QUANTUM REQUIREMENT FOR PHOSPHOPYRIDINE NUCLEOTIDE REDUCTION IN PHOTOSYNTHESIS [1, 2]

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Experiments will be reported concerning the number of absorbed light quanta needed to reduce 2 molecules of pyridine nucleotide (PN) in photosynthetic cells and spinach chloroplasts. We will call this number the quantum requirement of PN reduction. If CO_2 is reduced via reduced pyridine nucleotide (PNH), then 2 molecules of PNH are needed to reduce 1 CO_2 molecule. If the quantum requirement of PN reduction is large, compared to the quantum requirement of photosynthesis (defined as the number of absorbed quanta needed to reduce 1 CO_2 molecule), then this would indicate that the reduction of CO_2 does not mainly occur via PN; on the other hand if the number is of the same order of magnitude, then this would support the hypothesis that PN is an intermediate in the reduction of CO_2.

The reduction of PN was studied in intact photosynthesizing cells by making use of the long known fact that reduced di- or tri- phosphopyridine nucleotide (DPNH or TPNH) fluoresces in the blue region, when excited by near-ultra-violet radiation, while oxidized pyridine nucleotide does not. Photosynthetic cells, when excited by the mercury line at 366 mμ, show a blue fluorescence, which appears to increase upon illumination with red light. The red light causes photosynthesis to proceed at a much higher rate than the weak fluorescence exciting radiation, but does not cause blue fluorescence. Spectrofluorometric studies showed that the fluorescence increase was caused by PN reduction (5, 11). It was not established whether DPN or TPN was reduced. The observation that the steady state PNH concentration during illumination in Chromatium (the only species that was studied in this respect) was lower at higher concentration of CO_2 in the medium, suggested that PNH participated in the reduction of CO_2 (10).

Also results of other authors are consistent with this suggestion. Various workers demonstrated reduction of DPN or TPN by cell-free preparations from photosynthesizing organisms; recently even appreciable accumulation of PNH was reported (3, 8, 12).

Although it has been demonstrated that PN is reduced by illimunated extracts, that enzymes are present which are able to catalyze the reduction of CO_2 (at a slow rate) by TPNH in the presence of ATP (cf. 1, 16), and (by means of the fluorescence experiments), that PN is also reduced upon illumination of intact cells, it has not yet been proven that PNH participates in the reduction of CO_2. As will be shown, results of the experiments on the quantum requirement of PNH reduction in intact cells and chloroplast suspensions strongly support this hypothesis.

METHODS AND RESULTS

The rate of the reduction of added TPN by illuminated spinach chloroplasts was determined from a recording of the absorptance at 350 mμ. The absorptance (ratio of absorbed and incident light flux) was measured with a somewhat modified Zeiss spectrophotometer connected to a Varian recorder (2). The modified part of the apparatus is shown in figure 1.

The suspension was in a quartz vessel V of 1 mm thickness, placed in the vessel carrier of the spectrophotometer. The measuring beam from the monochromator, which is set at 350 mμ, passes a filter F_2, which transmits a narrow band around 350 mμ, but stops the red actinic light from a projector. Corrections for scattering of the suspension were made small by placing an opal glass plate O close to V (cf. 14) and close to a blank vessel, which was also placed in the vessel carrier. The correctness of the absorptance measurements was checked by adding small amounts of DNPH of known absorbancy.

The number of actinic quanta absorbed is equal to the number of incident quanta, multiplied by the absorptance of the actinic light. The number of incident quanta per sec per cm^2, was measured by moving a small calibrated silicon cell (Lange) into the actinic beam.

