ON THE MECHANISM OF CHLOROPHYLL-CYTOCHROME INTERACTION: THE TEMPERATURE INSENSITIVITY OF LIGHT-INDUCED CYTOCHROME OXIDATION IN CHROMATIUM*

BY BRITTON CHANCE AND MITSUO NISHIMURA

JOHNSON RESEARCH FOUNDATION, UNIVERSITY OF PENNSYLVANIA

Communicated November 24, 1959

Most of the reactions in biological systems that have been studied in detail are thermal reactions in which collisions of molecules are necessary for activation. This is particularly true in enzyme-substrate interactions. However, the increasing evidence for an ordered structure of biological systems, particularly in chloroplasts and mitochondria, has led to the consideration of alternative mechanisms, particularly those which would allow energy transfer between stationary protein molecules.

In the cytochrome chain, electron transfer can so far be explained by a restricted collision hypothesis. On the other hand, the intermolecular energy transfer from carotenoid to chlorophyll appears to involve inductive resonance processes. An intramolecular energy transfer occurs in aromatic amino acid-heme systems where light absorbed by the aromatic amino acids leads to a splitting of CO from heme. In this paper, we describe a light-induced intermolecular oxidation-reduction reaction between bacterial chlorophyll and a cytochrome of type c that is temperature independent (to 80°K) and that appears to require serious consideration of mechanisms for non-thermal oxidation-reduction reactions in living cells of this type.

Light-induced cytochrome oxidation in Chromatium has been shown to involve a number of cytochromes, as is true of R. rubrum. A quantum requirement of 2 per electron is found for the oxidation of a cytochrome of type c (cytochrome 423.5) by low intensity light absorbed by bacteriochlorophyll.

\[ \text{hv} \rightarrow \text{chl} \rightarrow \text{cyto} \quad (1) \]

Methods and Materials.—Kinetic measurements were made in the double-beam spectrophotometer which has been adapted for recording absorbancy changes as...
a function of time at liquid nitrogen temperatures. Identification of the component oxidized at low temperatures was made by the split-beam spectrophotometer which records absorbancy changes as a function of wavelength. Both instruments provide infrared illumination of the suspension from a tungsten source filtered through a Wratten 88A filter. The tungsten source is used at about $1/3$ rated voltage and under these conditions gives adequately rapid cytochrome kinetics. The kinetics of oxidation of cytochrome suggest that the infrared intensities used caused effects equivalent to the Na line of about $10^{-2}$ milli Einsteins/liter/sec. This corresponds to $10^{-10}$ Einsteins/sec. for the 0.1 ml volume. Chromatium was grown as described elsewhere and was concentrated by centrifugation.

Results.—Figure 1 shows the spectroscopic response of the bacterial suspension to infrared illumination at 300°K (left). An abrupt downward deflection of the trace occurred at the moment of illumination, as indicated by the arrow, corresponding to a decrease of absorbancy at 423 mμ, as measured with respect to 460 mμ, and an oxidation of reduced cytochrome. A cessation of illumination after the recording was terminated caused a dark reduction of the cytochrome (not completely shown). In a repetition of the experiment at liquid nitrogen temperatures (≈80°K) (the solution having been previously shielded from the measuring light until a few seconds before the infrared illumination was begun), infrared illumination again caused an abrupt downward deflection of the trace. In this case no reduction was observed on turning off the light for one hour (not shown). It is apparent from a comparison of the traces at 300°K and at 80°K (i) that the reaction proceeds to a substantial

![Figure 1](image-url)
extent at the lower temperature, (ii) that the half-time is not increased at the lower temperature, (iii) that the initial rate of the reaction, calculated as described in the figure, is no slower at the lower temperature, (iv) that the quantum requirement of the reaction is essentially the same at the lower temperature.

