The Role of Indophenol Dyes in Photoreactions of Chloroplasts*

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Indophenol dyes were shown to be effective in three different ways in photoreactions catalyzed by isolated chloroplasts. (a) The oxidized forms acted as uncouplers of all types of photophosphorylation. (b) The reduction of low concentrations of the oxidized forms was coupled to phosphorylation. (c) The reduced forms catalyzed cyclic photophosphorylation. 2,3',6-Trichlorophenol indophenol was found to be a more potent uncoupler than 2,6-dichlorophenol indophenol. According to these findings the recently proposed schemes of photophosphorylation should be modified. An ATP-synthesizing step must be located prior to the site of indophenol dye reduction. Furthermore, the site of ferricyanide reduction should be equivalent to that of the dye.

Indophenol dyes have recently been shown to be capable of supporting both cyclic and noncyclic photophosphorylation, when used in their reduced or oxidized states, respectively (Krogmann, 1960; Trebst and Eck, 1961; Avron 1961; Gromet-Elhanan and Avron, 1963). In addition, Avron and Jagendorf (1959) observed that the oxidized form of one of the indophenol dyes, TPIP,[1] completely inhibited ATP formation when added to a reaction mixture with phenazine methosulfate. This inhibition was maintained as long as the concentration of oxidized TPIP (which was continually reduced during the experiment) remained above a certain level. A similar inhibitory effect of oxidized indophenol dyes on photophosphorylation with ferricyanide was reported by Losada *et al.* (1961). Photophosphorylation of *Rhodospirillum rubrum* extracts was also found to be inhibited by DPIP (Geller and Lipmann, 1960). These observations, as well as our finding that high concentrations of indophenol dyes uncoupled the phosphorylation associated with their reduction (Gromet-Elhanan and Avron, 1963), prompted us to investigate the possibility that indophenol dyes may act as uncouplers of all phosphorylation reactions.

In this communication the effect of DPIP on all known types of photophosphorylation was followed in time course experiments. Evidence is provided that oxidized DPIP at concentrations exceeding 1×10^{-4} M is indeed an uncoupler of all types of photophosphorylation, including the one it mediates.

METHODS AND MATERIALS

Greenhouse-grown Swiss chard was used. Once-washed chloroplasts were prepared according to Avron (1960), but washed in a medium without ascorbate. The standard reaction mixture contained in μmoles: Tris, *p*H 7.8, 90; NaCl, 120; $MgCl_2$, 24; NaK phosphate, *p*H 7.8, 24 with adequate amounts of ^{32}P; ADP, 8; and water to a total volume of 6.0 ml. The amounts of ^{32}P, chlorophyll, and Hill oxidants or electron carriers used are indicated in each experiment.

The reactions were performed in an illuminated Aminco-Warburg apparatus, fitted with two rows of 300 w General Electric flood lamps (four per row), giving a light intensity at flask level of 20,000 lux. The reaction mixture was placed in 25-ml Erlenmeyer flasks, mounted on a metal rod connected to the Warburg shaker, and shaken at 100 rpm. The flasks were closed with stoppers fitted with glass tubing for gassing. They were allowed to equilibrate at 15° for 10 minutes before illumination. During the equilibration and reaction periods, water-saturated N_2 was continuously flushed via the glass tubing through the "Under N_2" flasks. The tank N_2 used was analyzed by mass spectrometry and found to contain no less than 99.95% N_2.

For measuring phosphorylation, samples of 0.5 ml were removed at specified times by means of a syringe fitted with a long thin needle which was inserted through the gas outlet. The samples were placed in test tubes containing 0.05 ml of 30% perchloric acid. After centrifugation 0.3 ml of the supernatant was analyzed for its ATP-^{32}P content. When studying the reduction of indophenol dye samples of 0.7 ml were removed as above and placed into cuvets (0.2 cm light path). Following measurement of the absorption at 620 mμ, the samples were placed in test tubes containing 0.07 ml of 30% perchloric acid and assayed for ATP-^{32}P.

ATP-^{32}P formation was assayed as previously described (Avron, 1960). The reduction of the indophenol dyes was followed by the change in optical density at 620 mμ (Jagendorf, 1956). The millimolar extinction coefficient of both DPIP and TPIP was taken as 20 at 620 mμ (Punnett, 1959; Minakami *et al.*, 1962). Chlorophyll was determined according to Arnon (1949).

Photosynthetic pyridine nucleotide reductase was purified from Swiss chard according to San Pietro and Lang (1958) as far as and including the acetone-precipitation step.

RESULTS

Photophosphorylation with Indophenol Dyes.—Figure 1 illustrates time-course studies of ATP formation in the presence of TPIP. It can be seen that oxidized TPIP afforded no ATP formation for over 3 minutes, and even inhibited the endogenous photophosphorylation under air. Following this lag photophosphorylation began with similar specific activities under air or nitrogen.

The lag could be eliminated either by reducing the dye with ascorbate prior to the illumination (Fig. 1), thus starting cyclic ATP formation catalyzed by reduced TPIP, or else by using low concentrations of the oxidized dye which mediated noncyclic ATP formation (see Gromet-Elhanan and Avron, 1963). The difference in the rate of the TPIP plus ascorbate mixtures under air and under nitrogen was the result of the very different concentration dependence of this

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[1] The following abbreviations are used: TPIP, 2,3',6-trichlorophenol indophenol; DPIP, 2,6-dichlorophenol indophenol; Tris, tris(hydroxylmethyl)aminomethane.

