ADDENDUM

A sentence was omitted from the manuscript entitled "Identification of the Immune System Responsible for the Specificity of Actively Acquired Tolerance in Mice," by G. Doria, which was published in the March issue of these PROCEEDINGS (volume 49, pp. 281–286, 1963). The sentence "Immediately after the intravenous injection, all groups of recipients were given intraperitoneally 1 ml of 1 per cent rat RBC." should be inserted between lines 23 and 24 of page 282.

ERRATUM

In the article entitled "Cohesion of DNA Molecules Isolated from Phage Lambda," by A. D. Hershey, Elizabeth Burgi, and Laura Ingraham, which was published in the May issue of these PROCEEDINGS (volume 49, pp. 748–755, 1963), the exponent in equation (1) should be 0.35. This equation appears on page 752.

COHESION OF DNA MOLECULES ISOLATED FROM PHAGE LAMBDA

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Aggregation of DNA is often suspected but seldom studied. In phage lambda we found a DNA that can form characteristic and stable complexes. A first account of them is given here.

Materials and Methods.—DNA was extracted from a clear-plaque mutant (genotype cb^+) of phage lambda[1] by rotation[2] or shaking[3] with phenol. Sodium dodecylsulfate, ethylenediaminetetraacetate, citrate, or trichloroacetate was sometimes included in the extraction mixture without effect on the properties of the DNA. Phenol was removed by dialysis, with or without preliminary extraction with ether, against 0.1 or 0.6 M NaCl.

Sedimentation coefficients were measured[4] at 10 μg DNA/ml in 0.1 and 0.6 M NaCl in aluminum cells at 35,600 rpm with consistent results, and are reported as $S_{20,w}$.

Zone sedimentation[5] of labeled DNA's[6] was observed in 0.1 M NaCl immobilized by a density gradient of sucrose. A sample, usually containing less than 0.5 μg of DNA in 0.15 ml of 0.1 M NaCl, was placed on 4.8 ml of sucrose solution, and the tube was spun for 5 or 6 hr at 28,000 rpm in an SW39L rotor of a Spinco Model L centrifuge at 10°C.

Solutions containing 5–40 μg DNA/ml in 0.1 or 0.6 M NaCl were stirred on occasion for 30 min at 5°C with a thin steel blade turning in a horizontal plane.[7] Since we used two stirrers of different capacities, stirring speeds given in this paper are comparable only within a context.

Salt solutions were buffered at pH 6.7 with 0.05 M phosphate.

Results.—Disaggregation and breakage: Solutions containing 0.5 mg/ml of lambda DNA in 0.1 M NaCl acquire an almost gel-like character on standing for some hours in a refrigerator. Diluted to 10 μg/ml, the DNA exhibits in the optical centrifuge an exceedingly diffuse boundary sedimenting at 40–60 s (Fig. 1A). If the diluted solution is aged for several days, the sedimentation rate may fall somewhat (not below 40 s), but the boundary remains diffuse and often appears double.

Stirring the diluted solution at 1,300 to 1,700 rpm yields a single component sedimenting at 32 s (Fig. 1B). The product so obtained is stable for a week or more in the cold in 0.1 M NaCl. We call this process disaggregation by stirring.

If samples of the diluted solution are stirred at increasing speeds between 1,800 rpm and 2,100 rpm, one sees a stepwise transition from 32 s to 25.2 s components, each by itself exhibiting a sharply sedimenting boundary (Figs. 1C and 1D). We call this phenomenon breakage. Broken DNA can form aggregates, but the characteristic 32 s species cannot be regained.

