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EFFECTS OF ILLUMINATION AND OXYGEN SUPPLY UPON THE LEVELS OF PYRIDINE NUCLEOTIDES IN *CHLORELLA* CELLS

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SUMMARY

1. Using intact cells of *Chlorella*, investigations were made on the effect of light on the levels of various forms of pyridine nucleotides, compared with that on the photogenic reducing agent (*R*).

2. No form of pyridine nucleotide was found of which the concentration was affected in the same manner as that of *R*, which has been known to be produced only in the light and eliminated completely in the dark.

3. Under both aerobic and anaerobic conditions, light decreased the level of DPN^+ and increased the level of TPN. Quantitatively, there was a correspondence between these effects.

4. When algal cells were transferred in the dark from anaerobic to aerobic conditions, a marked decrease of DPNH occurred, the levels of DPN^+, TPN^+ and TPNH remaining almost unchanged. The decrease of DPNH in the light (in N_2) was assumed to be brought about by the O_2 produced concomitantly with the photogenic formation of *R* and effecting the transfer of DPNH to some substance other than DPN^+.

5. It was concluded that (a) neither TPNH nor DPNH is the primary *R* and that (b) one of the effects caused by light is the conversion of DPN^+ to TPN.

INTRODUCTION

Since the work of CALVIN and his associates[1,2] it is now generally agreed that the reductive step involved in the photosynthetic cycle is the transformation of phosphoglyceric acid to 3-phosphoglyceraldehyde, an enzymic reaction that is known to

Abbreviations: DPN^+ and DPNH, oxidized and reduced diphosphopyridine nucleotide; TPN^+ and TPNH, oxidized and reduced triphosphopyridine nucleotide; total DPN = DPN^+ + DPNH; total TPN = TPN^+ + TPNH; PN^+ = DPN^+ + TPN^+; PNH = DPNH + TPNH; total PN = total DPN + total TPN.

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require DPN or TPN as a cofactor. The photochemical reduction of DPN^+ and TPN^+ added to chloroplast suspensions was first reported by VISHNIAC AND OCHOA[3], and reports to the same effect were later made from several other laboratories (reviewed by VISHNIAC[4]). Using suspensions of some photosynthesizing microorganisms, DUYSENS *et al.*[5,6] observed that upon illumination of cells with infrared or red radiation the absorption band at 350 mμ slightly increased and the cells emitted blue fluorescence. On the basis of these observations they concluded that the photochemical reduction of PN occurs in some earlier phase of the mechanism of photosynthesis. A fundamentally different phenomenon was reported by CHANCE AND SAGER[7] who, using cell suspensions of *Chlorella* and a mutant of *Chlamydomonas*, observed a photochemically induced spectral change which suggested the *oxidation* of PN caused by illumination.

The photo-induced oxidoreductive process occurring in green cells can be studied from an entirely different angle by performing the "pre-illumination experiment" using ^{14}C as a tracer, an experiment which was first worked out by CALVIN AND BENSON[8]. The unknown photogenic agent(s) found by CALVIN *et al.* to cause a markedly enhanced $^{14}CO_2$-fixation was found by us to react with, and to be consumed by, various oxidizing agents such as oxygen[9], quinone[10], hydrogen peroxide[11], and 2,6-dichlorophenol indophenol[12], and it was concluded that it is a reducing agent which, directly or indirectly, causes the reduction of phosphoglyceric acid in the mechanism of photosynthesis. It is, therefore, tempting to assume that the photogenic agent in question—which for convenience[9,13] we call *R*—might be either a reduced form of PN or a reducing substance causing the reduction of PN.

The time courses of photochemical formation of *R* and its decay process occurring in the dark can be followed with sufficient accuracy by measuring the amount of $^{14}CO_2$, added after darkening, fixed by *R*. The present experiments were undertaken in order to investigate whether and in what manner the change of *R* level under different conditions is correlated with the changes in quantities and oxidoreductive states of TPN and DPN in *Chlorella* cells. Quantitative determinations of oxidized and reduced forms of DPN and TPN were conducted by enzymic methods, to which we devoted a great deal of effort to obtain reliable results.