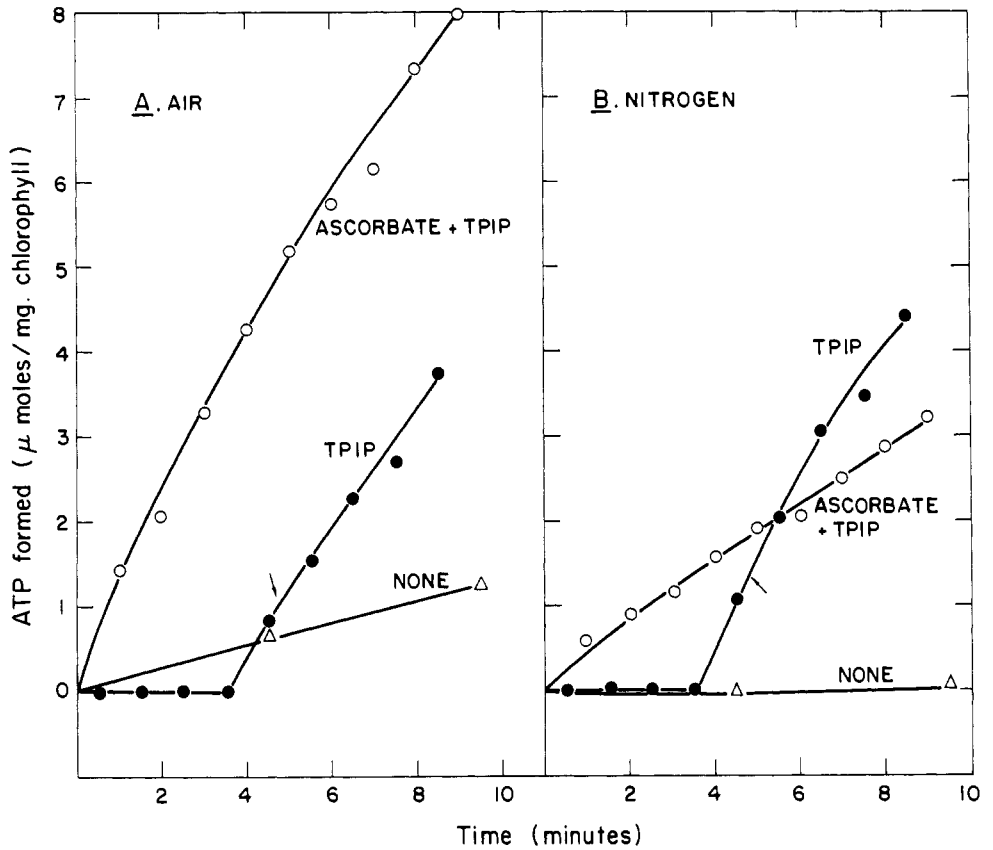

FIG. 1.—Time course of photophosphorylation with TPIP. The reaction mixture was as described under Methods and Materials and contained: once-washed chloroplasts containing 156 μg chlorophyll; ^{32}P, 4 × 10^6 cpm; TPIP, 1.5 μmoles; ascorbate, 60 μmoles. The arrow denotes the approximate time of color disappearance.

cyclic phosphorylation under the two atmospheres (Fig. 2).

When the reduction of TPIP and the formation of ATP were measured simultaneously, it was found that the lag in photophosphorylation persisted as long as the concentration of oxidized TPIP remained above 2 × 10^{-5} M (Fig. 3). Similarly, oxidized DPIP supported noncyclic ATP formation only when used at concentrations lower than 1 × 10^{-4} M, while higher concentrations were inhibitory (Gromet-Elhanan and Avron, 1963). The differences in the concentrations of DPIP and TPIP were the result of the different uncoupling potency of the two dyes, as will be discussed (Fig. 10).

It can be concluded, therefore, that oxidized indophenol dyes above a certain concentration inhibited the photophosphorylation induced by their presence without inhibiting their reduction. Thus they acted as uncouplers. However, since the concentration of the oxidized dyes continuously decreased in these systems their effect could be overlooked unless tested with time. The effect of oxidized DPIP on the time course of various types of photophosphorylation was therefore measured.

The Effect of Oxidized DPIP *on Photophosphorylation Catalyzed by Electron Carriers.*—The inhibitory effect of oxidized TPIP on phenazine methosulfate–mediated ATP formation, first observed by Avron and Jagendorf (1959), was confirmed in our system with oxidized DPIP (Fig. 4). The length of the pronounced lag of photophosphorylation was determined by the initial concentration of the oxidized DPIP. After the lag was overcome, ATP formation proceeded in a diphasic manner—a slow rate was followed by a more rapid rate. The slow rate was most probably due to phosphorylation coupled to the reduction of DPIP, while the more rapid rate was due to cyclic photophosphorylation mediated by phenazine methosulfate and reduced DPIP (see also Fig. 7). A similar diphasic behavior was previously reported for photophosphorylation catalyzed by a mixture of ferricyanide and phenazine methosulfate (Avron and Jagendorf, 1959).

Figure 5 summarizes simultaneous measurements of DPIP reduction and ATP formation in the presence of a mixture of DPIP and phenazine methosulfate. The inhibition of photophosphorylation ceased when the concentration of oxidized DPIP decreased below 7 × 10^{-5} M.

Figures 6 and 7 illustrate the effect of DPIP on photophosphorylation supported by flavin mononucleotide and menadione, respectively. Photophosphorylation in the presence of these carriers was inhibited by DPIP in a manner analogous to the inhibition of phenazine methosulfate–mediated phosphorylation.

The Effect of DPIP on Photophosphorylation Coupled to TPN Reduction.—The addition of DPIP to reaction mixtures with TPN (Fig. 8) also induced a lag in the time course, indicating that the inhibitory effect of oxidized DPIP on ATP formation is a general one. However, in this case the rate of DPIP-mediated photophosphorylation was usually similar to, and sometimes higher than, the rate of ATP formation with TPN. The rate with mixtures of both oxidants was higher than the rate with each one by itself.