Aggregation: Disaggregation, in contrast to breakage, is reversible, as shown by the following experiment. Lambda DNA at 40 μg/ml in 0.6 M NaCl was
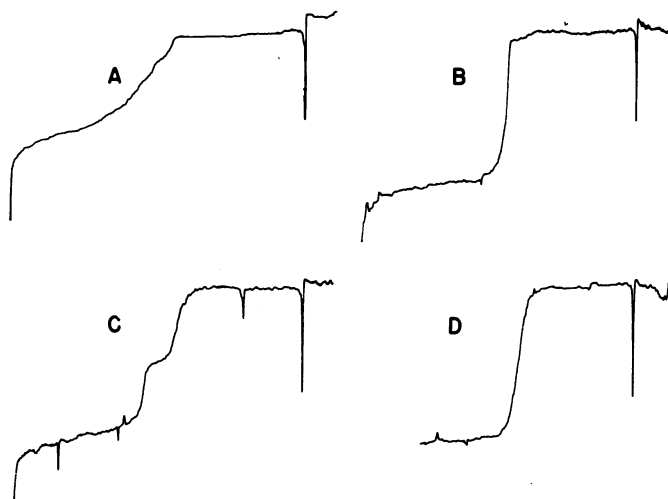
FIG. 1.—Sedimentation pattern of initially aggregated DNA after
stirring at several speeds. *A*, unstirred; *B*, 1,600 rpm; *C*, 1,900 rpm;
D, 2,000 rpm. The meniscus shows at the right.

disaggregated by stirring at 1,700 rpm, and samples at either 40 μg/ml or 10 μg/
ml in the same solvent were warmed to 45°C. After measured time intervals, the
tubes were chilled and their contents diluted to 10 μg/ml, if necessary, with cold
0.6 M NaCl. Sedimentation coefficients were measured over the course of some
hours. Unheated samples showed the same sedimentation rate at the beginning
and end of the series of measurements. The heated samples were analyzed in
random order, so that the results reflect mainly the duration of heating, not the
duration of subsequent storage.

The results, presented in Figure 2, show that the sedimentation rate of the
DNA increases rapidly on heating at 40 μg/ml, and less rapidly on heating at 10
μg/ml. The reversibility of disaggregation, and the dependence of rate of aggrega-
tion on concentration of DNA, justify our choice of language.

Similar experiments showed that heating in 0.1 M NaCl under the same condi-
tions does not cause appreciable aggrega-
tion. Aggregation occurs in that solvent
at higher DNA concentrations, however.
Thus, aggregation is accelerated by high
DNA concentrations, high temperatures,
and high salt concentrations.

Linear molecules: According to the
description given above, aggregated
lambda DNA can be reduced under shear
to a uniform 32 s product, which is evi-
dently the structure subject to breakage
at higher rates of shear. The maximum
stirring speed withstood by 32 s lambda
DNA is 1,800 rpm at 10 μg/ml. When

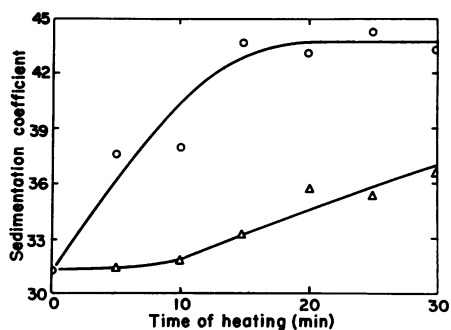FIG. 2.—Aggregation at 45°C. Circles, 40
μg DNA/ml in 0.6 M NaCl; triangles, 10
μg/ml. The scale on the ordinate refers to
observed sedimentation coefficients.

T2 DNA is stirred under the same conditions, it is reduced to fragments sediment-
ing at 31 s.   Thus, lambda DNA exhibits a fragility under shear that is appropriate
to linear molecules[7] sedimenting at about 32 s.   We therefore conclude that 32 s
lambda DNA consists of linear molecules. · These and other DNA structures are best
identified by zone centrifugation, as illustrated below.

   Linear molecules can also be prepared (irrespective of the initial state of aggrega-
tion of the DNA) by heating a solution in 0.1 or 0.6 M NaCl to 75°C for 10 min
and cooling the tube in ice water (Fig. 3A, solid line).   This procedure is effective
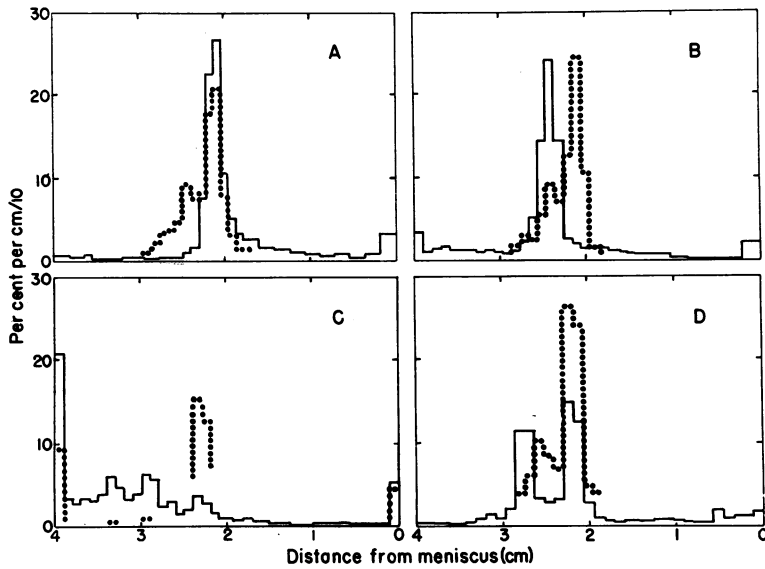at concentrations up to 10 μg/ml at least.

