IS THE PLASTID AN ENDOSYMBIONT?

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Cover Notes

SUMMARY (1979)

Hans Ris proposed in 1961 that chloroplasts might be highly derived endosymbiotic microorganisms, originally related to blue-green algae. Evidence from 1966 and earlier is reviewed to test this proposal. Elegant experiments using the unique genetic system offered by *Oenothera* (the evening primroses) clearly show that plastids carry heritable characters not under nuclear control. Two or even three distinctive kinds of plastids may coexist and retain their identities in a single line of heteroplastidic cells. The distinctive characters of a line of plastids were even maintained in contact with a foreign nuclear genome for more than 10 generations of reproduction of the host plant. Studies in *Epilobium*, corn, tobacco, and other plants further demonstrate the heritability and mutability of an independent plastid genome. Time-lapse micro-cinematography, electron microscopy, histochemistry, cell fractionation, tracer and biochemical studies, and DNA hybridization all show that plastids are reproduced only from pre-existing plastids, that they contain DNA differing in many traits from nuclear DNA, that they contain their unique ribosomes, and that even when isolated in vitro or in enucleated cells they still synthesize their own DNA, transcribe at least some RNA, and synthesize some protein. In all of these characters plastids more closely resemble complete blue-green algea than they do other parts of the eukaryote cell.

What do these findings imply for ideas about the origins and early evolution of cells? There are two major anomalies in the previously accepted dogma that all eukaryotes trace from phytoflagellates, which are supposed in turn to derive from blue-green algae:

1. the diversity of very simply organized free-living sarcodina is incompatible with their derivation from the vastly more complex and highly organized phytoflagellates.

2. There is an unbridgeable structural gap between blue-green algea and the simplest phyto-flagellates. There is a close relationship of cell structures of a blue-green algae and a single chloroplast. Nothing is left over to serve as evolutionary anlagen for the remaining structures of the eukaryote cell.

These anomalies vanish if chloroplasts evolved independently, and only secondarily united in a symbiosis with eukaryote cells. It follows that each type evolved independently from the primordial organic soup. Chloroplasts derive from a line of "producers" which evolved stereochemically complex systems of coupled electron transfer reactions to cope with a decline in the quality of the soup. Cytoplasmic motility would be incompatible with coupling the systems stereochemically. Eukaryotes trace from early "consumers" which evolved simple cytoplasmic motility to sop up adsorbed building blocks, and then graduated to phagocytosing "producers." The cytoplasmic shearing forces provided strong selection pressures for the evolution of nucleoprotein chromosomes and nuclear membranes. More complexly specialized motility apparatuses trace easily from a generalized cytoplasmic motility based initially on only a few different kinds of molecules.

Origins of the paper [1979]

The manuscript was first submitted in Hampton L. Carson's Genetics and Evolution course at Washington University, St. Louis., May 3, 1966. It was revised Summer, 1966, in hopes of finding a sponsor for its publication. It was shown at the cell biology meetings in Ames, Iowa, with no result, and since then I have had no time to update the presentation. The typescript includes 26 pages of text, and 66 references. Although old, and certainly not current with the literature, I have decided to try submitting the MS as is to Evolutionary Theory. Many of the ideas are still fresh and deserve further development.

Further comments [2005]

The 1979 abstract and comment was included on a version of the paper distributed with job applications for biology positions in 1979-80. As things transpired, I failed to find the kind of academic position that allowed me to continue my career in biology.

The version here was scanned, OCRed and converted to HTML in 2004 from a photocopy of the summer 1966 version included in 1979-80 job applicatoins. The only changes made from the raw OCRed text were to correct OCR conversion and spelling errors and to add HTML markup and links.

It is interesting to compare my 1966 MS with Lynn Sagan/Margulies' 1967 article, On the Origin of Mitosing Cells", J. Theoretical Biology, p. 225 and 1970 book, Origin of Eukaryotic Cells, Yale University Press. To me, the most significant indicator that chloroplasts had an independent genetic system was the phenotypic evidence for the inheritance of plastid features in ways that could not be explained by inheritance via genetic systems based on nuclear chromosomes. The other area where our analyses differed significantly was in the origin of the flagellar motility apparatus. My theory is that flagellar locomotion of eukaroyotic cells is a directly evolved extension of an already motile cytoplasm, where Sagan/Margules hypothesized that the flagella evolved through the endosymbiosis of spirochaete bacteria. There is now

overwhelming evidence that endosymbiosis of chloroplasts and mitochondria certainly occurred, and in fact, may have occurred more than once.

Had I succeeded in finding a sponsor for the paper at the 1966 cell biology conference, I have no doubt that it would have become a classic paper in cell and evolutionary biology. However, given that I had no perceptible qualifications in cell biology, that I was only a masters degree student in a university that had no accredited masters degree program, and that the study was a distraction my research program on chromosomal evolution and speciation in lizards, I did not persevere in trying to publish it following the lack of interest at the cell biology conference. It took all of my intellectual effort to move from the non-existent graduate program at Southern Illinois University, Edwardsville to Harvard University's PhD program.

IS THE PLASTID AN ENDOSYMBIONT?

