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Effect of Alkyldinitrophenols on Photophosphorylation in Chloroplasts*

K. S. Siow and A. M. Unrau

ABSTRACT: Both 2,4- and 2,6-dinitrophenol are known to exhibit uncoupling effects at lower pH, and inhibition of electron transport at higher pH. Alkylated dinitrophenols are much more effective both as uncouplers and inhibitors of photophosphorylation. Lipid solubility as well as dissociation were found to be key factors associated with the effect of alkyldinitrophenols on photophosphorylation in chloroplasts. The rate of adenosine triphosphate formation was essentially proportional to electron transport if the nonphosphorylating rate is substracted from the rate obtained in phosphorylating systems regardless of the over-all rate of reaction. This is interpreted as meaning that the phosphorylation reaction is exclusively associated with that part of electron transport stimulated by the presence of phosphorylating reagents. The stoichiometry (P/2e ratio) of the phosphorylation process therefore is probably 2.0. Since the inhibition of electron transport and its associated adenosine triphosphate formation by dinitrophenols is more severe as the light intensity is lowered, the site of inhibition by these compounds is assumed to be at or very close to the photoinduced reaction step.

2,4-Dinitrophenol is undoubtedly one of the best known uncouplers of oxidative phosphorylation although much stronger uncouplers are now available. In contrast, photophosphorylation was first shown to be relatively insensitive to this compound (Arnon *et al.*, 1954; Avron, 1960; Krogmann *et al.*, 1959; Whatley *et al.*, 1959) and it was at one time claimed that it was not an uncoupler but only an inhibitor of electron transport for chloroplasts. This led to the earlier speculations that the mechanisms for the electron transport coupled energy transfer in oxidative phosphorylation and photophosphorylation were basically different (Jagendorf, 1959). The observation of a true uncoupling effect of 2,4-dinitrophenol in photophosphorylation at lower pH values (Neumann and Jagendorf, 1964) and of a similar effect of 4-isooctyl-2,6-dinitrophenol (Baltscheffsky, 1965) seems to indicate that, at least to some extent, other factors may be involved in the action of dinitrophenols on chloroplast systems. In some earlier experiments in our laboratory involving carrot disks, a rather distinctly different action pattern was observed for alkylated 2,4- and 2,6-dinitrophenols. These observations prompted a more detailed investigation on the effect of some alkylated 2,4- and 2,6-dinitrophenols on photophosphorylation.

Experimental Section

Chemicals. The compounds used in this investigation were as follows: 2,4-dinitrophenol, 6-(1,2-dimethylbutyl)-2,4-dinitrophenol, 6-(1-methylpentyl)-2,4-dinitrophenol, 2,6-dinitrophenol, 4-(1,2-dimethylbutyl)-2,6-

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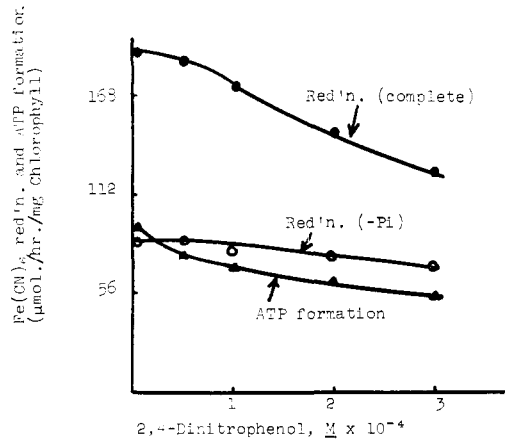

FIGURE 1: Effect of 2,4-dinitrophenol on photophosphorylation and ferricyanide reduction at pH 7.6 and saturating light intensity.