FIG. 3.—Zone sedimentation of several molecular forms.   Solid lines: P[32]-
labeled linear molecules (A), folded molecules (B), aggregates (C), dimers and
linear monomers (D).   Broken lines, a preparation of H[3]-labeled marker DNA
containing linear and some folded molecules.

   Linear molecules are obtained directly by extracting the DNA (by rotating, not
shaking, the tubes)[6] at 2 μg/ml into 0.1 M NaCl (Fig. 3A, dotted line).   Control
experiments showed that the mechanical operations involved in the extraction do
not destroy previously formed complexes in solutions diluted to 2 μg/ml.

   We conclude that the 32 s form of lambda DNA is analogous to more conven-
tional phage DNA's and is a typical double-helical molecule.

   Folded molecules: Another form of lambda DNA we usually prepare by heating
a dilute solution (5 μg/ml or less) in 0.6 M NaCl to 75°C for 1 min, and allowing
the container to cool slowly (0.4° per min at 65°) in the heating bath with the
heater disconnected.   The resulting product sediments as a narrow band moving
1.13 times faster than linear molecules in zone centrifugation (Fig. 3B).   The
expected sedimentation coefficient is 32 × 1.13 = 36.2 s.   Material prepared as
described and then concentrated by dialysis against dry sucrose followed by 0.6
M NaCl shows in the optical centrifuge a sharp boundary at 37 s.

The formation of 37 s material is equally efficient at several DNA concentrations between 5 μg/ml and 0.1 μg/ml (at higher concentrations it is obscured by simultaneous aggregation). The 37 s product, therefore, is composed of monomers that we shall call folded molecules.

When a dilute solution containing either linear or folded molecules in 0.6 M NaCl is heated to 75°C, one gets only linear molecules by rapid cooling and only folded molecules by slow cooling. Partial conversion of linear to folded molecules occurs on heating to 45°C for 30 min followed by rapid cooling, and nearly complete conversion at 60°C. Thus, at 75°C linear molecules are the stable form of lambda DNA. At low temperatures, folded molecules are more stable but the conversion is slow. The slow cooling from 75°C serves to find a temperature near 60°C at which the conversion to folded molecules is rapid and the product is stable.

Folded molecules are formed on heating and slow cooling in 0.1 M NaCl as well as in 0.6 M NaCl, but the conversion is not complete at the lower salt concentration. Some molecular folding also occurs when linear molecules are stored at low concentration and low temperature for a few weeks in 0.1 M NaCl or a few days in 0.6 M NaCl. This is the origin of the faster-sedimenting component of the tritium-labeled marker DNA whose sedimentation pattern appears in Figure 3.

Folded molecules can be converted back into linear molecules by stirring as well as by heating, though the margin between the stirring speed required to accomplish this and the speed sufficient to break linear molecules is rather narrow.

It should be added that heating DNA at 10 μg/ml and 45°C in 0.6 M NaCl produces many folded molecules whose formation competes with the simultaneous aggregation. For this reason the dependence of rate of aggregation on DNA concentration is not truly represented in Figure 2.

Folded molecules themselves do not aggregate. Solutions concentrated for analytical centrifugation continue to yield sharp boundaries after aging in 0.6 M NaCl. Neither do folded molecules form complexes with linear molecules. This was shown by mixing P^{32}-labeled folded molecules with unlabeled linear molecules (20 μg/ml) and aging the mixture in 0.6 M NaCl for 4 days at 5°C. A similar mixture containing labeled linear molecules served as control. Zone centrifugation of each mixture with added H^3-labeled marker DNA showed that the labeled linear molecules but not the folded molecules had formed complexes with the unlabeled DNA.

