzymes of respiration and coupled phosphorylation as part of its structure. This integration of enzymes and membrane probably explains why the isolation of the former has met with so little success. To quote Lehninger, 'our limited information on respiratory carriers and coupling enzymes as isolated molecular entitles, has figuratively had to be carved out of solid rock.' Working with mitochondrial fragments obtained from isolated mitochondria by shattering with sonic vibrations, Lehninger showed that the content per mg. of protein nitrogen of cytochrome oxidase, β–hydroxybutyric dehydrogenase, succinoxidase and ATPase was fairly constant for any one fragment. These results indicated a membrane structure of protein and lipid molecules alternating with enzyme molecules in a distinct and repeating pattern, this pattern reflecting the intricacy of the respiratory process. The formation of cristae can be seen as the response to the need for a relatively large reaction surface. Unlike the respiratory carriers, the enzymes of the Krebs cycle are apparently randomly mixed in 'solution' in the internal matrix.

RNA content

Reports of the presence of RNA in mitochondria come from several sources, but as pointed out by Novikoff (1957) precautions must be taken against contamination from RNA-containing particles (microsomes) in investigations of this kind. In mitochondria isolated from rat liver homogenates he found the RNA content decreased with successive washings. Harel *et al* (1959), Kuff and Dalton (1961), and McLean *et al* (1958) rule out the possibility of contaminations in their experiments and conclude that although of small amount, RNA is intrinsic to mitochondria. One of the difficulties in accepting this view was the apparent insensitivity of intact mitochondria to ribonuclease (the enzyme that has RNA as substrate), but Kalf *et al* (1959) have shown that fragments of calf heart mitochondria produced by sonic vibrations are sensitive to this enzyme. Rendi (1959) obtained fractions of rat liver mitochon-

dria by deoxycholate treatment and estimated the RNA content to be 15% and found its ability to incorporate C^{14}–leucine into protein was imparied by ribonuclease. Presumably, permeability factors are operating in intact mitochondria but in ruptured membranes the substrate RNA is available. The evidence then favours the existence of mitochondrial RNA, a view that is now generally shared by workers in this field. That it has genetic continuity and may have a role in protein synthesis is claimed by some investigators (see below).

DNA content

Several claims have been made that mitochondria contain DNA and the first to be considered is that of Mahler and Pereira (1962). They isolated the crystalline mitochondrial enzyme yeast lactic dehydrogenase-cytochrome b_2 (YLDH) from dried yeast and found it to have an associated polydeoxyribonucleotide consisting of 33 deoxyribonucleotides per enzyme particle. This YLDH DNA does not conform to the usual Watson-Crick double-helical configuration, its bases being mainly unpaired. The possibility that the association of nucleic acid and enzymes is due to a non-specific polyanion-polycation interaction and therefore of an artefactual nature, is ruled out on several counts. For example the complex between YLDH and YLDH DNA is precipitated out from a mixture of proteins and both ribo- and deoxyribonucleotides demonstrating the selective nature of the interaction. Evidence is also presented by these authors making it appear unlikely that the unusual properties of YLDH DNA results from enzymic or chemical change in 'normal' DNA during the reaction process. The conclusion then seems well founded that the association of YLDH and a single-stranded DNA is a naturally-occurring entity, and not fortuitous. Even more convincing would be a repetition of these results with isolated mitochondria as a starting point. Single-stranded DNA is known to carry genetic information in the virus X174 (Sinsheimer, 1959) and in *Vaccinia* virus (Pfau and McCrea, 1962) and it is a reasonable speculation that YLDH DNA also has a genetic function.

Nass and Nass (1963) claim that characteristic DNA fibres are clearly visible in mitochondria from many organisms when viewed in thin section in the electron microscope. From thier observations

of the fine structure of these fibres after various fixations, their stabilization as 20 Å fibrils, the action on them of chelating agents and electron stains, they conclude that the DNA fibres of mitochondria are similar in different organisms as regards their structure. Furthermore, the fibres appear to be highly hydrated and not bound to histones.

In autoradiograph studies of fern egg cells, Bell and Muhlethaler (1963) have followed the uptake of tritiated thymidine into structures they consider to be developing mitochondria, indicating the presence of DNA in these organelles (Fig. 4, *after p.* 8).

Although not providing proof, these various reports make it likely that DNA is a constituent of mitochondria. Presumably, it has a genetic function. The demonstration of mutability of the postulated DNA would go some way towards establishing this latter point and could come from genetic analysis of heritable deficiencies in mitochondria. Before considering the genetic evidence, the position concerning the continuity of mitochondria should be discussed particularly in relation to the cytological findings.