In all the cases described above the addition of oxidized indophenol dyes to either electron carriers or Hill oxidants always induced an initial lag of ATP formation. The length of this lag depended on the concentration of the oxidized dye, and hence could vary in different chloroplast preparations which main-

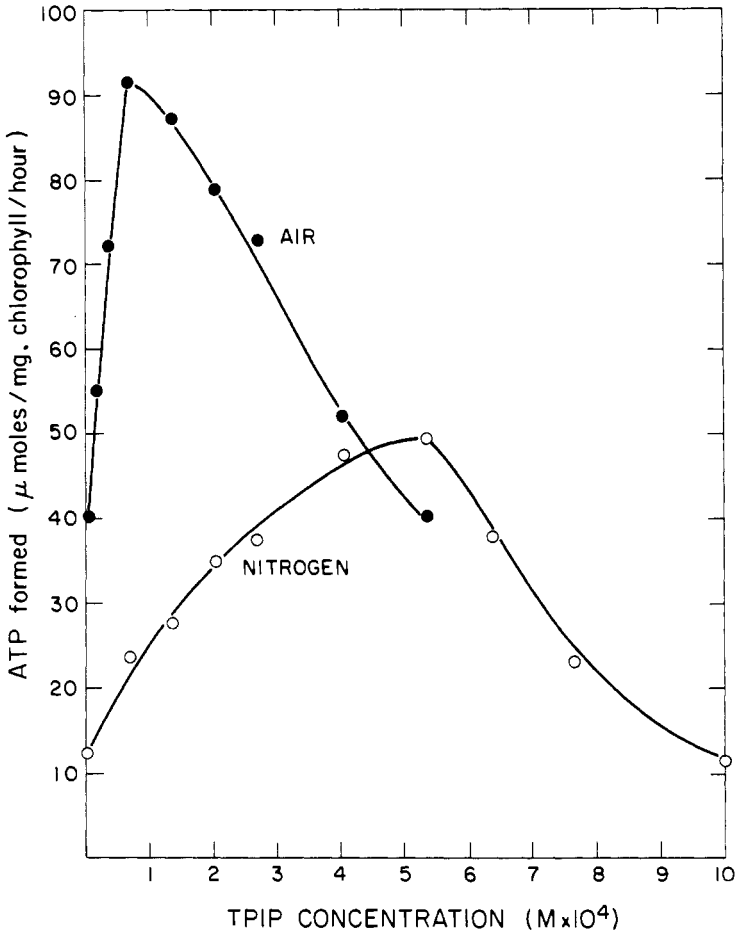

FIG. 2.—Concentration dependence of photophosphorylation with reduced TPIP under air and under nitrogen. The reaction mixture was as described under Methods and Materials and contained: once-washed chloroplasts containing 102 μg chlorophyll; ^{32}P, 6 × 10^5 cpm; ascorbate, 120 μmoles. Reaction time, 5 minutes; 1.0 ml was taken for ATP-^{32}P determination.

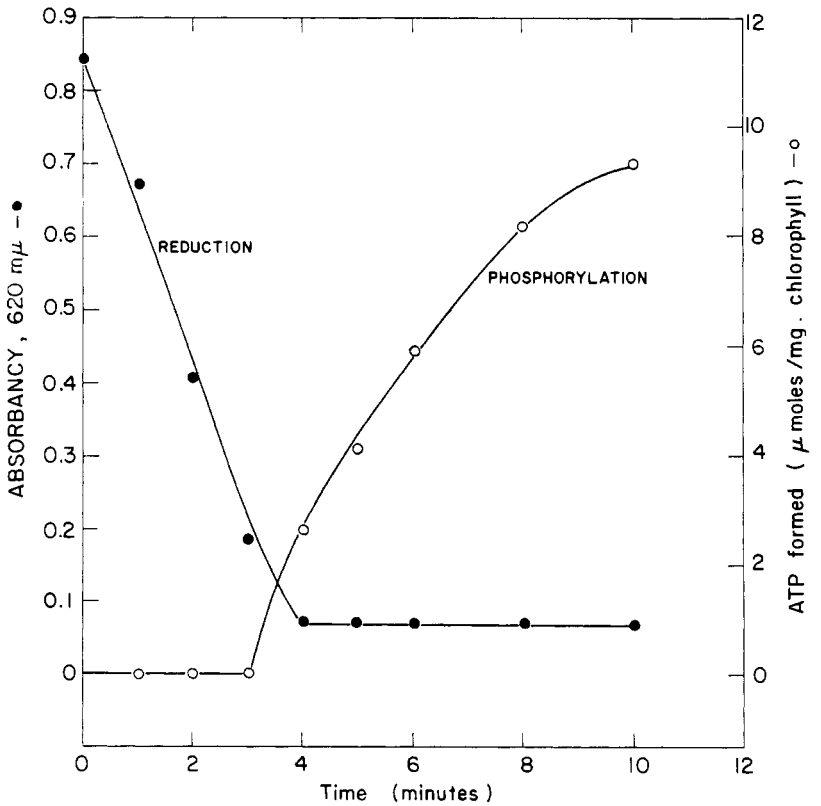

FIG. 3.—Time course of reduction and phosphorylation with oxidized TPIP. The reaction mixture was as described under Methods and Materials and contained: once-washed chloroplasts containing 132 μg chlorophyll; ^{32}P, 2 × 10^6 cpm; TPIP, 1.2 μmoles. Gas phase, nitrogen.