It should be remarked in advance that the results obtained in our experiments were in many respects contrary to our expectations. In short, we could not find any parallelism between the change of the *R* level and that of TPNH or DPNH. The effect of light upon the PN system appeared to lie, in the main, in causing the transformation of DPN^+ to TPN. In addition, a complicated change in the proportions of the PN-system was brought about also by the presence or absence of oxygen. While we can hardly reject the possibility of participation of the PN system in some important part of the photosynthetic cycle, the inevitable conclusion we arrived at was that the photogenic reducing agent *R* is neither a pyridine nucleotide itself nor a substance governing *directly* the oxidoreductive states of these nucleotides in photosynthesizing cells.

METHODS

Material and apparatus

The experimental organism used was the "dark cells"[14] of *Chlorella ellipsoidea* which had been grown photo-autotrophically by the method reported earlier[15].

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Experiments were performed using Apparatus I described in one of our previous papers[9]. The main part of the apparatus consisted of a round flat illumination vessel ("lollipop") and an exchangeable "dark vessel" attached to it. 100 ml of algal suspension (0.002 *M* K_2SO_4 containing 3.1 ml packed volume of algal cells) were placed in the lollipop and, with constant bubbling of N_2 or CO_2-free air, either illuminated or kept dark according to the experiment. At various intervals during this treatment, aliquots (7.5 ml) of cell suspension were transferred to dark vessels, each of which contained 1.9 ml HCl or NaOH (final concn., 0.1 *N*) or $NaH^{14}CO_3$ (final concn., $3 \cdot 10^{-3}$ *M*). The samples in the dark vessels with acid or alkali were promptly warmed up to 100° in a boiling-water bath, and after cooling they were analysed for DPN and TPN. The vessels provided with $NaH^{14}CO_3$ were kept dark for 30 sec after introduction of algal cells, and the ^{14}C incorporated by the effect of *R* was measured by the method already described[9]. The experimental temperature was 23°, and the light intensity applied in the illuminating experiments was about 2 × 30,000 lux.

Determination of reduced and oxidized forms of DPN and TPN

The principle of the methods adopted was that of GLOCK AND MCLEAN[16], who, using the rat liver and other animal tissues as materials, determined the quantity of DPN^+, DPNH, TPN^+ and TPNH separately by the following procedure. Taking advantage of the difference in stability between PN^+ and PNH towards acid and alkali—PN^+ being stable towards acid but labile towards alkali, while the converse is true for PNH—PN^+ was separated from PNH, and with each portion TPN and DPN were determined by measuring their coenzymic activities displayed in specific enzymic systems. A system consisting of glucose-6-phosphate, glucose-6-phosphate dehydrogenase, cytochrome *c*, and TPNH-cytochrome *c*-reductase was used for TPN, and a system consisting of ethanol, alcohol dehydrogenase, cytochrome *c*, and DPNH-cytochrome *c*-reductase for DPN. In both cases the rate of reaction was determined by measuring the rate of reduction of cytochrome *c*, which was followed spectrophotometrically at 550 mμ.

Spectrophotometric readings were made at intervals of 6–11 min taking as a reference a reaction mixture containing only water and oxidized cytochrome *c*. For each run, parallel experiments were performed using known quantities of standard preparations of TPN or DPN, and on the basis of the relationship thus found between the amounts of nucleotides and the rate of reduction of cytochrome, the quantities of the nucleotides contained in the test samples were estimated.

Preparations

Glucose-6-phosphate, commercial preparation (Sigma Co.); cytochrome *c*, prepared from horse heart by the method of KEILIN AND HARTREE[17]; DPNH-cytochrome *c*-reductase, isolated from the pig heart (homogenized and centrifuged at 750 × *g* for 15 min) and purified according to the method of MAHLER *et al.*[18] up to Step 3 in their procedure (neutralized ammonium sulfate fractionation); glucose-6-phosphate dehydrogenase, alcohol dehydrogenase, and TPNH-cytochrome *c*-reductase, prepared according to the methods described by GLOCK AND MCLEAN[16].

DPN was prepared from the sheep liver according to the method of LE PAGE AND MUELLER[19], and purified by ion-exchange chromatography on Dowex-1-formate[20]. The preparation contained 70 % DPN but no TPN. TPN used was a commercial

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preparation (Sigma Co.) which was found to contain 76 % TPN but no DPN. The reduced forms of both DPN and TPN were prepared by enzymic reduction.