The absorbancy of the suspension for the actinic light was measured, after removing filter F_2, and setting the monochromator at wave length 668 mμ of

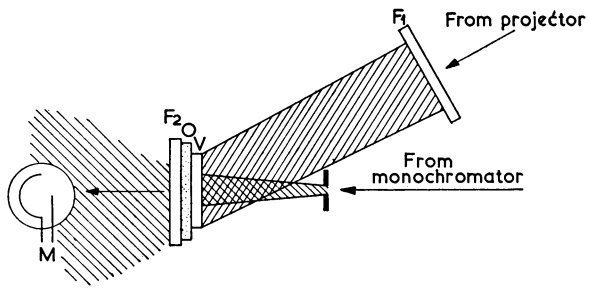

FIG. 1. Schematic diagram of part of the apparatus for measuring the rate of PN reduction by a chloroplast suspension, which is illuminated by filtered light from a projector.

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the actinic light. The apparent rather small absorbancy at 720 mμ, which is probably due to scattering, since chlorophyll does not absorb at this wave length, was subtracted from the measured absorbancy at 668 mμ, and the result was taken as the "true absorbancy" of the suspension. Since the true absorptance was close to 90 %, serious errors appear unlikely in this measurement.

The quantum requirements were determined for intensities of actinic light, at which the rate of TPN reduction increased approximately linearly with light intensity. Also DPN was reduced, but at a lower rate than TPN, especially at the higher light intensities. The data indicated (2) that, at very low light intensities, the rates and thus the quantum requirements of DPN and TPN reduction approached each other, and also that TPN and TPNH strongly inhibited DPN reduction.

The following quantum requirements for TPN reduction were found with 5 different chloroplast preparations from commercially obtained spinach: 12.8 11.6 11.6 11.0 14.0.

Application of the absorption method for measuring PN reduction in vitro is difficult, at least in normally colored photosynthesizing organisms, because of the smallness of the effect, and because of the uncertainty, caused by absorption changes by other cellular components, such as cytochromes. The increase of fluorescence in the blue region upon illumination of suspensions of photosynthesizing cells was found to be a more unique and sensitive indication of PN reduction (5), and was taken as a measure of the number of PN molecules reduced. The blue fluorescence was calibrated by measuring the increase in fluorescence, caused by adding a known amount of DPNH to the suspension. If we assume that a molecule PNH formed inside the living cell has the same fluorescence yield as a molecule DPNH outside, then the number of reduced PN molecules can readily be calculated. However, this assumption is probably not correct, since the shift of the maximum of the fluorescence difference spectrum in vivo from about 460 to 440 mμ (5) indicates that an appreciable part of the PNH molecules formed in the light must be bound to cell constituents. Binding of DPNH to alcoholdehydrogenase was found to cause not only a shift in the maximum from 460 to 440 mμ, but also a pronounced increase in fluorescence (6, 15). This phenomenon has been observed for binding of PNH to various other enzymes (cf. 15, 19). If all PNH molecules in vivo possess an equally high fluorescence yield as those bound to yeast alcoholdehydrogenase (6), then the number of molecules PNH calculated on the above mentioned assumption has to be divided by a factor of about 4. We do however not yet know the fraction of PNH, which is bound in vivo, nor its precise fluorescence yield. The increase in fluorescence yield of PNH bound to various enzymes does not appear to be higher than 4. We therefore have multiplied the originally calculated numbers by a factor between 1 and one fourth. For chloroplasts, which are able to produce TPNH from added TPN in an amount, which is in excess of what can be bound by cell constituents, the uncertainty is smaller.

The actinic light used was a narrow band in the red or, for Chromatium, in the near infra-red region. The incident intensity in ergs (and thus in quanta) per cm^2 per sec was determined by means of a calibrated thermopile. The vessel containing the suspension was the same as that described above and its absorptance was measured in the same way.

Upon onset of photosynthetically active actinic light, the PNH level increases steadily during the 1st second or seconds. The initial rate of increase is higher at higher intensities of actinic light, but approaches a maximum.

The quantum requirement was determined for the initial rate, at an actinic intensity below that, needed for saturation of the initial rate. The quantum requirements for PNH reduction were between the following limits (cf. 2). For Chlorella: 8.6 and 34: for the purple bacterium Chromatium: 5.6 and 22.4: for suspensions of spinach chloroplasts to which TPN had been added: 8.6 and 18.