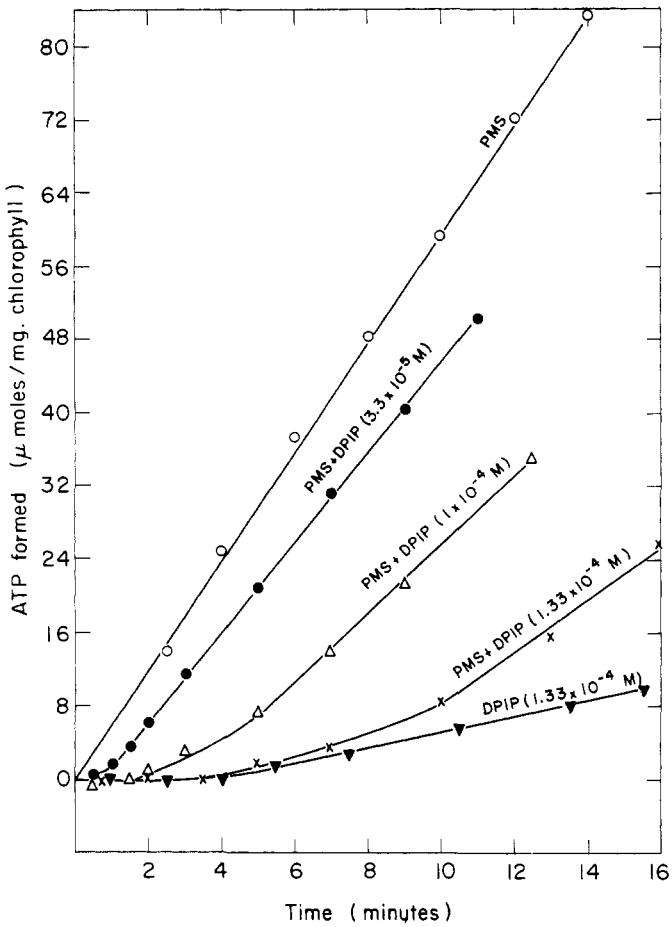

FIG. 4.—The effect of DPIP on photophosphorylation catalyzed by phenazine methosulfate (PMS). The reaction mixture was as described under Methods and Materials and contained: once-washed chloroplasts containing 42 μg chlorophyll; ^{32}P, 2×10^6 cpm; phenazine methosulfate, 0.2 μmole. Gas phase, nitrogen.

tain different rates of indophenol dye reduction, or with different amounts of chlorophyll.

The Effect of DPIP on Photophosphorylation Coupled to Ferricyanide Reduction.—The behavior of mixtures of ferricyanide and DPIP proved to be of special interest. Ferricyanide can reoxidize reduced DPIP nonenzymatically owing to the difference in their redox potentials (see also Losada *et al.*, 1961). Therefore the concentration of the oxidized DPIP added at the beginning of the experiment remained constant, and the inhibition of ATP formation was not overcome. This led to linear rather than the lag-type time-course curves obtained with other carriers or oxidants (Fig. 9). The slope of the curves decreased with increasing DPIP concentrations. In this case increasing amounts of chlorophyll did not change the slope of the curves, since the amount of ferricyanide used (five to forty times more than DPIP) was enough to compete even with the highest rates of DPIP reduction over the measured reaction periods.

Comparison of DPIP and TPIP.—The potency of DPIP and TPIP as uncouplers on the ferricyanide-dependent photophosphorylation was compared in a manner analogous to that described in Figure 9. It can be seen (Fig. 10) that 50% inhibition was attained at about 6×10^{-5} M DPIP or 6×10^{-6} M TPIP. Thus TPIP was about 10-fold more potent than DPIP in uncoupling photophosphorylation.

DISCUSSION

Indophenol dyes proved to be effective in three different ways in photophosphorylation. Under appropriate conditions they could effect either cyclic or noncyclic ATP formation or act as uncouplers of all photophosphorylations.

When photophosphorylation was measured starting with a high concentration of the oxidized dye, all three functions of the dye came into play. There was first a lag in ATP formation caused by inhibition by the uncoupling concentration of the oxidized dye. Later, as the dye was being reduced, the concentration of its oxidized form decreased, and consequently the lag in ATP formation was overcome. Then followed a period of noncyclic photophosphorylation accompanying the reduction of the nonuncoupling concentrations of the oxidized dye. Finally, when enough reduced dye had accumulated, cyclic ATP formation with the reduced form occurred (see Fig. 3).

These diverse functions complicated the interpretation of results of photophosphorylation with the indophenol dyes. However, by various treatments of the dyes their effects could be separated. Thus, by first reducing the dyes prior to illumination, a cyclic type of ATP formation occurred (Trebst and Eck, 1961; Avron, 1961; Gromet-Elhanan and Avron, 1963). On the other hand, the dyes could be kept oxidized throughout a whole experiment by adding a terminal electron acceptor. In this way, using low concentrations of the oxidized dyes, noncyclic photophosphorylation was obtained (Gromet-Elhanan and Avron, 1963). The use of high concentrations of the oxidized dyes in short-term experiments afforded an uncoupled system, where the dyes were continuously reduced at a high rate without ATP formation. DPIP and TPIP have been the most commonly used indo-