William P. Hall, III

Introduction

In 1961 Hans Ris proposed that chloroplasts may be highly evolved and modified derivatives of ancient endosymbiotic microorganisms related to the photosynthetic monera (the blue-green algae and bacteria). The hypothesis revived ideas expressed by Altmann, (l890), Mereschkowsky, (1905), and Famintzin, (1907). In this and another paper (Ris and Plaut, 1962) Ris cited genetic evidence for partial plastid autonomy (Rhoades, 1955; Granick, 1955), cytochemical and cytological work from his lab (Ris and Plaut, 1962), and comparative studies on blue-green algae (Ris and Singh, 196l) as new support for the hypothesis.

Following Ris's papers, there has been an almost exponential increase in the publication of information about the autonomous nature of the chloroplast; yet, to my knowledge, only one writer (Swift, 1965) and his co-workers (Kislev, et. al., 1965) supported, or even mentioned, the possibility that chloroplasts may be derived from endosymbionts. Yet, many investigators have cited the Ris papers for other reasons. No one has publicly discussed the important and far reaching implications of the Ris hypothesis.

My review will show that the Ris hypothesis certainly provides a valid explanation for a large mass of data concerning the genetics, chemistry, and physiology of the plastid, which cannot be readily explained in other ways. After this evidence is presented, some of the more obvious implications of the hypothesis will be discussed. An

examination of these implications will also expose several independent avenues of approach calling for the same conclusion.

Evidence derived from plastid studies, indicating that these bodies arose as independent organisms, is particularly strong in five categories:

1. Experiments demonstrate that at least part of the plastid's inheritance is independent of nuclear control.
2. Several cytological studies show that plastids are derived by division from previously existing plastids and not from any other cellular source.
3. There is strong evidence that the partially autonomous genetic system contained within the plastid is based on its own unique species of DNA.
4. This unique DNA is synthesized within the plastid, and this synthesis is independent of nuclear control.
5. The plastid uses this unique DNA to control the synthesis of proteins by a plastid specific ribosomal system.

In this paper I will pay particular attention to the first category of evidence, since it has the longest history and shows most clearly the independent nature of the chloroplast. The genetic experiments are supported by other evidence showing that the plastid does, in fact, possess the physical and biochemical pre-requisites expected of a genetically unique, self-reproducing organism.

THE GENETIC EVIDENCE

A large mass of genetic evidence has been published showing that some plastid traits are not controlled by nuclear genes. This information was reviewed by several authors (Caspari, 1948; Weier and Stocking, 1952; Rhoades, 1955; Granick, 1955; and von Wettstein, 1961). The elegant studies of plastid inheritance within the genus *Oenothera*, and some of the more recently published work will be discussed here as examples of the available evidence.

To understand the studies of *Oenothera* plastid inheritance, it is necessary to understand the unique nuclear inheritance system found in these relatives of the evening primrose. This genetic system was reviewed by Cleland (1962). Many *Oenothera* races have two distinct haploid sets of chromosomes. A specific haploid chromosome set is called a Renner complex which has a specific haploid genotype called a genome. The complex and its associated genome is usually given a Latinized name. One Renner complex differs from others by a series of reciprocal, whole-arm translocations, arranged so that all of the chromosomes of two different complexes pair in meiosis to form a single complete circle. Meiotic disjunction is not random; each Renner complex segregates as an intact unit into a meiospore. Most complexes possess one or more balanced gametophytic or zygotic lethals that effectively prevent the formation of individuals homozygous for a specific complex. Two different Renner complexes form a single linkage group of two genomes which are generally heterozygous for most loci. Because of the balanced lethals, this is the only propogatable genotype in many lines. Therefore, the line is "true-breeding," even

though heterozygous. The genetic system of *Oenothera* is particularly useful for the study of plastid inheritance, because single Renner complexes enter given gametes. This allows the production of hybrids with accurately known and reproducible combinations of nuclear genomes.

The comments on *Oenothera* plastid genetics are primarily based on Cleland's review (1962). Added information was derived from the other reviews previously cited.

In 1913, De Vries observed anomalous inheritance of plastid traits in some *Oenothera* hybrid crosses. Reciprocal crosses of *Oe. hookeri* (homozygous for the ^{h}hookeri Renner complex) and *Oe. lamarkiana* (a balanced heterozygote with the gaudans and velans Renner complexes) produced an un-explainable distribution of progeny. When ^{h}*hookeri* was used for the female parent and *lamarkiana* provided the pollen, all classes of progeny had normal chloroplasts. When the reciprocal cross was made, with *lamarkiana* as the female, the progeny of the gaudans and ^{h}hookeri complexes were normal, while the velans.^{h}hookeri progeny had defective yellow plastids. However, according to the *Oenothera* genetic system, the ^{h}hookeri.velans progeny from the first cross and the velans.^{h}hookeri from the second should have had exactly the sane phenotypes. The two crosses were identical except that different species were used for the female parent.

Later, Renner (1924) reported that about 15% of the yellow F_1 hybrids from similar crosses of *lamarkiana* and *hookeri* had green flecks or sectors. Self fertilized flowers from green sectors of the hybrid produced green plants, while selfed flowers from the yellow areas produced only yellow plants. A re-examination of the reverse cross showed the reciprocal pattern. Similar observations were made in hybridization experiments with many other *Oenothera* species. Renner offered the following hypothesis to explain this strange situation. He proposed that plastids derived from one race or species could differ in genetic quality from plastids derived from another race.[1] Renner further suggested that *Oenothera* plastids were generally inherited along with the egg cytoplasm although on occasion a few male plastids might be introduced with the sperm nuclei. Once in the egg, the male plastids would segregate randomly with the female plastids during embryogeny which would lead to the formation of sectoral chimeras or localized areas where the plastids differed in functional ability. The evidence for this process of segregation will be discussed in detail below.

