some. The proband's abnormal chromosome 1 was therefore somewhat similar to the duplication chromosome shown in Fig. 2A.

The inverted insertion hypothesis is somewhat more parsimonious than the hypotheses advanced by Sparkes *et al.* While three breaks would be required to produce an inverted inversion as opposed to two for a paracentric inversion, two additional breaks would be required to produce the deleted chromosome from the chromosomes in an inversion loop (their figure 2, B and C). In addition, it is unlikely that the deleted chromosome was produced by the mechanism proposed by Sparkes *et al.* in their figure 2C. That figure shows two breaks occurring in the loop at the point where the "inner" chromosome folds back over itself. In the most probable three-dimensional pachytene configuration of an inversion loop (Fig. 2B), that point does not exist (*4*). It may be only an artifact produced when the loop is rendered in two dimensions.

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4. Of 15 surveyed genetics and cytogenetics texts, only Dobzhansky (*2*) shows inversion loops in three-dimensional perspective. J. Sybenga [*Meiotic Configurations* (Springer-Verlag, New York, 1975), p. 63] differentiates between diagrammatic representation and microscopic appearance of inversion loops.

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Protein and Nucleic Acid Sequence Data and Phylogeny

Having examined the data and methodology on which Schwartz and Dayhoff's (*1*) proposals on the phylogeny of pro- and eukaryotes are based, I find the credibility of their conclusions somewhat limited.

Schwartz and Dayhoff present three evolutionary trees based on, respectively, ferredoxins, 5*S* ribosomal RNA (rRNA), and c-type cytochromes sequences. They then combine data from the three individual trees into a composite tree.

The major objection to phylogenetic conclusions drawn from the ferredoxin and cytochrome trees is that they are based on a set of probably homologous but certainly not entirely orthologous proteins. Schwartz and Dayhoff recognize this for the *Chlorobium* ferredoxins and *Pseudomonas* cytochromes; but they do not allow the fact that while some microorganisms, such as *Chlorobium limicola*, possess two closely related ferredoxins, others possess very different ones; for example, both 8Fe-8S and 4Fe-4S occur in *Rhodospirillum*, and 8Fe-8S and 2Fe-2S occur in *Azotobacter* (*2*). Schwartz and Dayhoff's ferredoxin tree may thus represent a gene phylogeny without significance in the interrelationship of organisms.

Similarly, with the cytochrome tree one cannot draw conclusions from the finding that blue-green algal and chloroplast cytochrome c_6 stand on a different section of the tree from the mitochondrial cytochrome c and the cytochrome c_2 of purple nonsulfur bacteria.

The differences in properties and sequences between c_6 and c_2 cytochromes, and the finding that prokaryotes may possess several c-type cytochromes [such as the c_{551}, c_5 as partially illustrated in figure 5 of (*1*)] are first indications that c_6 cytochromes may not be orthologous with c_2. This possibility becomes a quasi certitude when one considers that c_6 is only one among three soluble c-type cytochromes of blue-green algae (*3*). Of those, the most likely candidate for orthology with c-c_2 is the not yet sequenced cytochrome c_{552}, which has a basic isoelectric point and an α band at a lower wavelength than c_{554} (c_6).

The importance of using only orthologous genes when one wants to infer evolutionary relationships between organisms has been emphasized (*4*). Who would consider seriously a phylogeny of vertebrates drawn from a comparison of myoglobin of some species and hemoglobin from others? The species for which myoglobin is used will cluster together far away from related species for which hemoglobin is selected (*5*). Similarly, given the doubts on the orthology of those cytochromes, a comparison based on the use of cytochrome c_6 to represent blue-green algae and chloroplasts, and cytochromes c_2 and c for purple nonsulfur bacteria and mitochondria, should not be used to demonstrate a separate symbiotic origin of eukaryotic organelles.

From their cytochrome tree Schwartz and Dayhoff concluded that since separate branches leading to the two blue-green algae intermix with eukaryotic algae, chloroplasts must have a polyphyletic origin. This appears to be another hasty conclusion.

The main problem here is the reliability of evolutionary reconstructions based on sequence data. The model in (*1*) is likely to give a false impression of the reliability of the matrix method used. It does indeed show that for distant sequences, the matrix method is more accurate than the ancestral sequence method, but how accurate it is when applied to real data cannot be inferred. There is still no absolute way to define the accuracy of such techniques and even if one succeeds in finding the most parsimonious tree for a set of sequences (*6*), this only represents a probabilistic estimate of evolutionary history for which the confidence limit is unknown.