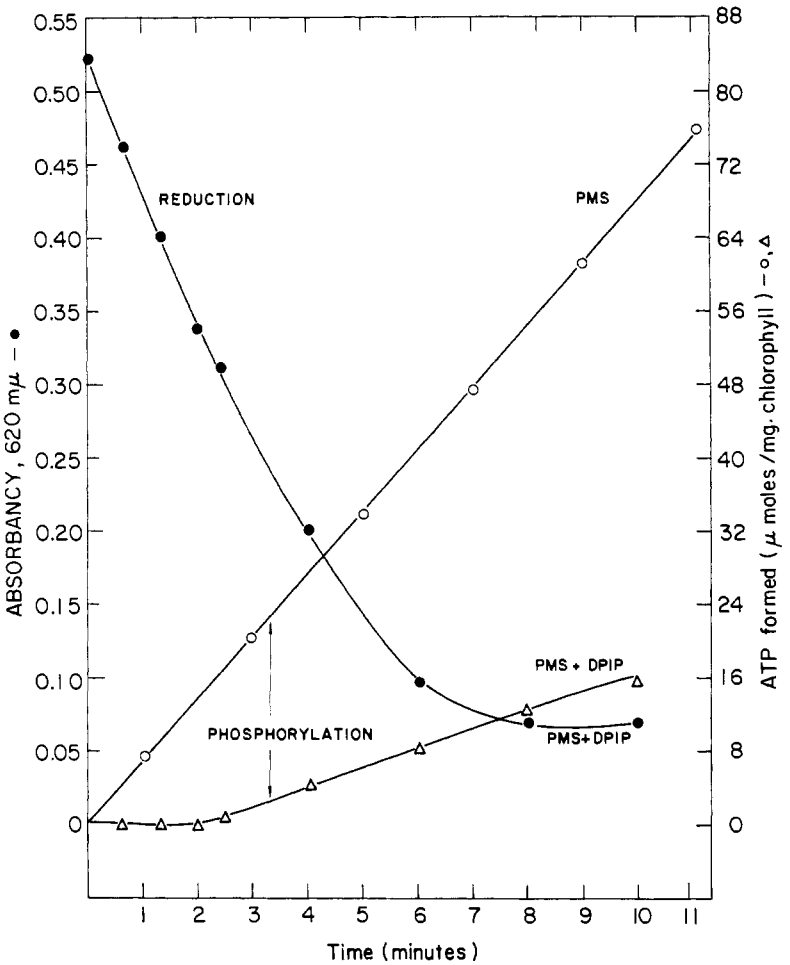

FIG. 5.—Time course of DPIP reduction during photophosphorylation catalyzed by phenazine methosulfate (PMS) plus DPIP. The reaction mixture was as described under Methods and Materials and contained: once-washed chloroplasts containing 50 μg chlorophyll; ^{32}P, 2 × 10^6 cpm; phenazine methosulfate, 0.2 μmole; DPIP, 0.8 μmole. Gas phase, nitrogen. The residual absorbancy of 0.07 is due to the chloroplasts.

phenol dyes. However, TPIP was found to be a much more potent inhibitor of photophosphorylation than DPIP (Fig. 10). This explains the effect of different concentrations of DPIP and TPIP previously reported (e.g., Avron and Jagendorf, 1959; Losada *et al.* 1961).

In the light of the results presented in this communication it can be concluded that the oxidized forms of the indophenol dyes are uncouplers, in the sense that they inhibit ATP formation without affecting the rate of electron transport. The uncoupling effect of the indophenol dyes is reversible, since the inhibition of ATP formation disappears when the oxidized dye is photoreduced in the reaction system.

Only a difference in the concentration of the oxidized form determines whether the dyes will act as uncouplers or mediate photophosphorylation. Therefore their uncoupling effect might be due to an unfavorable redox potential induced in the reaction system by the addition of the high concentrations of the oxidized dyes. Inhibition of phosphorylation reactions by unfavorable redox potentials were also observed by Newton and Kamen (1957), Wadkins and Lehninger (1959), Geller and Lipmann (1960), Vernon and Ash (1960), and Bose and Gest (1963).

The multiple function of the indophenol dyes in mediating or inhibiting photophosphorylation reactions sheds a new light on the interpretation of many of the results which have previously appeared. Thus, the notion that the reduction of indophenol dyes by chloroplasts is not accompanied by phosphorylation, was based on experiments using high concentrations of oxidized TPIP (e.g., Krogmann and Vennesland, 1959). It is evident that in these experiments the absence of ATP formation was due to the use of uncoupling concentrations of oxidized TPIP (Fig. 10). The accepted erroneous interpretation of such experiments led Vernon (1962) to suggest a special site for the reduction of TPIP and to exclude the possibility of ATP formation along the pathway leading to oxygen evolution and TPIP reduction. It is evident that these conclusions must be revised.

Losada *et al.* (1961, Table 3) reported that "the addition of indophenol dyes to the photophosphorylation system with TPN did not suppress photophosphorylation and has, in fact, slightly increased it." However, since they used high concentrations of chloroplast fragments and measured the photophosphorylation only at one time after 12 minutes, they must have overlooked the inhibitory effect of the indophenol dyes. In a time-course experiment conducted under conditions similar to those used by Losada *et al.*, we found the same general features seen in Figure 8. The effects of the higher chlorophyll concentration were exhibited only in a lower specific activity and in a shorter lag period. It should also be pointed out that it is impossible to discern at the present time whether the phosphorylation obtained in the presence of both an indophenol dye and TPN was due to that coupled to TPN reduction, to that