In the specific example under consideration, it was proposed that a few *hookeri* plastids entered the *lamarkiana* egg with the sperm nuclei. Once present in the egg cytoplasm, the *hookeri* plastids segregated randomly into meristamatic cells during embryogeny. These cells eventually formed the green areas in the adult plant. The *hookeri* plastids could become green, since they were able to function properly in association with the velans.^{h}hookeri nucleus; although the *lamarkiana* plastids were defective in this same association.

Renner (1936) further concluded that differences between plastid races must be caused by genetic differences within the plastids themselves, and not by any other factors in the cytoplasm. Many different crosses showed that plastids derived from either pollen or egg, from species A, were defective in combination with specific hybrid genomes formed with species B. This was irrespective of which species provided the egg cytoplasm to the hybrid zygote. It was proposed that these genetic

differences between plastid types must have come about by mutation. As evidence, Renner reported finding six separate instances of naturally occurring plastid mutations. These mutants were incapable of becoming green in any genetic environment to which they could be transferred. These were definitely plastid mutations rather than nuclear gene mutations since each was shown to be inherited exactly as were the plastid differences previously discussed. Renner estimated that these mutations were found in .0005 of the examined plants.

Stubbe (1957) provided further evidence that *Oenothera* plastids differ genetically from one another in the same cellular environment. He made cytological studies of the early developmental stages of hybrid plants, when mixtures of plastid types could still be found within single cells. The two plastid types could be easily distinguished when they occurred in the same cell, or after segregation into different cells. In one experiment he was able to produce a hybrid possessing three distinct plastid types. In this plant Stubbe found areas where all three plastid types could be seen in the same cell. The existence of several plastid classes in the same cell, under the identical cytoplasmic conditions after many cell divisions, certainly indicates that different genetic determiners reside within individual plastids.

The long term genetic stability of *Oenothera* plastids was elegantly proved by Schwemle et al. (1938). The experiment was begun by crossing a particular plastid type into association with a foreign genome where the plastids became defective. This produced an obviously weak plant. These weak hybrid plants were selfed and propagated sexually for up to l4 generations of the host plant. After a few generations the hybrid lines gradually became fully green and recovered vigor. One might have assumed that the defective plastids adapted to the new genome, but suitable outcrosses showed that it was actually the hybrid genome which adapted to the plastids! Plastids transferred from the adapted line into the same, but not adapted, genome produced a phenotype identical to the the original weak hybrid. Plastids carried for several generations with the hybrid genome also immediately recovered their normal appearance when crossed back into their normal nuclear association, thus indicating that the plastid's genome was not changed after thousands of duplications during long association with a foreign nuclear genome.

The independent nature of the *Oenothera* plastids has also been used as a taxonomic tool for the classification of plastid classes and for the determination of nuclear genome relationships. Schotz (1954) used a fortuitous plastid mutation which was incapable of becomming green in any nuclear combination as a standard for comparing division rates of naturally occurring plastid classes. In some tests two different plastid classes could be compared to the mutant type in association with identical nuclear genotypes. Different characteristic rates of division were found for different classes of plastid in this study.

Stubbe (1959) recognized five distinct classes of plastid after studying more than 500 genome combinations involving l4 distinct *Oenothera* races. These races were grouped into "superspecies" based on their characteristic plastids He also found that the genomes of the Renner complexes could be placed in three classes according to the effects combinations of them had on the five plastid classes.

I believe the material just presented is entirely adequate to prove that plastids of the genus *Oenothera* have inheritable factors which are clearly independent of the nuclear genome. This inheritance must also depend on genetic information found within each of the individual plastids. However, before this conclusion is entirely accepted, can any alternative explanations be found, or can the work be invalidated?

One might question the reliability or precision of the investigators, or the care with which the genetic experiments were performed. Although I am not able to criticize the original work, since most is in German, I don't think this would be reasonable. I think it would be difficult for several independent investigators to reach the same conclusion or to make the same mistakes. Erroneous conclusions are particularly doubtful in the light of the many control experiments reported.

Some experiments were specifically designed to rule out any cytoplasmic factors that might influence inheritance of the plastid phenotype. Also, it does not seem likely that any possible external or internal influence could cause phenotypically distinct plastids to be inherited, particularly when these types could be found in the same cell.

It is remotely possible that phenotypic effects like those observed could be caused by viruses. However, the reported results would seem to require every plastid in all *Oenothera* lines to be infected by virus in order to explain the taxonomic distribution of plastid phenotype classes. If present, these hypothetical viruses would necessarily seem to be temperate in nature since there is no indication that viruses of one cell ever infect plastids in another cell, or even other plastids in the sane cell.[2] Finally, if temperate viruses were actually present in all of the chloroplasts, they would seem to require the presence of an endogenous genetic system for transmission. Since none of these alternatives provide reasonable explanations for the data, there is no alternative but to assume that *Oenothera* plastids are genetically distinct entities.

If plastids are really genetically distinct, they should be mutable, and the mutant plastids and their progeny should segregate at each cell division and not just in meiosis as do nuclear genes. Some of the *Oenothera* experiments seemed to indicate mutation and mitotic segregation, but much better evidence is available.