Preliminary experiments

A series of preliminary experiments were performed to test the applicability of GLOCK AND MCLEAN's method to *Chlorella* cells. To 7.5 ml of *Chlorella* suspension containing 0.23 ml (packed volume) of algal cells, varying amounts of oxidized and reduced DPN or TPN were added, and immediately thereafter the suspensions were treated with NaOH or HCl (final concn., 0.1 *N*) at 100° for 2 min. After the treatment, the samples were rapidly cooled to 0°, then centrifuged in a refrigerated centrifuge, and the supernatants obtained were neutralized and assayed for DPN and TPN. As a control, an algal suspension without the addition of PN was treated in the same way. It was found from these experiments that the acid treatment led to a complete or almost complete destruction of DPNH and TPNH but left DPN^+ and TPN^+ quite intact, while the alkali treatment completely eliminated DPN^+ and TPN^+ but left TPNH practically unaffected. Contrary to the statement by GLOCK *et al.*, however, about 50 % of DPNH was destroyed by the alkali treatment (see the results given in Table I). With the blank test using a *Chlorella* suspension (without added PN) it was confirmed that the extraction process lasting for 2 min at 100° was sufficient to remove practically all PN existing in algal cells. It was also observed that the extract of *Chlorella* cells caused, even in the absence of the substrates of dehydrogenases, some reduction of cytochrome *c*. The rate of this "endogenous" reduction was found to be unaffected by the addition of TPN or DPN. In all subsequent experiments, therefore, a correction was made for this endogenous reduction.

In another series of preliminary experiments it was found that the extract of *Chlorella* cells contained a certain substance which affected more or less strongly the enzymic reactions used for the PN-assay. Data of experiments showing this effect are presented in Table I. In these experiments the extracts of *Chlorella* cells which were obtained by acid or alkali treatment were, after being neutralized, added to a known amount of DPN or TPN, and the enzymological assay was performed using as a control the samples without the addition of the extract. As may be seen from the data given in the table, the reaction for TPNH was more than 50 % inhibited by the substance in question, while the reactions for DPNH and TPN^+ were inhibited by 5 %, and that for DPN^+ remained unaffected. In other experiments not shown it was found that the degree of inhibition of each nucleotide test remained the same in different algal samples—either pre-illuminated or kept dark—provided that the same amount of algal cells were extracted with a definite amount of acid or alkali solution. In all subsequent experiments, therefore, fixed amounts of extracts which were obtained from fixed amounts of algal cells (see our earlier description) were used for the enzymic assay to keep the inhibiting effect of the unknown substance constant. Taking into consideration the degree of decomposition caused by acid or alkali treatment and the effect of the unknown substance interfering with the enzymic assay, estimations of various forms of pyridine nucleotides contained in algal cells were made by multiplying the experimental values (obtained by comparison with the control values shown by standard PN preparations) by the following factors: for DPN^+ 1.0, for TPN^+ 1.05, for DPNH 2.1, and for TPNH 2.2.

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TABLE I

EFFECTS OF ACID AND ALKALI TREATMENT UPON VARIOUS FORMS OF PN, AND THE INTERFERENCE OF SOME SUBSTANCE CONTAINED IN ALGAL CELLS IN THE ENZYMIC ASSAY OF PN

(All the experiments were performed under the specific conditions described in the text.)

Extracting medium	*Forms of PN*	*Recovery (in %) of added PN after acid- or alkali-treatment*	*Recovery (in %) of added PN in direct readings in the presence of cell extract*	*Factors for multiplication of direct readings*
0.1 *N* HCl	DPN^+	100	100	1.0
	DPNH	0		
	TPN^+	100	95	1.05
	TPNH	3		
0.1 *N* NaOH	DPN^+	0		
	DPNH	50	95	2.1
	TPN^+	0		
	TPNH	100	45	2.2

Determination of the concentration of R

The level of *R* in algal cells was determined by measuring the amount of $^{14}CO_2$ fixed in 30 sec in the dark, and taking into consideration the specific radioactivity of $^{14}CO_2$ applied, the molar quantity of fixed CO_2 was calculated. Using the latter value, the molar quantity of *R* in algal cells was calculated on the basis of the following relation: $R + 3/5\ CO_2 \longrightarrow P$ (oxidized form of *R*) + 1/5 Phosphoglyceric acid (see our previous paper[13]).