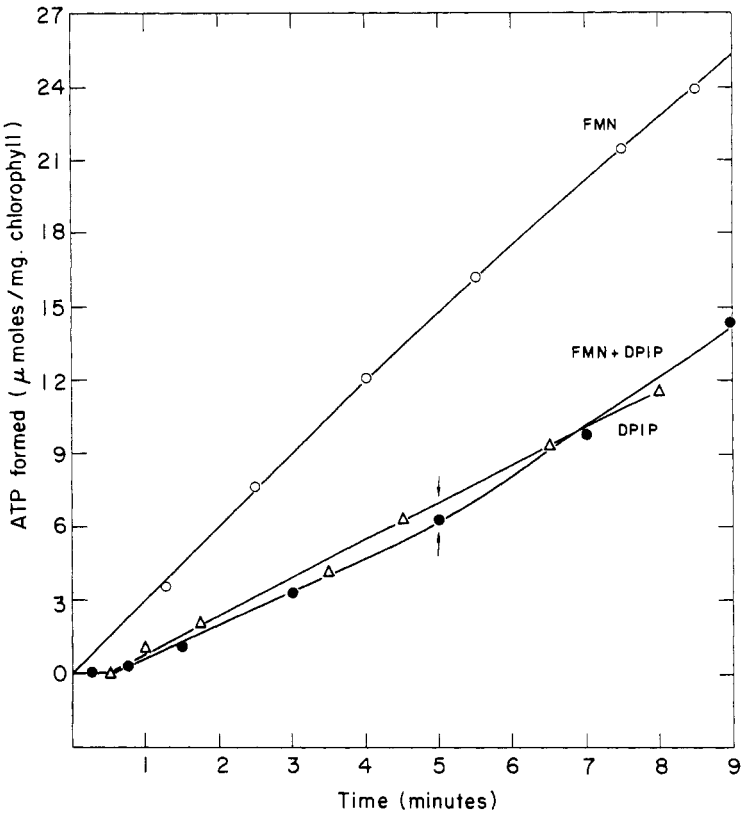

FIG. 6.—The effect of DPIP on photophosphorylation mediated by flavin mononucleotide (FMN). The reaction mixture was as described under Methods and Materials and contained: once-washed chloroplasts containing 60 μg chlorophyll; ^{32}P, 2×10^7 cpm; FMN, 0.6 μmole; DPIP, 0.8 μmole. Gas phase, nitrogen. The arrow designates the approximate time of color disappearance.

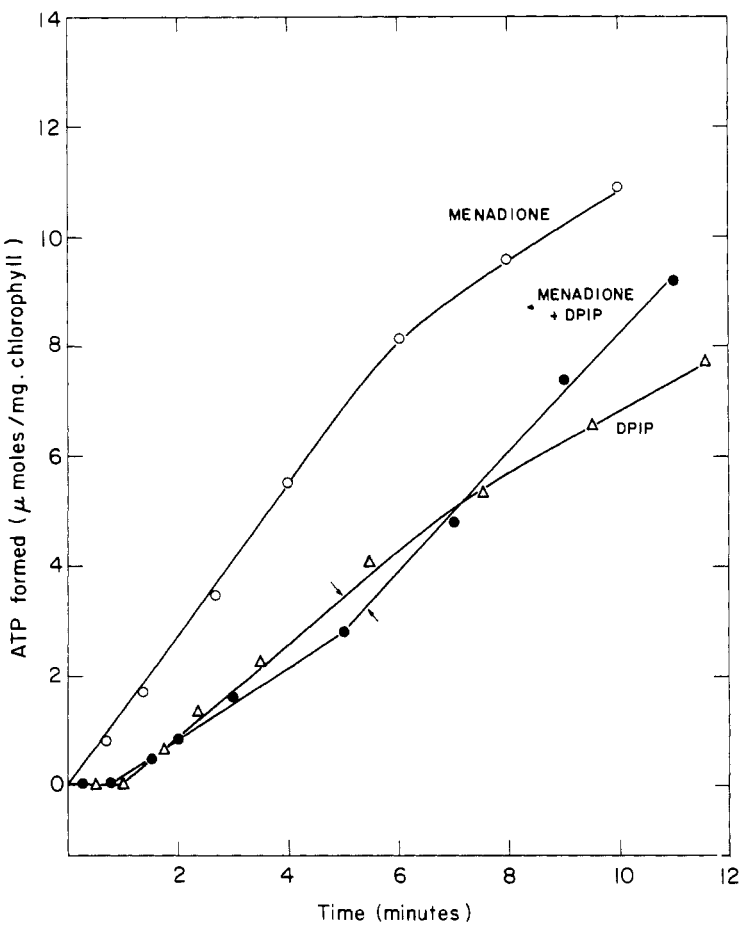

FIG. 7.—The effect of DPIP on menadione-catalyzed photophosphorylation. The reaction mixture was as described under Methods and Materials and contained: once-washed chloroplasts containing 40 μg chlorophyll; ^{32}P, 1.5×10^7 cpm; menadione, 0.2 μmole; DPIP, 0.6 μmole. Gas phase, nitrogen. The arrow denotes the approximate time of color disappearance.

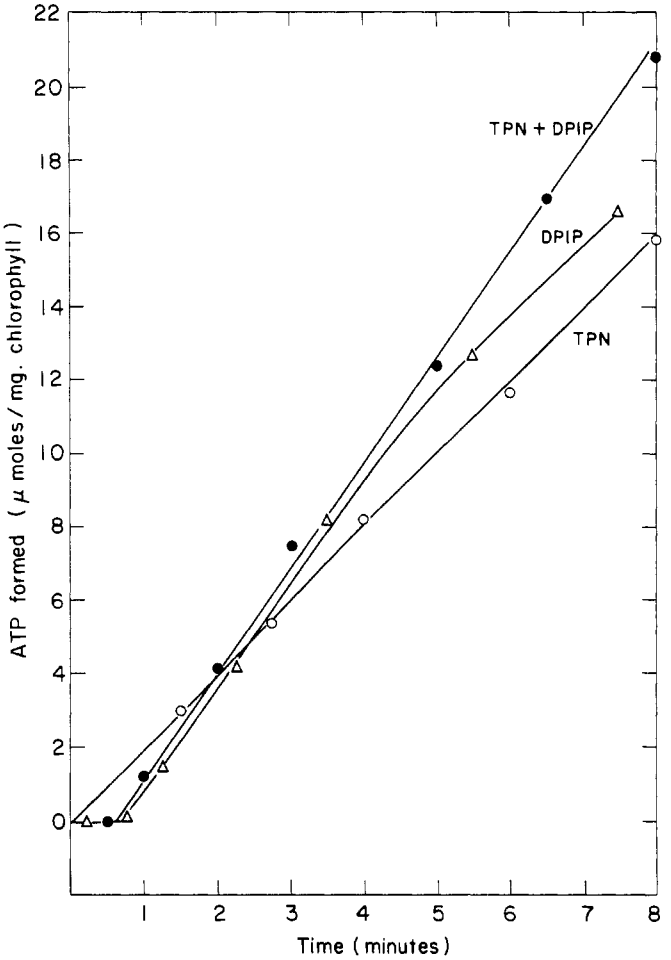

FIG. 8.—The effect of DPIP on TPN-mediated photophosphorylation. The reaction mixture was as described under Methods and Materials and contained: once-washed chloroplasts containing 40 μg chlorophyll; ^{32}P, 1×10^7 cpm; TPN, 4 μmoles; DPIP, 0.6 μmole. Gas phase, nitrogen. The color of DPIP disappeared in between 3 and 4 minutes.