Recently, Michaelis (1959) reviewed some of his studies of the inheritance and segregation of cytoplasnic mutations in *Epilobium*, a genus related to *Oenothera*. The inheritable nature of *Epilobium* plastids had already been demonstrated (Michaelis, 1949; 1958b). In the 1959 paper, Michaelis discussed statistically tested cytological observations which were made on the distribution and segregation of mutant plastids during the growth of individual plants. Since the natural mutation rate for plastids in this species was low (.0008 in 68,699 cultivated plants), plastid mutations were artificially induced by exposing experimental plants to ionizing radiation which was enough to raise the plastid mutation rate by a factor of 10 (Michaelis, 1958c).

Plastid mutations were studied in 172 plants where the radiation had induced a chlorophyll variegation. Wherever the abnormal plastid condition reached the flowers of the variegated plant, the inheritance of the trait was tested. In all of these cases, the mutation was inherited only maternally, indicating that it was non-nuclear. A statistically tested examination of the distribution of leaf variegation in 128 of the studied plants showed that areas of mutated chloroplasts were randomly distributed in

all but seven cases. This was taken to indicate that specific developmental processes were not involved.

Michaelis was unable to distinguish cytological differences between mutant and normal plastids until they began to become green in the maturing leaf. Cytological study of mature leaves from all of the 172 variegated plants showed 50 where individual, cells contained both plastid types, 92 where plastid differences were cytologically indistinguishable, and 30 plants where no mixed cells could be found. In this latter case all plastids in a given cell had either the mutant or normal phenotype. Assuming random assortment of plastid types at each mitotic division, statistical models predicted that sister cells should have similar ratios of mutant to normal plastids, and that deviations from this ratio should follow certain distributions. Cytological examination . of leaves containing mixed cells showed that the plastid types were distributed as expected. Thus, implying that genetically distinct plastids were segregating at random.

Starting with different numbers of segregating units, statistical predictions were made of the distribution of homoplastidic normal, heteroplastidic, and homoplastidic mutant cells to be expected after various numbers of mitotic divisions. For low numbers of segregating units, the theory predicted that the cell divisions of one individual plant should be adequate to produce fixation of plastid types in most cells. In theory, these predictions would allow the discrimination of effects caused by chloroplast mutations from effects possibly caused by different segregating factors in the cytoplasm. However, for the test to be useful, the point of mutation and the number of subsequent cell divisions must be known. Naturally, practical application of the theory is difficult, since it is usually impossible to localize the point of a mutation.

However, Michaelis found a particularly fortunate event. A back mutation was observed in a leaf growing from a purely mutant sector of a variegated plant. It was determined that the mutation event must have occurred during one of the leaf primordium's first cell divisions. It was also known, within an order of magnitude, how many cell divisions were required for the formation of the mature leaf. Cell families were studied to determine how many cell divisions were required to sort out the progeny of the mutant plastid. The cytological observations were then compared with the statistical tables. The comparison indicated that more than 10 and less than 20 segregating units must have been assorted. The average number of plastids in meristematic cells of this species was observed to be 12, with a range of 5 to 20; which was certainly within the limits of the statistical prediction. Plastids were the only cytoplasmic units present in so few numbers. Mitochondria were present in the next lowest numbers, but were so common that segregation of a mitochondrial mutant would have taken much longer. Michaelis concluded that segregation of a mutant plastid could be the only cause of the observed varigation.

After this test Michaelis examined the thirty cases of plastid variegation where no mixed cells were found. He proposed that mutant and non-mutant plastids were still segregating in early mitotic divisions. But, in these instances, the plastids within the sane cell might interact through the exchange of diffusible substances. He suggested two possible ways the plastids could influence one-another. First, the mutant plastid might manufacture a substance that damaged the normal plastids during the maturation process. In the other type of interaction, the normal plastid might

manufacture a substance that would allow the mutant form to develop normally. Theory predicted that the two types could be distinguished clearly by an examination of the distribution of mutant and normal cells. Michaelis found clear examples of both types of mutation in his material.

Burk, et. al. (1964) reported a similar study of the somatic segregation and histogenesis of a plastid controlled variegation in tobacco. These authors made a detailed study of the propagation and genetics of a naturally occurring plastid mutation. They found that the heteroplastidic condition seemed to be preferred in meristematic tissue--i.e. few cells tended to become homoplastidic. Also the mutant condition dominated the normal plastid during the maturation process and suppressed the normal plastid's phenotype if the mutants were present in excess.

The Burk article is also important because they thoroughly studied the distribution and fixation of the mutation in the various histogenic layers of the plant. Burk et. al. noted that Michaelis did not consider this particular aspect in his 1958 study. Also many other studies of variegation in a wide variety of plants were reviewed. They concluded that many variegations could be explained best by the early fixation of plastid mutations in one or more histogenic layers. This is why so few examples of the mutations have been confirmed by the finding of heteroplastidic cells. They remarked that it would be unlikely for many mutants to be favored in the heteroplastic condition.[3]

It should also be mentioned scattered evidence exists for plastids of the lower eukaryote plants (those plants that have nuclear membranes and divide by mitosis) also show genetic continuity. Granick (1955) reviewed most of the important breeding and cytological studies concerning the lower plants. More recent plastid studies in these plants have generally been aimed towards finding the physical and chemical basis for plastid inheritance.

A summation of the plastid inheritance studies provides a reasonably clear picture of the functional nature of the plastid associated genetic system.