RESULTS

Comparison of the time courses of R*-level and concentrations of various forms of PN as they changed in the light and dark in* N_2 *atmosphere*

To begin with, the fates of *R* and various forms of PN were followed under the conditions that have been shown to cause a characteristic rise of *R* level in the light and its decay in the dark. The cells were first kept dark for 20 min in N_2 to make them adapt to darkness; then they were illuminated under continued bubbling of N_2, and at intervals aliquots were removed to measure the amounts of *R* and various forms of PN. After 50 min of illumination when the concentrations of all factors attained their stationary levels, the light was turned off and the changes occurring in subsequent 32 min were followed quantitatively. The results obtained are presented in Figs. 1 and 2.

Several interesting facts emerged from these results. While both TPN^+ and TPNH increased during the light period, there occurred at the same time a distinct decrease of both DPN^+ and DPNH. Increase in the light of TPN^+ and TPNH followed to some extent the same course as the increase of *R* in the light, but quantitatively the former was far less significant than the latter. On turning off the light TPN^+ decreased almost to its original level, but TPNH increased to some extent, while *R* decreased very rapidly to the zero level. The level of DPNH increased, but that of DPN^+ was unchanged. As a whole, the most pronounced changes occurring under light and dark conditions were those of total DPN, and especially of DPNH, and correspondingly

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the quantity of total PN decreased in the light and increased in the dark as shown in Fig. 2. In any case, there was no form of pyridine nucleotide which behaved like *R* under alternate light and dark conditions. It should also be noted that even the maximum concentration of PN attained was far less than the stationary level of *R* established in the light.

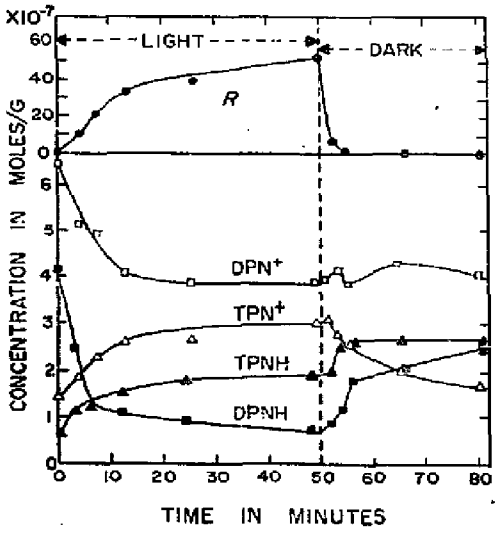

Fig. 1. Comparison of the time courses of *R*-level and concentrations of various forms of PN in the light and dark in N_2. The cells had been preincubated in the dark.

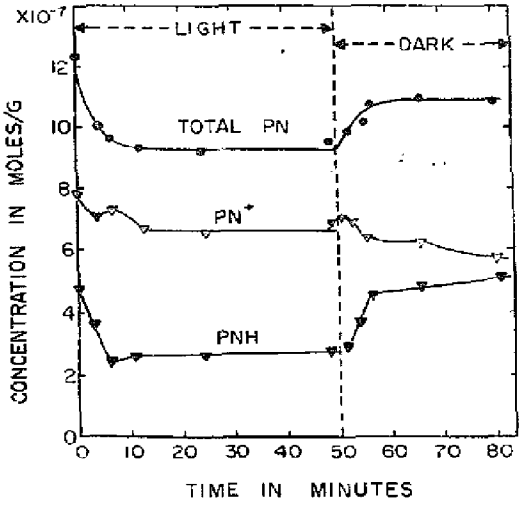

Fig. 2. Fates of total PN, PN^+ and PNH in the experiment shown in Fig. 1.

TABLE II

CHANGES IN QUANTITY OF VARIOUS FORMS OF PN OCCURRING ON TRANSFERENCE OF CELLS FROM DARK-ADAPTED TO LIGHT-ADAPTED CONDITIONS (IN N_2 ATMOSPHERE)

	Stationary concentrations (mole × 10^{-7} g) in the		*Difference*
	dark	*light*	
DPN^+	6.4	3.9	— 2.5
DPNH	4.1	0.8	— 3.3
Total DPN	10.5	4.7	— 5.8
TPN^+	1.4	3.0	+ 1.6
TPNH	0.7	1.9	+ 1.2
Total TPN	2.1	4.9	+ 2.8
Total PN	12.6	9.6	— 3.0

The fact that in the light DPN decreased while TPN increased leads us to speculate that light had the effect of causing phosphorylation of DPN to TPN. In Table II are presented the figures regarding the changes in amounts of DPN and TPN occurring when the cells were brought into the light for 50 min after they had been kept in darkness for 20 min. As is apparent from these figures, the increase in the total TPN in the light was approximately equal to the decrease of DPN^+ or of DPNH and about

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one-half that of the decrease of total DPN in the light. These data make it plausible to assume that there occurred, at least partially, a photochemical conversion of DPN into TPN under the conditions used in our experiment.