The similarity between the conditions, other than DNA concentration, controlling formation and destruction of folded molecules, and formation and destruction of aggregates, suggests that similar cohesive forces are involved in both phenomena. The folding implies that each molecule carries at least two mutually interacting cohesive sites, which join to form a closed structure. The uniformity of structure of folded molecules, indicated by the narrow zone in which they sediment, suggests that there are not more than two cohesive sites, and that these are identically situated on each molecule.

Dimers and trimers: Aggregated DNA often shows multiple boundaries in the optical centrifuge and always shows multiple components in zone centrifugation. An example, prepared by heating linear molecules for 30 min at 45°C and 40 μg/ml in 0.6 M NaCl, is shown in Figure 3C. Since the characteristic folding seen in

monomers is incompatible with aggregation, as already described, it is likely that some of the differently sedimenting products of aggregation are polymers differing in mass rather than configuration.

One form of aggregate can be obtained in moderately pure state by allowing aggregation to occur during a day or so in the cold at 100 μg/ml in 0.1 M NaCl (Fig. 3D). Such material contains a fraction of the molecules in linear form, and presumably contains in addition mainly the smaller and more stable aggregates. One of these, as shown in the figure, always predominates, and we assume that it is a dimer. It sediments 1.25 times faster than linear molecules.

In a study of zone centrifugation to be reported separately, we found a relation

$$\frac{D_2}{D_1} = \left(\frac{L_2}{L_1}\right)^{0.25} \tag{1}$$

between molecular lengths (L) and distances sedimented (D) of two DNA's, which is valid for linear molecules. According to this relation, dimers are about twice as long as linear molecules of lambda DNA. The only alternative compatible with the sedimentation rate is a second form of folded monomer, which is ruled out by the requirement for high DNA concentrations during formation. Therefore, dimers are tandem or otherwise open structures. (For definitions of "open" and "closed," see hereafter.)

In more completely aggregated material (Fig. 3C) one sees few or no linear molecules, a very few folded molecules (fewer the more concentrated the solution in which aggregation occurred), a considerable fraction of dimers sedimenting 1.25 times faster than linear molecules, and another characteristic component sediment-ing 1.43 times faster than linear molecules. According to its sedimentation rate, the last component could be a tandem trimer or a folded or side-by-side dimer. We believe that it is an open trimer for the following reasons.

A folded dimeric structure is ruled out because material sedimenting at rate 1.43 does not form when a dilute solution containing dimers (similar to that shown in Fig. 3D) is aged for two weeks in the cold in 0.1 or 0.6 M NaCl, or is heated in 0.6 or 1.0 M NaCl at 45°C. At high DNA concentrations, trimers do form under these conditions. At low DNA concentrations, dimers and trimers are stable and one sees only the conversion of linear to folded monomers.

A side-by-side dimeric structure can be ruled out on the basis of susceptibility to hydrodynamic shear. Figure 4 shows the result when samples of a mixture of trimers, dimers, and folded and linear monomers are stirred at increasing speeds. Trimers disappear first, being converted to dimers or linear molecules or both. Next to go are dimers. Folded monomers are much more resistant, but can be reduced to linear monomers at stirring speeds just insufficient to break the mole-cules. Thus, trimers, as expected if they are open structures, are more fragile than open dimers, whereas closed dimers should be more stable. We note, how-ever, that a small amount of the material sedimenting at the rate of trimers is relatively resistant to stirring and could signify a minority of closed dimers.

We note also that destruction of dimers and trimers does not liberate any folded monomers, a result consistent with the evidence from sedimentation rates for an open polymeric structure, and with our finding that folded monomers do not form

complexes. The fact that aggregation and folding are mutually exclusive processes implies that both utilize the same limited number of cohesive sites, which must be small in size to account for the open polymeric structure. As already suggested by the unique configuration of folded monomers, there may be only two sites per molecule.