In order to identify the substance which is affected in the oxidation-reduction reaction at low temperatures and to show that it was converted from a ferrous to a ferric form, it is necessary to demonstrate the difference spectrum of the absorbancy changes at low temperature. For this purpose, the wavelength scanning split-beam spectrophotometer has been used, and a result for the visible region is shown in Figure 2. In this case, the absorbancy changes which occur on infrared illumination at room temperature are indicated by the open circles. It is seen that an absorption band having a maximum at \( \sim 553 \text{ m\(\mu\)} \) disappears upon illumination. At the reduced temperature, the trace following the solid circles indicates the disappearance of an absorption band having an \( \alpha \) peak at approximately 550 m\(\mu\) with a corresponding \( \beta \) band at approximately 524 m\(\mu\). It is of considerable significance that the absorbancy change shows only one \( \alpha \) and one \( \beta \) peak. The low temperature spectra show a single Soret band at about 420 m\(\mu\). These results identify a single cytochrome involved in the light reaction at low temperature.

There is a high probability that an electron spin resonance signal accompanies this light-induced oxidation of a reduced cytochrome. Calvin\(^{15}\) has obtained such a signal at low temperatures from the related bacterium \textit{Rhodospirillum rubrum}. We have observed a room temperature signal from Chromatium but have not verified its existence at \( 80^\circ \text{K} \).\(^{16}\) It is unlikely that such a signal is associated with the cytochrome\(^{17, 18}\) and more likely that it is associated with an unpaired spin in chlorophyll.\(^{15}\)

\textit{Discussion.}—On the basis of these preliminary data, we conclude that infrared illumination of chlorophyll in the intact Chromatium cell initiates a temperature-insensitive electron or proton transfer reaction between bacterial chlorophyll and a closely associated cytochrome of type c (423.5 m\(\mu\)). In this reaction, the cytochrome is oxidized from the ferrous to the ferric state with an over-all quantum requirement of about 2 per electron.\(^{10}\) The reaction is not measurably slowed
from 300°K to 80°K, although the possibility that the reaction may be sensitive to temperatures below 80°K has not yet been eliminated.

The failure of this reaction to slow between 300°K and 80°K suggests that a special reaction mechanism between chlorophyll and cytochrome should be considered. For some time before these data were available, there had been considerable speculation on various reaction mechanisms; a suggestion based partially on inductive resonance was put forward by Kamen in 1957 but even then it appeared unlikely that an inductive resonance phenomenon between chlorophyll and cytochrome was involved in view of the very small absorbancy of the reduced cytochrome in the infrared region of the spectrum. Current thinking now tends towards proton migration or charge transfer mechanisms. Explanations along these lines are described by Kamen and Hill. Calvin has described semi-conductor mechanisms. These data at low temperatures now require a serious consideration of such possibilities.

The illumination of chromatophore of Chromatium would result in the absorption of photons in bacterial chlorophyll. If there were no cytochrome associated with the chlorophyll, there could follow an inductive resonance energy transfer to a particular chlorophyll molecule having cytochrome associated with it. The concentration of such chlorophyll-cytochrome complexes is not known, but a rough calculation, based on the magnitude of the observed absorbancy change at room temperature and the total chlorophyll absorption using ε values of about 100 cm\(^{-1}\) mM\(^{-1}\), suggests that about 3 per cent of the chlorophyll could be so bound. The chlorophyll could then be transformed to an excited state and would accept an electron or a proton from the cytochrome, effectively forming an oxidized cytochrome and a reduced chlorophyll intermediate. Experiments on the detection of such a chlorophyll intermediate are currently in progress. Alternatively, an exciton would be transferred from chlorophyll to cytochrome. The mechanism of the reaction is not known, but the quenching of chlorophyll fluorescence by paramagnetic ions and the charge transfer mechanism proposed by Linschitz are surely to be considered. Although the particular mechanism described here may not be appropriate, our attention is called for the first time in a biological system to experimental evidence for a temperature-independent intermolecular oxidation-reduction reaction.