In the approaches used until now, that confidence limit can only be estimated if, in a model of the type used to compare the relative accuracy of different techniques, the data are closely comparable to those under study (*7*). But the Dayhoff model does not fit many of the data treated, especially the c_6 cytochromes. The reason is that the model deals with two clear-cut pairs of sequences: a member of each pair has a distance, measured along the tree, of 3 L with any member of the other pair, whereas the distance between members of the same pair is only 2 L. In other words, the distance between the two nodes of the network is equal to the distances from the tips to the closest node. In such a situation, it is logical that the dendrogram derived from the comparison of extant sequences gives an incorrect representation (erroneous cladogram) of phylogenetic relationship only in extreme cases; that is, either when an exceptional number of convergent mutations occurred or, as illustrated by Schwartz and Dayhoff, when the distance between the sequences is such (high value of L) that the residues of phylogenetic significance are so few in number that they are overshadowed by random similarities.

With the c_6 cytochromes, however, the extant sequences are more or less equally distant one from another (they are mostly 42 to 56 percent similar), which probably implies short internodal distances in comparison to the distances from the tips to the closest node. This is exactly what appears in the Schwartz and Dayhoff tree where, with the exception of the *Porphyra-Alaria* pair, the distances from the tips to the closest node are about two to six times greater than the internodal distances.

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That, with such increased distances from the tips to the nodes relative to the internodal distance (increased arm length), the reliability of the phylogenetic conclusions will decrease is clearly expressed in figures 8 and 9 of Peacock and Boulter (*7*). Short internodal distances suggest that permutation of branches of the tree will imply small differences in overall length. The probability that, because of a few convergent mutations, the most parsimonious tree will not be the evolutionary correct one will thus be higher.

The only way to increase reliability in such a situation is to enlarge the sample so that random convergences between distant sequences are neutralized. Numerous pairings of closely related sequences assure then that the comparison with more distant sequences will occur at a level where convergences are weeded out.

We have no definite way a priori to tell how large the sample need be, but I and the authors who determined many of the c_6 sequences (*8*) maintain that the present set is insufficient. However, one advantage of this restricted set of data is that it can be easily examined in detail and many calculations performed on it even without the help of a computer. I counted the minimum number of mutations needed (229) to account for the cytochrome c_6 trees of Schwartz and Dayhoff and found that to invert the branch leading to *Euglena* and *Plectonema*, that is, to create homogeneous prokaryotic and eukaryotic clusters, one would require only one more mutation (0.4 percent). Further, if, on this same tree with a homogeneous eukaryotic cluster, the *Euglena* branch is joined to the *Monochrysis* branch, the tree is mutationally equivalent to the Dayhoff tree. In such circumstances, even though my calculations are based on a different alignment (*9*) and refer to mutation distances given by the genetic code (whereas Schwartz and Dayhoff use their "accepted point mutation per 100 residue" system), I doubt that anybody would accept any far-reaching phylogenetic conclusions drawn from this tree.

I doubt any biologist, even one unaware of the limitations inherent to the data used, would give much credit to a scheme that postulates not only a polyphyletic origin of chloroplasts but also of the chloroplasts typical of the chromophytes (brown, yellow, and related algae) present in *Alaria* and *Monochrysis*. Schwartz and Dayhoff's figure 4 does not leave any alternative other than to consider that either a photosynthetic apparatus with phycobilins and isolated thylakoids, as present in *Plectonema* and *Porphyra*, is convergently derived from one with chlorophyll c and thylakoids grouped by three as in *Alaria* and *Monochrysis*, or the reverse.

The 5*S* rRNA tree also requires comment. Schwartz and Dayhoff in 1976 (*10*) were aware of the limits of the interpretation of the 5*S* rRNA data and did not commit themselves on the order of divergence of major groups. I am not convinced that the addition of four sequences in 1978 makes the sample large enough to allow a definitive answer. Further, Hori (*11*) has proposed a different tree based on those same RNA's, which shows how equivocal the interpretation of those data can be.