The spread in these values is due, as we have explained above, to the uncertainty in the fluorescence yield of PNH in the cells, as compared to that of DPNH in water solution.

Discussion

The quantum requirement for TPN reduction found for spinach chloroplasts with the absorption method, is not inconsistent with that found with the fluorescence method for different species, although the latter values have a great uncertainty. The quantum requirement for spinach chloroplasts was found to be about 12, as compared to 32, observed by other authors (13). The explanation for this large discrepancy is not clear. It is possible that in the latter investigation the rate of TPN reduction decreased during the 10-minute period of illumination, after which the TPNH formed was determined. In our calculations, the initial rate was used, since the rate decreased in time, perhaps because of TPNH formation. Another possibility might be that the kind of spinach used or the preparation of chloroplasts may have been the source of the discrepancy. The quantum requirements of 12 is not very different from the quantum requirements for CO_2 reduction by intact organisms as found by many other investigators, in the first place by Emerson and co-workers (cf. 7).

Most quantum requirements for CO_2 reduction were between 7 and 12, and according to a critical review (7), the "true value" may be between 7 and 9. The lowest quantum requirement reported in literature was about 3 (17). It may however be argued that a quantum requirement lower than about 4 contradicts the second law of thermodynamics. The argument is briefly as follows (4). If the absorbed light energy were completely converted into chemical energy then about 3 quanta would suffice to reduce 1 molecule of CO_2 to the level of carbohydrate and to "oxidize"

2 molecules of water to O_2. However, light energy like heat, is not, as is often believed, a form of freely transformable energy. The maximum efficiency with which light energy can be converted into "high grade energy" depends, amongst others upon the "structure" of the light, in an analogous way as the maximum efficiency with which heat can be transformed depends, amongst others, upon its "structure." For instance the maximum efficiency, E, with which temperature radiation emitted by a source of T_1 degrees Kelvin, can be used to produce chemical or other high grade energy in a reaction vessel, kept at T_0 degrees Kelvin, according to a consequence of the second law of thermodynamics, equals $E = (T_1 - T_0)/T_1$, which is a number smaller than 1. If $T_1 = 1100$ and $T_0 = 300$ °K (room temperature), then $E = 0.73$. When the reaction vessel is placed inside a "black" body of this temperature, and the light energy passes through a spherical filter, which surrounds the vessel and transmits a band between 660 and 680 $m\mu$, and reflects all other light, then the intensity incident on the vessel is 0.17 mW/cm². This light cannot be converted into high grade energy with an efficiency better than 73 %, whether the vessel contains a photosynthesizing suspension or a photoelectric cell. Since all investigators seem to agree that the quantum requirement of a suspension of photosynthesizing algal cells in this light (possibly aided by "catalytic" amounts of light of shorter wave length) is not higher than for any other light regime, it follows that the quantum requirement for algae and higher plants must be equal to or higher than 3/0,73 or about 4. The same considerations can be applied to all "partial" reactions of photosynthesis, following light absorption, and may be helpful, when analyzing these reactions.

The quantum requirements determined by us, are consistent with the hypothesis that the reduction of CO_2 proceeds via PNH. The most precisely determinded one for TPN reduction in spinach chloroplasts is somewhat higher than those generally found for algae and for CO_2 reduction in Chlorella, but this may be due to the difference between unicellular algae and higher plants. The quantum requirement of TPNH reduction for living cells must be determined more precisely, and be compared with simultaneously determined quantum requirements for CO_2 reduction, before it can be stated with more certainty whether the quantum requirement of PN reduction is equal to or different from that of CO_2 reduction. The present data, however, indicate that at least an appreciable part of the CO_2 reduction occurs via PN. If this is true, then our data are consistent with the statement that the minimum quantum requirement of CO_2 reduction is appreciably higher than 4.

The quantum requirement for PN reduction in the purple bacterium Chromatium 5.6 to 22.4 may be compared to that for CO_2 reduction 8.5 to 13 (18), and that for the oxidation of cytochrome c 553, which appears to be about 8 (within rather wide limits of uncertainty) per 4 cytochrome molecules oxidized (9). These values suggest that, in purple bacteria, the reduction of CO_2 is mediated by PN, and, as Olson (9) concluded, the oxidation of substrate is mediated by a cytochrome.