The number of electrons transferred from cytochrome to chlorophyll would appear to be one electron per two quanta. This conclusion is based on the resemblance of the absorbancy changes to those for the one-electron oxidation of ferrocyanochrome c to ferricytochrome c. Since the former has dominant absorption bands, our spectra identify the disappearance of this form but do not positively identify the oxidation state of the product. If a hypothetical higher oxidation state of cytochrome c is formed which has the same spectrum as the ferric state, our method would not detect it, and more than one electron could have been transferred per two quanta (two quanta/electron represents only 13 per cent efficiency). The fact that an isolated cytochrome, probably identical to that in Chromatium, appears to have three heme groups would make such a higher oxidation state rather unstable because of the possibility of electron transfers between the hemes.

In summary, our experiments do not eliminate the possibility that cytochrome is oxidized beyond the ferric state provided that such a state does not differ spectro-
scopically from the ferric state. However, the presence of other hemes in the cytochrome c molecule would render such a higher oxidation state unstable with the result that a net ferrous-ferric oxidation reaction would be observed.

In our studies of light-induced optical changes in "green" and "purple" photosynthetic systems, \(^{12,26,27}\) the evidence for the formation of oxidizing equivalents at the level of cytochrome has always been consistent, but evidence for the formation of reducing equivalents has been equivocal.\(^{26}\) Following Duysens\(^{28}\) and Olson's\(^{29}\) work, however, we have found a significant discrepancy in the time relations of the formation of oxidizing and reducing equivalents in Chromatium at 300°K. This supports the idea presented here that the separation of oxidizing and reducing equivalents occurs between cytochrome and chlorophyll (i.e., oxidizes the former and reduces the latter) instead of between chlorophyll and pyridine nucleotide, and explains how the oxidizing equivalents may regularly be observed in cytochrome and irregularly at the level of pyridine nucleotide.

Others have claimed to be studying primary events in photosynthesis because of the rapidity with which they occur following intense flashes of light.\(^{30}\) We present another criterion for a "primary" reaction: if the reaction occurs at a high quantum efficiency and with no change of rate at extremely low temperature, it may be a "primary" event. The reaction for Chromatium, described here, satisfies this criterion.

The generality of this reaction in Chromatium is not yet known, and the chlorophyll cytochrome interaction may differ in various types of cells. The ability to detect an infrared light-induced oxidation of cytochrome depends upon the dark cells containing some reduced cytochrome at low temperatures. This in turn depends upon the relative rates of decrease of the dark oxidase and reductase activities with temperature, which may differ in \(R.\ rubrum\) and Chromatium. We have not yet been able to detect this reaction in \(R.\ rubrum\) at 80°K. However, \(R.\ rubrum\) gives a free radical signal at low temperatures; it is therefore more likely that such a signal is associated with the acceptance of reducing equivalents by chlorophyll and not with cytochrome oxidation. Alternatively, the free radical signal may not correspond to a chlorophyll reaction at all, but to some unknown process; the electron spin resonance signal is very unspecific and does not identify the molecule involved as does the optical method.

The temperature-independent chlorophyll-cytochrome reaction observed in Chromatium is also of interest with respect to energy transfer between cytochromes. Whereas at room temperature several cytochromes are known to be affected by infrared illumination,\(^{16}\) at liquid nitrogen temperatures no evidence for the participation of additional cytochromes is obtained, even with long intervals of illumination at high intensities. It must, therefore, be concluded that, whereas the electron transfer reaction between chlorophyll and one cytochrome may be non-thermal, that between cytochromes involves temperature-sensitive diffusion processes, as postulated for the operation of the carriers of the respiratory chain of mitochondria.\(^{8}\)

These results, considered in the light of current interest in life at low temperatures,\(^{31}\) indicate that a portion of the photosynthetic process in the immediate vicinity of chlorophyll occurs under very adverse environmental conditions. Investigations of the extent of formation of reduced substances such as pyridine nucleotide at these temperatures will be of interest.
The authors are grateful to Drs. M. D. Kamen and J. M. Olson for their stimulating communications and helpful suggestions on this manuscript.

* This research has been supported in part by grants from the National Science Foundation and the U.S. Public Health Service.

16 Chance, B., M. Nishimura, and L. Fiette, unpublished observations.