Comparison of the time courses of R*-level and concentrations of various forms of PN as they changed in the light and dark in the presence of oxygen*

An experiment similar to the one described above was performed under aerobic conditions (under constant bubbling of CO_2-free air in the algal suspension), which has been known to cause a marked lowering of the steady level of R[9]. In Figs. 3 and 4 are shown the results obtained, from which it may be seen that there was again no form of PN whose course of change in the light and dark corresponded to that of R.

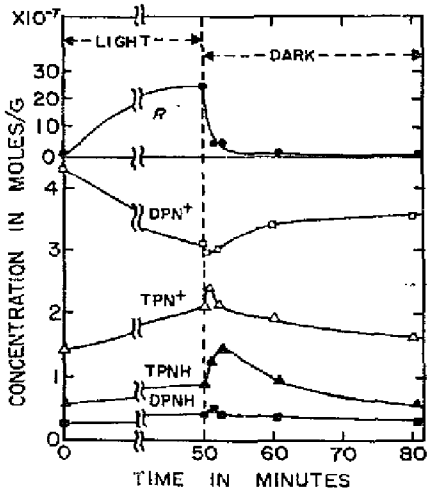

Fig. 3. Comparison of the time courses of R level and concentrations of various forms of PN in the light and dark under aerobic conditions.

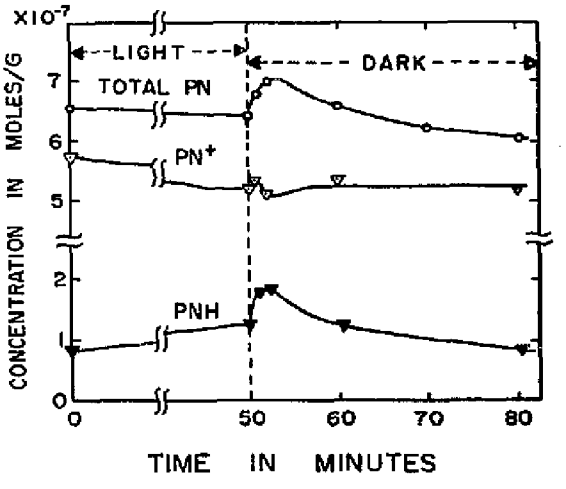

Fig. 4. Fates of total PN, PN+ and PNH in the experiment shown in Fig. 3.

The cells had been pre-incubated in the dark under aerobic conditions. The experiment was performed under constant bubbling of CO_2-free air through the algal suspension.

Some differences may be noticed between the results of the present experiment and those of the preceding one. The most distinct difference was that in the present experiment the level of DPNH remained—both in the light and dark—very low, and that the decrease of DPN+ (and not of DPNH) in the light was almost equal to the increase of total TPN, as is apparent from the data presented in Table III.

Effect of oxygen upon the levels of various forms of PN in the dark

Since the experiments described above suggested that the pattern of the PN system in algal cells was modified by the effect of oxygen, the following experiment was performed. The algal cells, which had been adapted to anaerobiosis (by bubbling of N_2) in the dark for 30 min, were suddenly exposed to air (by bubbling of CO_2-free air), and after the pattern of the PN system attained a steady state, the cells were again subjected to anaerobiosis, the whole experiment being performed in complete darkness. The changes in concentrations of various forms of PN observed during these treatments are illustrated in Figs. 5 and 6. As may be seen, the levels of DPN+,

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TABLE III

CHANGES IN QUANTITY OF VARIOUS FORMS OF PN OCCURRING ON TRANSFERENCE OF CELLS FROM DARK-ADAPTED TO LIGHT-ADAPTED CONDITIONS (IN CO_2-FREE AIR)