1. This genetic system is very stable-being propagated for thousands of replications without observable change.
2. At a minimum, the genetic system regulates certain chemical and morphological aspects of chloroplast development.
3. The genetic system is subject to occasional mutations, which may affect developmental or growth processes in diverse fashions.
4. The genetic system is discrete and located within each of the plastids.
5. The plastid traits seem to show clonal inheritance and segregation within the cellular environment.
6. The plastid's genetic system is completely isolated from the nuclear genome.[4]
7. The plastid genomes can, and do, undergo evolutionary changes, like the formation of species, apparently as an adaptive response to an evolving cytoplasm.

This report has presented good experimental verification for all of these aspects of the plastid specific genetic systems. However, it is realized that the experiments reviewed here certainly do not represent all of the available work, and it is also realized that

much of the published work is ambiguous or misleading. Finally, it is known that some of the papers contradict the statements just made. Yet the evidence presented still seems to be valid, and being valid, still needs an explanation.

Other Evidence

Many investigators, particularly before good cytological techniques were developed for the electron microscope, have claimed that chloroplasts are derived from the cell nucleus. Or, they have claimed that chloroplasts are the specialized progeny of mitochondria, which were derived from the nucleus. Other authors claim a *de novo* origin. Weier (1963) and Granick (1963) refute these ideas. In the first place, the genetic evidence indicates that the plastid is inherited independently. In the second place, good cytological studies (where proplastids and mitochondria were distinguished) never show any merging of nucleus and plastid or of mitochondria and plastid. Recent cytological studies have shown that proplastids and promitochondria are always distinctly different objects in good electron microscope preparations. Jensen (1965) clearly showed distinct proplastids and mitochondria in the plant egg cell. Other papers show plastids in the pollen tube (Hanson, 1965 [missing reference]). Many published electron micrographs show plastids in the process of division. Green (1964) took clear timelapse micro photographs of the division of *Nitella* plastids covering more than one replication cycle. In short, there is abundant cytological evidence that the plastids are in fact derived from previously existing plastids by fission, and not from any other source.

Since the genetic stability and low mutation rate which are characteristic of the plastid genomes are also characteristic of the genetic systems of free-living organisms, one would expect both of these systems to have the same chemical foundation. Since all. known organisms carry their hereditary information on DNA, one would expect to find DNA within the isolated plastid if this unit is actually a functioning organism. This has been demonstrated many times with a great variety of techniques. Swift (1965) and Gibor (1965) reviewed earlier experiments. More recent studies demonstrating plastid DNA were done by Hotta, et. al. (1965) and Shipp, et. al. (1965).

The earliest experiments attempting to demonstrate the presence of DNA in isolated chloroplasts were generally poorly controlled and inconclusive. Recent works have been very precise and carefully controlled. For instance, control experiments are frequently conducted to rule out the possibility of bacterial or mitochondrial contamination, which generally involve the addition and subsequent separation of known contaminants. Studies have been made on plastids taken from organisms ranging from the phytoflagellates to spinach and tobacco. All of the recent experiments have conclusively shown that the plastid DNA is, in fact, DNA; that it generally differs from the nuclear DNA in buoyant density and base pair ratio x, and that it does not hybridize with the nuclear DNA. This last experimental technique proves there are few areas where the base sequences (or the genetic code) are similar (Shipp, et. al. 1965).

In addition to the studies of isolated chloroplasts, there are also several electron microscope cytochemical studies (Ris and Plaut, 1962; Kislev, et. al, 1965). These show the physical presence of DNA strands within plastids taken from a variety of sources.

Studies have shown that chloroplasts can synthesize their own DNA in intact cells, in anucleate cells, and also when the plastids are isolated in vitro. The incorporation of various radioactive or isotopically tagged compounds into DNA within the plastid has been demonstrated. DNA synthesis has been observed while various antibiotics and other chemical inhibitors were used to insure that no information could be transferred to the plastid through the cytoplasm. Kislev, et. al. (1965), Hotta, et. al. (1965), Janowski (1965), and Shephard (1965) all performed experiments of this nature. Endogenous DNA synthesis was also demonstrated in the following, more complex experiments.

The endogenous synthesis of DNA, RNA, and proteins within a variety of plastids has been shown by several investigators (Goffeau and Brachet, 1965; Schwartz, et. al. 1965; Sissakian, et. al. 1965; and Sheppard, 1965). The line of information transfer from the plastids endogenous DNA to the production of specific proteins has been traced. No part of this line depends on information transferred from the cell's nucleus. Plastid ribosomes have been isolated from the cell's chloroplast faction (they generally have a different sedimentation rate or buoyant density than the cytoplasmic ribosomes) and used for the transcription of specific RNA messages into specific proteins. Interestingly enough, in one such experiment (Schwartz, et. al. 1965) the plastid ribosomes could accurately transcribe the message carried by a coliphage virus RNA, while they only synthesized nonsense when programmed by a tobacco mosaic virus RNA. This may be a very interesting result, since it is my understanding that TMV RNA generally operates with cytoplasmic ribosomes of a eukaryote cell, while the colliphage RNA would be adapted to operate with the ribosomes of a moneran cell.

Discussion

The papers I have reviewed show that plastids do in fact have many attributes of independent organisms. This is compatible with the idea that chloroplasts are endosymbiotic descendants of primitive free-living photo-synthetic monerans, now living in the cytoplasm of eukaryote plants. However, it is practically certain that plastids are incapable of an extracellular existance, whatever their evolutionary origin nay have been.

If plastids are desendents of free-living monerans, would one expect to find the close metabolic relationships between plastids and eukaryote cells that are seen today?