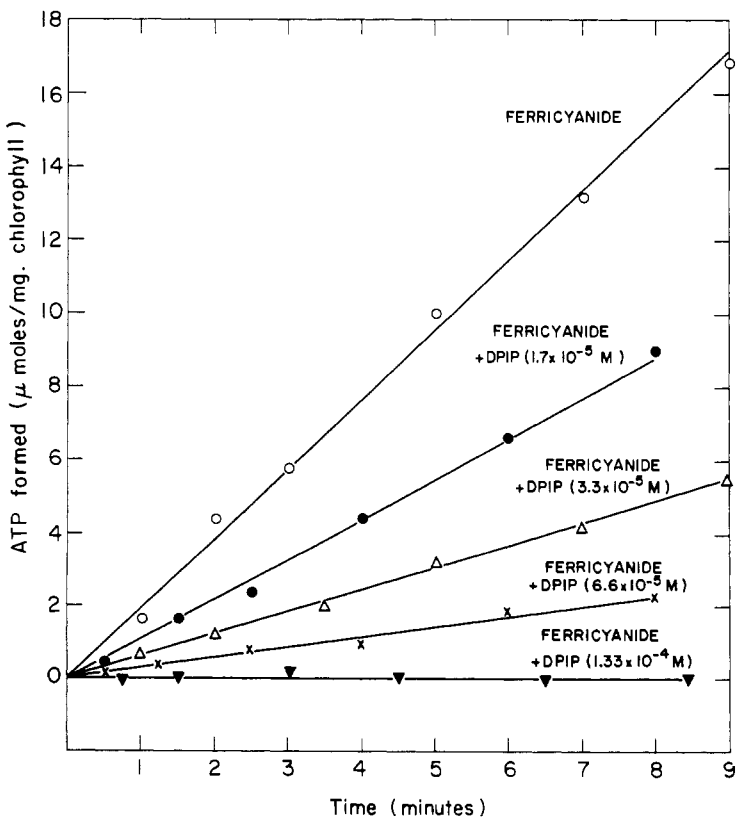

FIG. 9.—The effect of DPIP on ferricyanide-mediated photophosphorylation. The reaction mixture was as described under Methods and Materials and contained: once-washed chloroplasts containing 66 μg chlorophyll; ^{32}P, 3.5×10^6 cpm; ferricyanide, 4 μmoles. Gas phase, nitrogen.

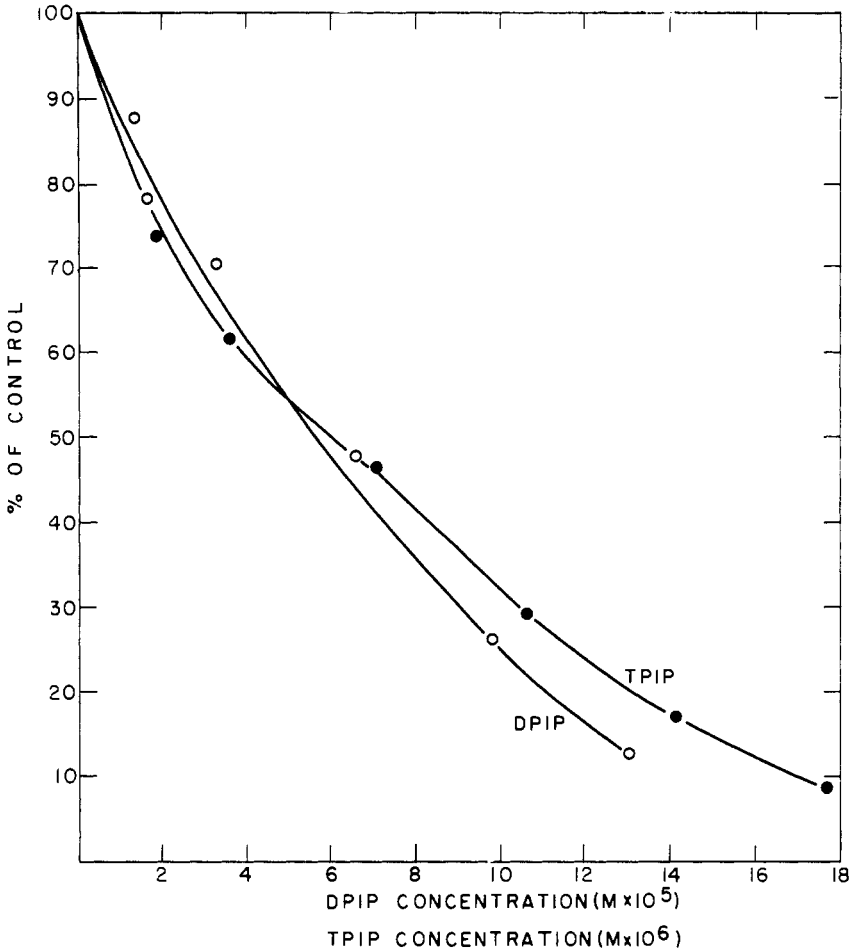

FIG. 10.—A comparison of the effect of DPIP and TPIP on ferricyanide-mediated photophosphorylation. The reaction mixture was as described under Methods and Materials and contained: once-washed chloroplasts containing 50 μg chlorophyll; ^{32}P, 1 $\times$ 10^7 cpm; ferricyanide, 4 μmoles. Gas phase, air. Reaction time, 3 minutes.

mediated by the indophenol dye, or to both. This also holds for the restoration of phosphorylating activity by the addition of DPIP and ascorbate to a TPN-photoreducing system poisoned by *p*-chlorophenyldimethylurea (Table 1, Losada *et al.*, 1961). The above interpretations rather strongly modify the conclusions of Losada *et al.* regarding the position of the phosphorylating step and the effect of indophenol dyes on TPN or ferricyanide-mediated phosphorylations.