The composite evolutionary tree [figure 5 in (*1*)] encompasses all the weaknesses of the individual trees. If those had dealt with the same organisms one could have hoped to find in the composite tree mutually supportive data. Unfortunately, not only is there not a single organism present on all the individual trees, but there are not even six species appearing on at least two trees as claimed (*1*, p. 400). Only *Spirulina maxima*, *Chlorobium limicola*, and *Clostridium pasteurianum* fulfill that requirement; the other groups are genera represented by different species on different trees (*Pseudomonas* and *Bacillus*) or even the group "plants." This makes it difficult to decide if individual trees are compatible with one another. The composite tree is thus essentially an addition of information drawn from the individual trees and does not provide corroborative evidence. For example, the pattern interrelating blue-green algae and aerobic and anaerobic bacteria, which is used to suggest that aerobic bacteria are older than blue-green algae, is essentially derived from the 5*S* rRNA tree only, the reliability of which is suspect.

In any event, the conclusion that aerobic life preceded oxygen release by blue-green algae is not only based on weak data but on a phylogenetically incorrect assumption concerning the nodal organisms. As Cronquist (*12*) says: "The nature and proper taxonomic position of the hypothetical past organisms that represent the branch points in the scheme cannot be determined solely from the phylogenetic relationships of modern species as deciphered from the amino acid sequences" (or nucleotide sequences in the case of figure 3). For example, in Schwartz and Dayhoff's figure 3, one cannot tell if the common ancestor of *Anacystis* and *Pseudomonas* was more *Anacystis*-like or *Pseudomonas*-like or whatever else, and again for the node that joins this ancestor to the one of *Photobacterium* and *Escherichia*, and so on. Neither the composite nor any of the individual trees is incompatible with the idea that nodal organisms posterior to the clostridia and some photosynthetic bacteria were blue-green algae. From those, aerobic bacteria and eukaryotes could have been derived—eventually polyphyletically if one follows figure 3. While Schwartz and Dayhoff are in favor of the idea that "the main trunk of this tree represents a continuum of photosynthetic forms" (*1*, p. 401), apparently they consider that those forms performed bacterial rather than blue-green algal photosynthesis. Nothing in the sequence data justifies this assumption, which must be derived from undiscussed outside evidence. I think that the most compelling outside evidence that might be introduced here is the commonsense idea that a significant amount of oxygen produced by blue-green algae is a prerequisite for the evolution of aerobic bacteria (*13*). From this I propose that (i) the pattern of figure 3 based on very short internodal distances is erroneous; or (ii) node organisms anterior to the appearance of aerobic bacteria were oxygen-releasing; or (iii) respiration has been developed polyphyletically from a stock ancestral to blue-green algae but later in time than their appearance [the unequal rates of molecular evolution admitted by Schwartz and Dayhoff make this possible, and both Margulis and Broda (*13*) are favorable to a polyphyletism of aerobes]. The fourth possibility, chosen by Schwartz and Dayhoff, is that some nodal organisms were aerobic and anterior to, but distinct from the blue-green algae. This possibility, which leads to the rejection of the well-founded arguments of Margulis *et al.* (*14*) for the biological origin of atmospheric oxygen, seems the less reasonable.

With regard to the origin of eukaryotic cells, the claim that the data support a symbiotic origin is also unfounded. Only the cytochrome tree can be interpreted as showing a distinct origin for chloroplasts and mitochondria. We have seen how this is based on the very doubtful postulate that c_6 cytochrome is orthologous to c_2. The significance of the 5*S* rRNA tree for the origin of eukaryotes is as doubtful as for the origin of aerobes, and ferredoxins and plastocyanin trees just show that chloroplasts are certainly related to blue-green algae, but are perfectly coherent either with a symbiotic origin or with an origin of the whole eu-

karyotic cell from the blue-green algae. To see this problem resolved through sequence analysis one will have to wait for perfectly comparable data coming from the three eukaryotic cell compartments (for example, partial sequences of the large rRNA's). If they show clearly incompatible cladistic patterns, the symbiotic theory will then really be favored; if not, the autogeneous theory.

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Demoulin raises a number of interesting questions that we address in order below. The major conclusions we drew from the sequence data (*1*), that the eukaryote mitochondria and chloroplasts were acquired by protoeukaryote host organisms through symbioses and that oxygen-releasing photosynthesis evolved in one line late in the proliferation of the major bacterial groups, remain very probably correct and are bolstered by new sequence data.