Summary

By means of absorption spectrophotometry the number of light quanta, absorbed by a suspension of spinach chloroplasts, required to reduce 2 triphosphopyridine nucleotide molecules, was found to be about 12. The analogous quantum requirements for phosphopyridine nucleotide (PN) reduction by living cells, as determined by fluorescence spectroscopy from the initial rate of PN reduction upon illumination, were found for intact cells of the purple bacterium Chromatium and for Chlorella, to be between 5.6 and 22.4 and between 8.6 and 34 respectively. These quantum requirements are of the same order of magnitude as those found by various authors for CO_2 reduction, which indicates that phosphopyridine nucleotide participates in the reduction of CO_2.

It was argued that a quantum requirement for CO_2 reduction by algae of less than 4 contradicts the second law of thermodynamics.

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PRIMARY PHOTOCHEMICAL AND PHOTOPHYSICAL PROCESSES IN PHOTOSYNTHESIS [1]

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I. EXCITONS AND THEIR MIGRATION

In photochemical reactions in condensed systems, intermediary steps can occur between light absorption and the primary photochemical reaction: the photophysical processes of energy or charge transfer. Either the excited electron, or the hole it leaves in the absorbing molecule, may move, to be found at the end of the excitation period in a site far removed from the original locus of excitation. If the electron and the hole travel separately, the final effect is separation of charges—which can be also described as internal oxidation-reduction.

The electron-hole pair is often referred to as an exciton. When the excited electron and the hole are associated in a single molecule, the (perhaps superfluous) term "intramolecular exciton" is used. Figure 1 illustrates the different modes of transfer of intramolecular and intermolecular excitons (1).

The binding energy of the exciton may be so small that it can dissociate under the influence of thermal motion, or under the pull of an applied electric field (photoconductivity). If impurities are present in the system, or adsorbed on the surface, they may trap the electron, or the hole, or both.

Recently, it has been pointed out that the concentration of pigment molecules in the photosynthetic organelles is so high as to justify approaching photosynthesis from the point of view of phenomena in the solid state, making the above considerations relevant.

II. STRUCTURE OF CHLOROPLASTS

All chloroplasts contain lamellae—flat or involuted, thin extended layers, about 20 mμ thick. They are found also in chloroplast-free cells of the blue-green algae. The lamellae either fill out the whole body of the chloroplast (lamellar chloroplasts), or form cylindrical grana (granular chloroplasts). Figures 2 and 3 show both types of chloroplasts in the same plant. Figure 3 shows that in granular chloroplasts, the lamellar structure extends into the intergranular stroma; the grana are merely approximately cylindrical volumes in which the lamellae are denser and more numerous.

The high optical density of the lamellae in preparations fixed with osmic acid (as in figs 2 and 3) is due to higher concentration of precipitated osmium; and this in turn seems to be due to the presence of lipoids. Consequently, the lamellae must contain the greatest concentration of lipoids, while the stroma is more predominantly proteinaceous. In granular chloroplasts, absorption and fluorescence microscopy confirm that the (lipophilic) pigments are located in the grana.

Chlorophyll, whose molecule consists of a flat, slightly polar, colored conjugated ring system (chlorin) and a long, colorless tail (phytol), should accumulate on interfaces between the hydrophilic and the hydrophobic layers. Thomas and co-workers (2) compared the number of chlorophyll molecules in the chloroplasts of 8 species with the total surface areas of their lamellae. The results varied between 1.8 mμ^2 and 3.8 mμ^2 per chlorophyll molecule, while the amount of chlorophyll per chloroplast varied by a factor of 5×10^3.

Artificial chlorophyll surface layers (3, 4) exist in two forms: crystalline and amorphous. In the first form, the area requirement is about 0.45 mμ^2 (suggesting a 2-molecular layer), and the red absorption

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