The initial plastid-eukaryote association must have taken place in the Cambrian period or earlier, since the earliest multicellular green algae and vascular plant fossils are known from the late Cambrian. This would allow at least $5x10^8$ years for refinement of the symbiotic association. The presymbiotic plastid ancestors must have

been competent to synthesize most, if not all, compounds needed for their structural development and growth. After entering eukaryote cells, the plastid ancestors would have a readily available and concentrated source of structurally and metabolically important compounds surrounding then. The eukaryote cell would probably depend on the plastid for energy fixation, and possibly for some complex synthetic activities. Under these circumstances natural selection would probably favor increased specialization of the partners. Mutations that increased the energy fixing abilities of the primitive plastids would probably be most favored by selection, while there would seem to be little selective value for the retention of metabolic pathways for the synthesis of compounds also manufactured by the eukaryote cytoplasm. In fact, photosynthetic efficiency could probably be increased most easily by the selective elimination of "useless" metabolic machinery with the concurrent expansion of the photosynthetic apparatus. In this reduction, the plastid would be likely to retain only the genetic information needed to specify and control its specialized functions. This could lead to an associated reduction in the amount of plastid DNA.

The elimination of "useless" plastid functions night give selective advantages to the host cell which must be responsive to the external environment. If the plastids incorporated compounds manufactured by the host cytoplasm, then the host nucleus would have genetic control over these compounds. Therefore, environmental natural selection operating on the nuclear genome, could directly affect plastid morphology and function. Since most eukaryote organisms have sexual processes allowing rapid evolution, which are apparently lacking in the plastids[5] and the hypothetical plastid ancestors (cyanophycae) transfer of genetic control to the host nucleus could have considerable selective value.

Considering the selective factors just discussed, and assuming a moneran origin, a highly evolved plastid should have little or no endogenous genetic system, and it should contain, at most, only a few structures not directly active in its primary functions. If no plastid genetic system remained, there would be no way to prove that plastids ever had endogenous genetic systems. However, the papers previously cited have shown that even the plastids of modem plants have enough of an hereditary mechanism to be observed in genetic experiments and by cytochemical techniques.

The smallest estimate of the amount of chloroplast DNA (Gibor and Izawa, 1963) was $1x10^{-16}$ gm per plastid from *Acctabularia*. Other studies cited by Gibor and Granick (1964) provide estimates of $1x10^{-16}$ to $10x10^{-16}$ gram DNA per higher plant plastid. Gibor and Granick using data from the literature, calculated that Euglena plastids each had $40x10^{-16}$ grams of DNA. Edelman, et. al. (1964), using their own data, calculated that *Englena* chloroplasts each had a minimum of $12x10^{-16}$ grams of DNA. Gibor and Granick observed that a DNA content of $1x10^{-16}$ was characteristic of some of the more complex viruses. Edelman, et. al. remarked that an *E. coli* cell carried around $16x10^{-16}$ grams of DNA. This data suggests that the chloroplasts of the most primitive flagellates probably carry almost as much genetic information as do some of the plastid's free living relatives. The more highly evolved organisms, which probably posses more highly evolved plastids, show a considerable reduction in the amount of DNA that they carry. This is precisely the picture that would be expected if the chloroplasts were derived from a free-living moneran ancestor.

Of course the acceptance of this hypothesis would cause some unique taxonomic problems. Dillon, (1962), Lwoff (1951), Hutner and Provasoli (1951), Goodwin (1964), as well as many others, propose that phytoflagellates and therefore higher plants also, are derived as a whole (they didn't think of the other possibility) from the blue-green algae [monerans lacking nuclear membranes], because of many chemical and structural similarities between their photosynthetic systems. Since no other properly photosynthetic forms are known, almost no one has considered any alternative possibilities. Obviously, the idea that only the photosynthetic part of the eukaryote cell is derived from the photosynthetic moneran is incompatible with the idea that the whole eukaryote cell is derived from that source. Since there is so much evidence supporting the endosymbiotic nature of the plastid, perhaps there is something wrong with those taxonomical assumptions.

Acceptance of the moneran origin of the plastid would also cause difficulties for the protozoan taxonomist. The viewpoint of many protozoologists (Kudo, 1954, Hall, 1953, Pitelka, 1963, Lwoff, 1951, Hutner and Provasoli, 1955, etc.) is that eukaryote heterotrophic protozoans were initially derived from the photosynthetic eukaryote cells. This viewpoint, of course, leaves the eukaryote cell without any point of origin. Therefore, if the Ris hypothesis is accepted for consideration, it must be assumed that the protozoans represent an independent line of evolution, separate from the photosynthetic monerans. Then it would seem reasonable that this line provided the host cells colonized by the presumptive plastids.

Where and how might this second line have started? (Don't ask the modern protozoologists though. Apparently they haven't seriously considered the problem since it was settled during the 30's and 40's (Lwoff, 1951).) Before 1931, protozoologists used a simple rule-of-thumb to decide which of two organisms had the most primitive characteristics:[6] The simpler of the two organisms was considered to be the closest to the primitive condition. As presently understood, the laws of thermodynamics and information theory would probably provide theoretical support for this viewpoint (Margalef, 1963).

Before the 1930's almost any protozoologist would agree that the amoeba-like protozoans, because of their simplicity, must have been closer to the ancestral animal condition than any other organism which could be examined with the light microscope. In addition to the visible simplicity there are many other valid reasons, for accepting this hypothesis.