Specificity of aggregation: If tracer amounts of P^{32}-labeled lambda DNA are mixed with unlabeled lambda DNA at 25 $\mu g/ml$ in 0.6 M NaCl, and the mixture is brought to 75°C for 1 min and allowed to cool slowly, subsequent zone sedimentation with added H^3-labeled marker shows that most of the P^{32}-labeled DNA has been converted to aggregates and a small remainder to folded molecules. When the same procedure is followed with H^3-labeled or unlabeled T5 DNA substituted for the unlabeled lambda DNA, the T5 DNA sediments (at its normal rate) 1.20 times faster than the P^{32}-labeled lambda DNA, which now consists entirely of folded molecules. Thus, lambda DNA shows no tendency to form complexes with T5 DNA, T5 DNA itself does not form stable aggregates, and T5 DNA does not inhibit molecular folding in lambda DNA. The cohesive sites in lambda DNA are therefore mutually specific, as our model requires.

Role of divalent cations: Divalent cations probably do not play any specific role in the phenomena described in this paper. In NaCl solutions, molecular folding and aggregation are not inhibited by added citrate or ethylenediaminetetraacetate. Neither are these processes appreciably accelerated, in the presence of NaCl, by added calcium or magnesium ions. In a solution of 0.01 M $MgCl_2$, 0.01 M $CaCl_2$, and 0.01 M tris (hydroxymethyl) aminomethane, pH 7.2,[8] linear monomers at 10 $\mu g/ml$ are about as stable as they are in NaCl solutions.

Interpretation of sedimentation rates: Equation (1) shows that if two identical DNA molecules were joined end to end their sedimentation rate would increase by the factor 1.27, evidently owing to the loss of independent mobility. Perhaps the result would be about the same whether they were joined end to end or to form a V, a T, or an X. Thus, we are led to the definition of an *open dimeric structure* as one formed by the joining of two linear molecules at a single point, recognizable by a 1.27-fold increase in sedimentation rate. The principle of independent mobility of parts suggests that, as the structure departed from the tandem arrangement, its sedimentation rate
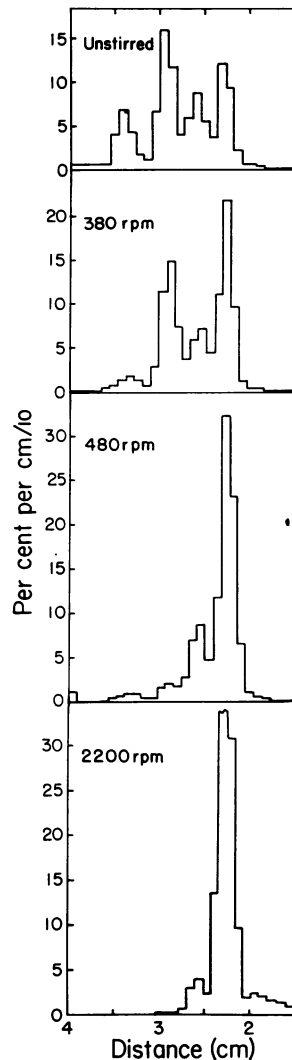

FIG. 4.—Successive destruction by stirring of trimers, dimers, and folded molecules, as seen by zone centrifugation. Linear molecules began to break at 2,400 rpm in this series. The starting material was prepared by heating a sample of DNA, already containing some spontaneously formed folded molecules and dimers, for 30 min at 45°C and 40 $\mu g/ml$ in 1.0 M NaCl, and diluting to 5 $\mu g/ml$ in buffered water.

could only increase, not decrease, and in the order V, T, X.

Our results also show that molecules of lambda DNA undergo some sort of folding, apparently as the result of bonding between two cohesive sites lying at some distance from each other on each molecule. If that interpretation is correct, it would appear that when the molecule (regarded as two halves joined end to end) forms an additional point of attachment between its parts, the sedimentation rate increases by an additional factor of 1.13, evidently owing to a further loss of independent mobility of parts. Thus, we are led to the definition of a *closed dimeric structure* as one formed by joining two linear molecules at two points. Such a structure ought to sediment 1.27×1.13 or 1.43 times faster than the linear monomer. We have not found closed dimers, but the question remains how the factor 1.13 would depend on the point of closure of a threadlike molecule. The principle of independent mobility of parts suggests that the sedimentation rate would approach or pass through a maximum as the fraction of the molecular length contained in the loop increased. In some measure it may be possible to answer such questions empirically by determining the locations of cohesive sites on the molecules.