Krogmann and co-workers (Krogmann, 1958, 1960; Krogmann and Vennesland, 1959) have shown that indophenol dyes can mediate an oxygen-dependent photophosphorylation. They suggested that "the photophosphorylation . . . occurs when the reduced dye is oxidized and not during the photoreduction of the dye." Figures 1 and 2 in this communication were performed essentially under the conditions described by Krogmann and Vennesland (1959). It is evident that there was always sufficient time in their experiments to overcome the lag in ATP formation. It seems, therefore, that the results of Krogmann and co-workers, when considered in the light of our findings, do not support their postulation of a new type of photophosphorylation which is coupled to the oxidation of reduced indophenol dyes. The oxygen dependence observed in their experiments was probably due to the different concentration dependence in air or nitrogen of cyclic phosphorylation mediated by indophenol dyes (see Fig. 2). This interpretation agrees with their findings that 3-(3,4-dichlorophenol)-1,1-dimethylurea did not inhibit the reaction in the presence of reducing agents (see also Table 3 of Gromet-Elhanan and Avron, 1963).

In the light of the presently available evidence, and assuming only one site for ATP formation, the previously proposed schemes of photophosphorylation (e.g. Losada *et al.*, 1961; Vernon, 1962), should be modified in two main points: (a) The site of ATP formation should be relocated prior to the site of indophenol dye reduction, and (b) the site of ferricyanide reduction should be equivalent to that of the indophenol dyes, rather than to that of TPN. These proposed alterations are based mainly on the following experimental observations: (1) ATP formation is coupled to the photoreduction of indophenol dyes (Gromet-Elhanan and Avron, 1963). (2) The photoreduction of indophenol dyes and most probably also ferricyanide involves only a part of the electron transport system which participates in TPN photoreduction (Levine and Volkmann, 1961; Levine and Smillie, 1962; Govindjee *et al.*, 1962; Mayne and Brown, 1963; Allen and Murchio, 1963; see also Bishop and Whittingham, 1963). (3) The participation of two photochemical reactions in the photoreduction of TPN (Govindjee *et al.*, 1962; Amesz and Duysens, 1962).

ADDED IN PROOF

After submission of this article for publication, two papers appeared which confirm and extend some of

the observations reported herein: (1) Keister, D. L. (1963), *J. Biol. Chem. 238*, PC2590. (2) Shen, G. M., Yang, S. Y., Shen, Y. K., and Yin, H. C. (1963), *Sci. Sinica Peking 12*, 685.

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Studies on the Nonenzymic Hydrogen Exchange Between Nicotinamide Adenine Dinucleotides*

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The components of the couple NAD^+-NADH interact with each other. Similar interactions were demonstrated with the components of $NADP^+$-NADPH and (1-*n*-propyl nicotinamide chloride)-(1-*n*-propyl-1,4-dihydronicotinamide) systems. In each case, there is a nonstereospecific hydrogen exchange with no net oxidation-reduction and the formation of a reversible complex. Another colored complex is formed also, but there is no evidence that the formations of these two complexes are dependent upon each other.

Of the numerous enzyme-catalyzed reactions involving NAD^+ and $NADP^+$, the transhydrogenase systems are of biological importance (Colowick *et al.*, 1952; Kaplan, 1961). In these reactions, a direct and stereospecific hydrogen exchange is observed (San Pietro *et al.*, 1955). Among the nonenzymic reactions, the reduction of 1-benzyl-3-acetylpyridinium chloride by NADH and NADPH was described as a model system for the transhydrogenases (Cilento, 1960). A direct and nonstereospecific hydrogen transfer was demonstrated in the nonenzymic reduction of the 3-acetylpyridine analog of NAD^+ by NADH (Spiegel and Drysdale, 1960; Drysdale *et al.*, 1961). The observed oxidation-reduction was attributed to the reactivity of the 1-substituted pyridinium ring for hydride abstraction. Similar systems, wherein a net difference between the E_0 values of the couple dinucleotide systems was observed, were suspected to exchange hydrogen with a net oxidation-reduction. Thus a mixture of NADH and 2-aldehydepyridine adenine dinucleotide gave similar results to those found with the 3-acetylpyridine adenine dinucleotide and 1-benzyl-1,4-dihydronicotinamide reduced 1-methyl-3-acetyl pyridinium iodide (Spiegel and Drysdale, 1960). Recently, PrNDH[1] was shown to rapidly reduce NAD^+ to NADH (Ludowieg and Levy, 1962). While

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[1] Abbreviations: NAD^+(T), NADH(T), oxidized and reduced nicotinamide adenine dinucleotide containing tritium at C-4 of the nicotinamide ring; $NADP^+$(T), NADPH(T), oxidized and reduced nicotinamide adenine dinucleotide phosphate containing tritium at C-4 of the nicotinamide ring; $PrND^+$, 1-*n*-propyl nicotinamide as a cation; $PrND^+$-iodide and $PrND^+$-chloride as halides of $PrND^+$; $PrND^+$(T), 1-*n*-propyl nicotinamide with tritium at C-4 as a cation; $PrND^+$(T)-iodide and $PrND^+$(T)-chloride as halides of $PrND^+$(T); PrNDH, 1-*n*-propyl-1,4-dihydronicotinamide; PrNDH(T), tritium labeled PrNDH at C-4; Tris, tris(hydroxymethyl)aminoethane; DEAE-cellulose, diethylaminoethyl-cellulose; GSSG, GSH, oxidized and reduced glutathione.