Demoulin's major objection to the conclusions we draw from the ferredoxin and c-type cytochrome trees is that each is based on a set of proteins "that are not entirely orthologous." The only published sequence data we could find for Demoulin's examples are the 45 amino-terminal residues of the 8Fe-8S ferredoxin from *Azotobacter vinelandii* (*2*). This sequence is consistent with our tree. Some general comments about gene duplications need to be made. In deducing the position of each branch on a tree, it is sufficient that the sequence used be directly descended from the ancestral gene in the earliest ancestor on the branch. If there have been gene duplications in the descendants on the branch, any one of them will be useful, regardless of their multiplicity. Thus, if we were reconstructing a tree using bacterial globins (if such existed) and wanted to place the eukaryote line, a sequence of either myoglobin or hemoglobin would be useful, because the myoglobin-hemoglobin divergence occurred on the animal branch, much later than the first eukaryote. Recent sequence studies have revealed several duplications in the plant-type ferredoxins (*3*). Most of them are quite local; none affects the configuration of the main branches of our tree. In order for us to have been misled in placing the branch to the oxygen-releasing photosynthetic forms on the ferredoxin tree, a duplication would have to have occurred prior to their divergence from the *Bacillus-Desulfovibrio* branch, and we would have to have selected the wrong plant and blue-green algal ferredoxins. If so, the real divergence would have been more recent than that shown, not earlier. The blue-green algae would still share considerable evolution with the bacteria. A newly determined sequence, the 8Fe-8S ferredoxin from *Pseudomonas putida*, and experimental corrections to the sequence from *Desulfovibrio gigas* are now available (*4*). New calculations place the divergence of the *Pseudomonas* branch very close to the divergence of *Desulfovibrio* and *Bacillus*. The branch leading to the blue-green algae and eukaryote chloroplasts still cannot be accurately placed on this tree due to the large amount of difference between the sequences of these 2Fe-2S ferredoxins and the others with 4Fe-4S clusters.

None of the available sequence data contradict our supposition that the cytochrome c_2 and c_6 genes are descended from the same gene present at the time of the divergence of the chloroplast-blue-green algal line from the pseudomonad-Rhodospirillaceae-mitochondrial line.

Demoulin is correct in pointing out that the reliability with which the configuration of an evolutionary tree is inferred decreases sharply if branch lengths are very long compared with internodal distances. There is not sufficient information to be certain of the exact topology of the cytochrome c_6 subtree. However, if there was only one symbiosis to form a single ancestor from which all photosynthetic eukaryotes were descended, then *Plectonema* and *Spirulina* must be adjacent on one branch of the cytochrome c_6 subtree. In our calculations, the first configuration in which this occurs is tenth (of 105 possible) in order of tree size and includes a branch −4 PAM's (accepted point mutations per 100 residues) long. We therefore suggested that there may have been more than one symbiotic event. We certainly agree with Demoulin that many additional carefully chosen sequences are needed to sort out the early divergences of the blue-green algae and the eukaryote chloroplasts. In order to expeditiously investigate crucial sequences it is helpful to have a hypothesis regarding the evolutionary tree. This was one of our goals in writing the article.

A much more precise picture of chloroplast-blue-green algal evolution is emerging from the ferredoxin sequences than is possible from the cytochromes because there appears to have been a duplication early in the proliferation of blue-green algal types (*5*). This also allows us to place the earliest divergence in this subtree. The location of a *Porphyra umbilicalis* branch, although not compelling, is consistent with the symbiosis leading to the chloroplasts of the red algae being separate from that of the green algae and higher plants (*6*).

In constructing a tree based on *5S* ribosomal RNA (rRNA), Hori and Osawa (*7*) use a sequential methodology, adding a branch at a time; that is entirely different from the matrix method we used. Nevertheless, the only major difference in the configurations of the two trees is that Hori and Osawa place the *Escherichia-Photobacterium* branch on the *Pseudomonas* branch. In addition, we place the point of earliest time on the

branch to *Clostridium* in conformance with our ferredoxin tree.