As already noted, members of the order Amoebina have the simplest structure, on both visible and ultrastructural levels. (Pitelka, 1963) Virtually all protozoan groups can be derived by one, or at the most a few, steps of increasing specialization and complexity from an ameboid ancestor. Testaceans are more complicated because they have tests (Kudo, 1956). Actinopods are more complicated because randomly oriented motility molecules are rearranged into orderly polymers (Kitching, 1964). Some of the amoebina tend towards the development of polymerized, motile, pseudopodia which are not flagellar in nature (Bovee, 1964). Some past amoeba probably found a particularly successful spatial, arrangement of the polymer fibers leading to the famous 9+2 pattern of the flagellum. Interestingly enough, in the sarcodinan groups there are some other patterns reminiscent of flagellar structure in complexity (Kitching, 1964, Roth, 1964).

One very good clue to primitivencss is the absence of evidence hat any of the free-living, non-testate Amoebina reproduce sexually;[7] although sexual mechanisms are found, in one form or another, in most of the other protozoan types. This distribution would be expected if meiosis is a specialized and advanced type of mitosis.[8] As one further example, the flagellate mitotic and chromosomal apparatus seems to be much more closely related to that of the higher plants and animals, than it is to the genetic apparatus of some of the amoeba (Kudo, 1954, Cleveland, many papers, Saito, 1961, McClellan, 1959). The Sarcodina seem to have the greatest variety of unique mitotic apparatuses. One would expect to find just this experimentation with many forms in the most primitive group. The more advanced groups would show specializations of the more successful mitotic mechanisms.

In short, I think a little reflection will show that reasonably direct lines of advancement and evolution nay be drawn to the modern protozoan groups, if the amoeba is taken as the ancestral form. On the other hand, if the flagellates are assumed to be ancestral; complex series of advancements and regressions must be assumed in order to develop any reasonable protozoan phylogenies. The principle of William of Occam, sometimes known as parsimony, tells one to choose the simplest of the available alternatives.

For the sake of completeness, the large gap of structural complexity between the most advanced living monerans, and the simplest photosynthetic eukaryotes should be pointed out. Why are the lines of structural evolution so clear both above and below this gap? If the eukaryote organism [as a whole] is derived directly from the monerans, where are all of the intermediate structural forms? Dillon (1962) remarked on this absence, and mentioned the wide nature of the gap.

Taking stock--it now appears that there were at least two independent lines of evolution early in the phylogeny of life. The first line leading to the development of non-motile producers reached its culmination with the development of the complex synthetic apparatus characteristic of the blue-green algae. The second stock must have had many characteristics of the amoebas. However, the presence of motility compounds [i.e., macromolecules] in this line provided fertile substrates for the evolution of complex kinetic structures, such as filaments, mitotic spindles, microtubules, flagella and eventually muscle fibers.

Under many circumstances, the combination of advanced synthetic and kinetic abilities could have great selective value. A symbiotic association would have many obvious advantages over both the pure moneran or eukaryotc cell types.[9] It is also reasonable that this association, being so adaptive would have taken place many times. In fact, it still happens with fair frequency, as is seen by the number of symbionts known in metazoan animals (Barnes, 1963; Trager, 1960; Lederberg, 1952). Lederberg noted several recent cyanophyte endosymbioses in protozoans, although he rejected the Famintzin, Moreschowski idea. The diatoms may also be an independent line derived from an early sarcodinian offshoot, since its centriole is totally unlike that found in most eucells (Drumm and Pankratz, 1963).

To this point we have been looking at the evolution of the chloroplast from a viewpoint in the present. How would the situation appear if it were examined from a viewpoint preceding the origin of life?

Oparin has described the stage setting for this drama rather well. The atmosphere of the abiotic world was a reducing one, probably high in simple hydrocarbons and ammonia, with a liquid phase of water (Berkner and Marshall, 1965). This atmosphere was bombarded with high energy radiation, which activated the simple carbon and nitrogen compounds present. These activated compounds would then react to lose the excess potential energy they carried. This process would lead naturally and probably to the formation of large reservoirs of high potential, information rich, organic compounds. These would be reasonably stabile, since there would be practically no free oxygen available to attack them. It might be noted that the famous experiments of Calvin, Miller, Fox, etc. confirm the probability of this process. It is also interesting to note that Barghoorn (Barghoorn and Schopf, 1966) believes that he may have an electronmicrograph of the fossilized evidence of these organic compounds.

I think that almost anyone familiar with the Oparin hypothesis (1938) would agree that the first "living" organisms were necessarily very simple heterotrophs which fed on the geochemically produced biomolecules. Under these circumstances, the heterotrophs would eventually consume their abiotic food supply, which would favor the evolution of synthetic ability. If this path was actually followed, there may be several living remainders. The chemosynthetic and photosynthetic bacteria seem to be more primitive than the blue-green algae in their synthetic abilities.

It is interesting to note that none of these primitive producers have developed large size or a successful system of motility. Why?

It is suggested that complex arrays of specifically organized organic molecules, arranged in precise stearic configurations, are necessary for efficient photosynthesis. Any system of motility that might disturb this stearic configuration would obviously be maladaptive for an organism that depended on said configuration for its dinner. Apparently the bacterial flagellum and the bending motion of *Oscillatoria* have been the most successful attempts towards motility. Nowhere in these motile structures is there anything that could reasonably give rise to either a cytoplasmic ameboid notion, or to the highly complex and specific structural arrangement of the eukaryote flagellum. It also seems that cytoplasmic motility night be a prerequisite for the attainment of large cell size. The moneran cell size is probably limited by the internal diffusion rate of important compounds. Any cytoplasmic motility for internal circulation would probably disturb the stearic configuration of the synthetic machinery and therefore be maladaptive. Again, the moneran cannot reasonably be the ancestor of the [entire] eukaryote cell.