	Stationary concentrations (moles × 10^{-7}/g) in the		*Difference*
	dark	*light*	
DPN^+	4.3	3.0	— 1.3
DPNH	0.2	0.4	+ 0.2
Total DPN	4.5	3.4	— 1.1
TPN^+	1.4	2.1	+ 0.7
TPNH	0.6	0.9	+ 0.3
Total TPN	2.0	3.0	+ 1.0
Total PN	6.5	6.4	- 0.1

TPN^+ and TPNH showed only insignificant changes on transition from anaerobiosis to aerobiosis and *vice versa*. Striking changes were observed in the level of DPNH which decreased markedly on transition from anaerobiosis to aerobiosis and increased appreciably on transition in the reverse direction. Similar changes took place in the levels of PNH and total PN. The fact that the level of DPN^+ remained almost constant while that of DPNH changed markedly compels us to infer that under the conditions studied DPNH was produced from, and converted into, some unknown substance(s) other than DPN^+.

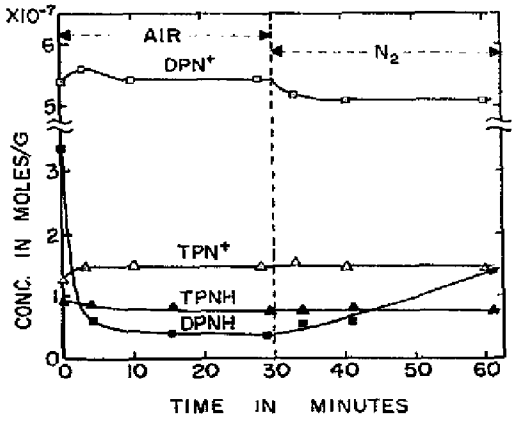

Fig. 5. Effect of oxygen upon the levels of various forms of PN in the dark. The cells had been pre-incubated in the dark under anaerobic conditions for 30 min.

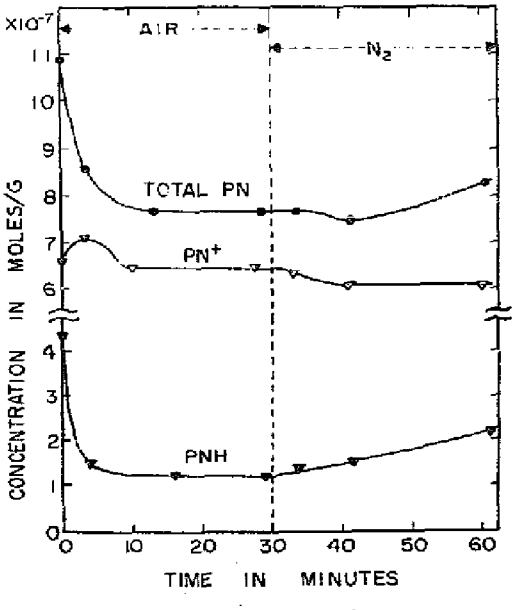

Fig. 6. Fates of total PN, PN^+ and PNH in the experiment shown in Fig. 5.

DISCUSSION

The data presented in Fig. 1 showed that in N_2 the level of DPNH in algal cells decreased in the light and increased in the dark, and that these changes were not

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accompanied by corresponding reciprocal changes of DPN^+. The state of affairs was similar to that observed in the experiment shown in Fig. 5, and it seems to suggest that the changes of DPNH observed in the illumination experiment (Fig. 1) were due, not to the direct effect of light, but rather to the effect of oxygen which was produced in the light and disappeared in the dark (see TAMIYA *et al.*[13]). That the decrease of DPNH in these cases was not accompanied by a corresponding increase of DPN^+ may suggest the occurrence of some complicated reaction such as; : DPNH + pyrophosphate $\longrightarrow$ reduced nicotinamide mononucleotide + ATP. Naturally, the situation is in need of clarification by further experimentation.

On inspecting the data presented in Tables II and III, it is found that both under anaerobic and aerobic conditions the decrease of DPN^+ in the light was approximately equal to the increase of total TPN in the light. Since no such correspondence was observed in the dark experiment using air and N_2 (Fig. 5), it seems likely that light had the effect of converting DPN^+ into TPN in algal cells. The question by what mechanism such a phosphorylating reaction could be brought about photochemically is left open to further investigations.

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