With respect to Demoulin's criticism of our composite tree [figure 5 in (*1*)], it is clear in our references that we had available to us sequences of 5*S* rRNA from *Bacillus stearothermophilus* and cytochrome c_{551} from *Pseudomonas fluorescens*. This brings the number of identical species appearing on two trees to five. We regret any confusion caused by our picturing only representative sequences on those two trees. For the "plant chloroplast" branch, sequences were not available from the same species. However, comparable data in the context of major phylogenetic branches mean that the sequences and the species in which they are found are sure to be directly descended from the same gene in the first ancestor on the branch. We assumed that the green algae and vascular plants share a common chloroplast ancestor and therefore used the c_6 sequence from *Euglena* and the ferredoxin from *Scenedesmus* to locate this branch in the composite tree. In combining the ferredoxin and 5*S* rRNA trees, we assumed that the two coccoid blue-green algae, *Aphanothece* and *Anacystis*, shared an ancestor more recently than the time of divergence of both from *Pseudomonas*.

Our suggestion that the development of aerobic respiration preceded that of oxygen-releasing photosynthesis is not proved by sequence data. However, our reasoning does not involve arguments about whether the ancestor of *Pseudomonas* and *Anacystis* is more like one or the other organism. Most of the Rhodospirillaceae, the pseudomonads, *Escherichia*, and some species of blue-green algae such as *Nostoc* sp. strain MAC can live heterotrophically in aerobic conditions. It therefore seems reasonable to suggest that their most recent common ancestor possessed a rudimentary form of aerobic respiration. On our composite tree, we place the development of some important components of a respiratory chain slightly earlier, near the divergence of the *Bacillus* and *Desulfovibrio* branch from the trunk of the tree.

Both of these organisms possess respiratory metabolisms. However, *Desulfovibrio* respires anaerobically using sulfate as the terminal electron acceptor, whereas *Bacillus* respires aerobically. It is certainly possible that aerobic respiration evolved separately in all of these lines. However, that is not the simplest explanation of our composite tree. Contrary to Demoulin's commonsense argument, Schopf (*8*) has pointed out that it is difficult to imagine the development of oxygen-releasing photosynthesis prior to the development in that line of a rudimentary mechanism for coping with oxygen, as oxygen is produced intracellularly in photosynthesis. This is not to say that aerobic respiration developed in anything like the present atmosphere. The high level of free oxygen in our present atmosphere is almost certainly due to oxygen-releasing photosynthesis.

Our composite tree clearly supports a symbiotic origin for the eukaryotes. It pictures the branches that contribute to the eukaryote host and organelles as distinctly separate, with each being closely related to contemporary free-living prokaryotes. Demoulin states that a resolution of the question of how eukaryotes originated "will have to wait for perfectly comparable data coming from the three eukaryotic cell compartments (for example, partial sequences of the large rRNA's)." We would welcome the elucidation of additional sequence data for its value in reconstructing a highly probable evolutionary schema. However, no information is perfect. A tree based on partial rRNA sequences might well suffer all the criticisms Demoulin has made here. If there is any suggestion of gene doubling in the sequences, the possibility that nonorthologous segments are being compared could be raised. One could raise questions about the accuracy of the tree in a small region and suggest that the accuracy of the overall tree is suspect. Alternative methods, whether or not sound, could be used to demonstrate the unreliability of any tree based on the segments.

In the nearly 3 years since we first presented our composite tree (*9*), we have found it an excellent working hypothesis with which to organize new sequence data and our ideas. We hope that it is as useful to others in the many disciplines for which it has implications.

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25 June 1979

Long-Term Choline Treatment of Memory-Impaired Elderly Patients

Davis *et al.* (*1*) and Sitaram *et al.* (*2*) have reported an improvement in human memory following the administration of single doses of certain cholinomimetic agents (1 mg of physostigmine, 4 mg of arecholine, and 10 g of choline chloride). In both studies, the enhancement of memory was demonstrated in normal volunteers by means of a verbal serial learning task. These results, coupled with evidence that reduced cholinergic function may be related to the memory decline of the elderly and senile, led the authors of both reports to suggest that treatment with cholinergic agents might benefit elderly patients with memory impairment.

Since there are no clearly efficacious treatments currently available for age-related memory impairment (*3*), we find the data in (*1*) and (*2*) to be encouraging and agree that the potential use of cholinergic agents in senility should be investigated. However, the likelihood of suc-

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