This leaves only one avenue to consider--one which has been completely forgotten about or ignored in the literature. What kind of metabolism did the first "living" organisms have?

These were heterotrophs, and probably had a fementative metabolism. These [first] organisms must have made their living by making small alterations on abiotic macromolecules in order to fit them to their needs. Initially this metabolism probably required only a few enzymes, since the organism was a direct product of the abiotic chemicals initially available. As the food supply became harder to obtain, *two* classes

of adaptive alternatives would be available. The first alternative; development of increasing enzymic specialization for synthesis has already been considered.

The second alternative would require only the development of a simple motility. I think this is the easiest and most probable line of evolution from a proto-heterotrophic organism. The only adaptive requirement needed for motility would be the development of a somewhat specialized membrane and a large number of a few kinds of [macro]molecules which could change shape when stimulated to do so by a change in, say, ionic concentration. Even a motility sufficient to allow the proto-heterotroph to roll around on the immediate substrate would have adaptive value, since this would allow biomolecules to be sopped up from a large substrate surface, rather than requiring diffusion to supply the food substances. This type of adaptation would certainly not require as much information (or consequently as many genetic mutations) as would be required for the development of the structural complexity of the autotrophic producer. As long as some organisms specialized for autotrophic production, the evolution of a motile heterotrophic line would seem to be a logical necessity. Once this second line started, easy modifications would lead to phagotrophy and the development of specialized kinetic structures, such as flagella and the spindle apparatus. Involvement of the cytoplasm in motility would put an immediate selective premium on the development of a system of internal membranes to protect the genetic structures, an arrangement having little obvious value for the reasonably akinetic monerans. A constantly changing and wearing membranous system would place a selective value on the development of areas of membrane manufacture, such as the dichtysomes or golgi bodies. In short, the evolutionary origin and development of a proto-heterotrophic line of organisms would be expected to lead to the evolution of just those organelles characteristic of the eukaryote cell.

The subsequent combination of these two lines to form the autotrophic eukaryote would also be probable. Cytoplasmic motility and complex biosynthesis were shown to be directly antagonistic functions. However, a combination of the two [within one cell] would have some obvious advantages over either of the original states. For instance, the combination of motility and photosynthetic ability must have opened the great surface areas of the primordial oceans to autotrophic organisms (Berkner and Marshall, 1965, Fischer, 1965). Before symbiosis, the autotrophic monerans, and hence the heterotrophic organisms feeding on them, were probably limited to substrate areas within the range of visual light and below the penetration of ultraviolet. With the symbiotic combination of biosynthesis and motility, pelagic communities night become possible or likely. This is a morphologically feasible combination, since the moneran's external membrane would protect the stearic configuration of its synthetic machinery from the kinetic sources of the eukaryote's cytoplasm.

Summary

I have just barely scratched the surface of the ideas released by the tentative acceptance of Hans Ris's hypothesis that the chloroplasts of higher plants are really not part of the plant at all, but are, in fact, endosymbiotic microorganisms. Even if the idea is totally wrong--which I doubt--it should be seriously considered for the new

and untried experimental approaches and viewpoints that it can so abundantly generate.

Perhaps a serious and considered study of the facts and understanding created by the acceptance of this hypothesis would lead to some reasonable ideas concerning the origin and evolution of the mitochondria, which also show some signs of genetic independence.

Notes

[1] It would seem that plastids from a given race might be able to function properly with the chemical substrates elaborated under the direction of some genome combinations, but become defective when placed in association with other combinations. This same explanation could apply equally well to inherited plastid defects under Mendelian control.

[2] Someone reported unsuccessful attempts to transfer the hypothetical virus, but I can't find the source at this writing.

[3] This is, of course, the idea of natural selection. When two species compete for the same habitat (in this case, within a single cell) one or the other will be eliminated. Both types would be expected to remain in the same cell only if they were mutually beneficial.

[4] Rhoades (1955) says that specific gene loci may induce plastid mutations, but once induced they are permanent. The iojap locus in corn provides an example. Regarding the iojap locus, one should remember that specific nuclear gene loci are known which increase the mutation rate at other nuclear loci.

[5]But see Gillham (1965). Gillham presented no evidence that these genes were located in the chloroplast. Assortment of the mutant traits suggested some 40 equivalent units were involved; perhaps suggesting that the genes night be located in mitochondria.

[6] This consideration necessarily excluded forms specialized for parasitism.

[7] With the possible exception of the coprozoic genus *Sappina* where part of the life cycle seems very reminiscent of the primitive sexual process described for the Bascidomyceete fungi (Darlington, 1958).

[8] This does not rule out the possibility that other quasi-sexual mechanisms may allow some genetic recombination to take place. There are several suggestions of possible mechanisms that may be discussed in a further paper.

[9] Fischer, (1965) discusses in detail the adaptive significances of this symbiotic arrangement. In his article he only saw this value in terns of a zoochlorellar or zooanthellar association, without considering what would happen to the endosymbiont

over the course of a proposed 2.7 billion years. In short, his ecological analysis was probably quite accurate, but its generality was missed.

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