Discussion.—Lambda DNA can exist in at least four characteristic forms that we call linear monomers, folded monomers, open dimers, and open trimers, which sediment respectively at the rates 1.0, 1.13, 1.25, and 1.43, expressed in arbitrary units. These structures are interconvertible with certain restrictions according to the scheme

<p style="text-align:center">open polymers ⇌ linear monomers ⇌ folded monomers</p>
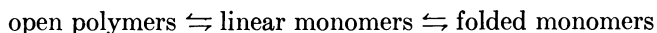
As the scheme indicates, linear monomers are subject to two distinct processes: aggregation, seen at DNA concentrations exceeding 10 μg/ml, and folding, seen at any concentration but forced to compete with aggregation at high DNA concentrations. Both processes are accelerated as the temperature is raised to about 60°C, beyond which only linear monomers are stable, and as the salt concentration is raised from 0.1 to 1.0 M. Both processes are rapidly reversed at 75°C or by hydrodynamic shear. All four structures are stable at low temperatures, low DNA concentrations, and low salt concentrations, except for a slow conversion of linear to folded molecules.

Since folded molecules exist in only one stable configuration, and since molecular folding and aggregation are mutually exclusive processes, we postulate that each molecule carries two cohesive sites in prescribed locations, and that these are responsible for both processes. To account for the considerable effect of molecular folding on sedimentation rate, the sites must lie rather far apart along the molecular length. To account for the moderate effect of dimerization on sedimentation rate, the cohesive sites must be small compared to the total molecular length.

According to the proposed model, one might anticipate two dimeric forms, open (that is, joined by one pair of cohesive sites) and closed (joined by two). We find only open polymers, though a minority with closed structures is not excluded. Failure to detect closed polymers may be explained, at least in part, by the fact that the rate of folding must decrease as the length of the linear structure increases.[9]

Whether all details of our model are correct or not, it is clear that lambda DNA forms a limited number of characteristic complexes, not the continuously variable series that might be expected if the molecules could cohere at random. The

limited number of mutually specific cohesive sites implied thereby suggests a specialized biological function, one that remains to be identified.

Summary.—The DNA of phage lambda undergoes reversible transitions from linear to characteristically folded molecules, and from linear monomers to open polymers. Some conditions favoring one state or another have been defined. It may be surmised that each molecule carries two specifically interacting cohesive sites.

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[3] Mandell, J. D., and A. D. Hershey, *Anal. Biochem.*, **1**, 66 (1960).

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[5] Hershey, A. D., E. Goldberg, E. Burgi, and L. Ingraham, *J. Mol. Biol.*, **6**, 230 (1963).

[6] Burgi, E., these PROCEEDINGS, **49**, 151 (1963).

[7] Hershey, A. D., E. Burgi, and L. Ingraham, *Biophys. J.*, **2**, 423 (1962).

[8] Kaiser, A. D., *J. Mol. Biol.*, **4**, 275 (1962).

[9] Jacobson, H., and Stockmayer, W. H., *J. Chem. Phys.*, **18**, 1600 (1950).

ERRATA: ROLE OF CHLOROPLAST FERREDOXIN IN THE ENERGY CONVERSION PROCESS OF PHOTOSYNTHESIS

BY K. TAGAWA, H. Y. TSUJIMOTO, AND DANIEL I. ARNON

Page 569: In the final printing, the graphs for Figures 1 and 2 were inadvertently transposed. The graph labeled "Fd-catalyzed photophosphorylations with and without TPN," is Figure 1, but was erroneously shown as Figure 2. The graph labeled "Fd-catalyzed cyclic photophosphorylation" is Figure 2, but was erroneously shown as Figure 1. The text of the legends for Figures 1 and 2 was correctly printed.

Page 571: The first sentence of the last paragraph should read, "It is interesting to note that, although reduced ferredoxin is nonenzymatically oxidized by oxygen,[5] an appreciable leakage of electrons to O_2 (O_2 is always present around the chloroplasts *in vivo*) is prevented by the strong affinity of reduced ferredoxin, (*a*) for the TPN-reducing system, and (*b*) for the grana-bound electron carriers of cyclic photophosphorylation."