Origin

Lewis and Lewis (1914) looking at animal cells in tissue culture in the phase-contrast microscope reported that increase in the number of mitochondria in the cytoplasm of non-dividing cells resulted from division of existing organelles into two or more daughter mitochondria. In dividing cells, on the other hand, mitochondria did not seem to reproduce but decreased in size and became evenly dispersed throughout the cytoplasm insuring a fairly equal distribution between the daughter cells at cell cleavage. According to those observations, the inheritance of mitochondria is a fortuitous process, the complement of any particular daughter cell depending on which side of the cleavage plane it happened to lie. More recently, Frederic (1958) has made critical studies of mitochondria in dividing fibroblasts under phase-contrast and reports that the decrease in size is a more complicated process than indicated by the Lewises observations. This decrease according to Frederic is accompanied by fragmentation into units and loss of optical density, these changes occurring just prior to nuclear division. At cell cleavage it is this modified form of

the mitochondrion that is passively inherited. Reconstitution of mitochondria from the fragments of 'constitutive elements in the cytoplasm' begins towards the end of nuclear division and continues into the stationary phase. The underlying mechanism of breakdown and reconstitution is unknown. According to Frederic, then, mitochondria as such are not self-duplicating, but continuity resides in certain basic structural units. Bell and Muhlethaler (1963) also describe disintegration of mitochondria during the maturation of the egg-cell of a fern (Fig. 4). In this case the breakdown seems to be complete and evidence is presented from electron micrographs to the effect that a new complement of mitochondria arises by evaginations of the nuclear membrane which are apparently budded off into the cytoplasm where they undergo the requisite modifications to become functional mitochondria. Origin from the nuclear membrane is also postulated by Brandt and Pappas (1959) in the amoeba *Pelomyra*, by Hartmann (1954) in motor nerve cells and by Hoffman and Grigg (1958). Other modes of mitochondrial origin have been suggested including their development from cytoplasmic particles or 'microbodies', from pinocytosis vacuoles, and from the cell membrane. These claims are critically evaluated by Novikoff (1961) who points out that the determination of movement of membranes and organelles from static pictures imposes a serious limitation on the method.

A somewhat different approach to the problem of the genesis of mitochondria in vegetative cells of the mould *Neurospora crassa* is seen in the experiments of Luck (1963). A choline-requiring mutant was grown on a medium containing radioactive choline. This substance is incorporated into lecithin, a major constituent of mitochondrial phospholipids. When cells had become fully labelled, they were transferred to non-radioactive medium. During the subsequent logarithmic growth period, purified mitochondrial fractions were isolated and the distribution of the label among individual mitochondria determined. An even distribution among all mitochondria was found, the average grain count per mitochondrion decreasing as a function of cell growth. This dispersive pattern of the label is interpreted as indicating that mitochondrial formation is by division of existing mitochondria. Presumably, mitochondrial growth by addition of lecithin and other units would precede division. To be

consistent with *de novo* synthesis, labelling would have to be confined to a certain proportion of isolated mitochondria, i.e., those formed during growth on radioactive medium, with this proportion decreasing on unlabelled medium as a logarithmic function of time. The main point is that mitochondria formed during growth on non-radioactive medium are labelled and although the above interpretation may account for this, it is just possible that old and therefore labelled, mitochondria disintegrate at cell division to a large extent as in dividing fibroblasts according to Frederic. Labelled breakdown products may then be incorporated in the synthesis of new mitochondria. Until cytological evidence is available Luck's interpretation may be tentatively accepted and the usefulness of quantitative radio autographic methods in the analysis of this kind of problem acknowledged.

Mitochondrial changes have been extensively studied in spermiogenesis in animals both vertebrate and invertebrate. A general feature is the tendency for mitochondria to form aggregates or to fuse together in the spermatid. Further modification of mitochondrial structure may be quite striking up to the point of degeneration as in the small *Testacella* (Andre, 1959), but in no case is a *de novo* origin reported or has multiplication by fission been seen. At the same time it must be admitted that a clear cytological demonstration of mitochondria reproducing by division in any cell has yet to be presented.

So far three possible mechanisms for the production of mitochondria have been considered: (i) by *de novo* synthesis from structures below the resolution of the electron microscope (ii) by elaboration of visible, non-mitochondrial structures including the nuclear membrane, and (iii) by subdivision of existing mitochondria, not necessarily involving a copying process beforehand. It is clear that the question is still an open one from the evidence so far presented. The incompleteness of our knowledge is not a reflection on the efforts of the investigators but rather serves to emphasize the difficulties of the problem. In any case, perhaps it is misleading to look for any one mechanism of mitochondrial formation and that any one of the three operates depending on the type of cell and the environmental circumstances.