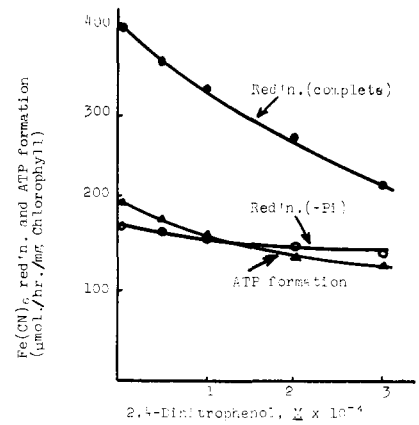

FIGURE 2: Effect of 2,4-dinitrophenol on photophosphorylation and ferricyanide reduction at pH 8.3 and saturating light intensity.

TABLE I: Physical Constants of Dinitrophenols.

Compound	p*K*	p*Q*
2,4-Dinitrophenol	4.1	−2.15
2,6-Dinitrophenol	3.7	−2.43
6-(1,2-Dimethylbutyl)-2,4-dinitrophenol	4.5	−4.56
6-(1-Methylpentyl)-2,4-dinitrophenol	4.6	−4.63
4-(1,2-Dimethylbutyl)-2,6-dinitrophenol	4.2	−4.45
4-(1-Methylpentyl)-2,6-dinitrophenol	4.2	−4.50

dinitrophenol, and 4-(1-methylpentyl)-2,6-dinitrophenol. The compounds were dissolved in methanol–ethylene glycol (2:3, v/v) to give solutions of desired concentrations and the final concentration of the above solvent mixture in the reaction mixture was constantly less than 2.5% by volume.

Chloroplasts. Whole chloroplasts were isolated from fresh spinach leaves. Spinach plants were grown under controlled light intensity, day length, and temperature in growth chambers. The isolation procedure and buffer systems were those found in the literature (Good *et al.*, 1966). Chlorophyll content was determined using a published procedure (Arnon, 1949).

Reaction Conditions. The standard reaction mixture contained whole chloroplasts (45 μg of chlorophyll and the following components in a total volume of 3 ml: sucrose, 600 μM; Tricine buffer adjusted to desired pH, 120 μM; $Na_2H^{32}PO_4$, 30 μM; ADP,[1] 3μM; $MgCl_2$, 15 μM; and $K_3Fe(CN)_6$, 1.2 μM. The basal reaction mixture contained the same components except $Na_2H^{32}PO_4$. The reaction mixture was routinely preincubated in a water bath (20°) in the dark before the light source was turned on. The intensity of light after passing through a red filter (transmission > 580 mμ) was well above the saturation level since increasing the intensity (removal of filters) gave no increase in reaction rate.

Reduction of Ferricyanide. The reduction of ferricyanide was followed spectrophotometrically by measuring the absorbance of the entire reaction mixture at certain time intervals at 420 mμ. Absorbancy changes were corrected using a dark control.

ATP Assay. The formation of ATP was assayed using a published procedure (Avron, 1960). The procedure basically involves measurement of $^{32}P_i$ incorporation into ATP.

Determination of Dissociation Constants. The p*K* values for the alkyl as well as parent dinitrophenols were determined using a published procedure (Blackman *et al.*, 1955) and are summarized in Table I.

Determination of Lipophilic Properties of Phenols. An estimation of the partitioning of the undissociated forms of the phenols was made in xylene–water systems. Aqueous solutions of the compounds, buffered with 0.2 M

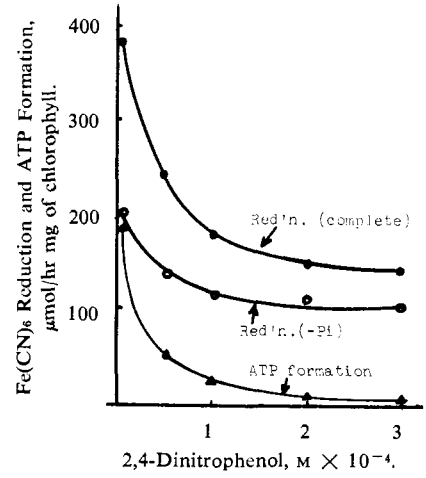

FIGURE 3: Effect of 2,4-dinitrophenol on photophosphorylation and ferricyanide reduction at pH 9.0 and saturating light intensity.

[1] Abbreviations as listed in *Biochemistry 5*, 1445 (1966).

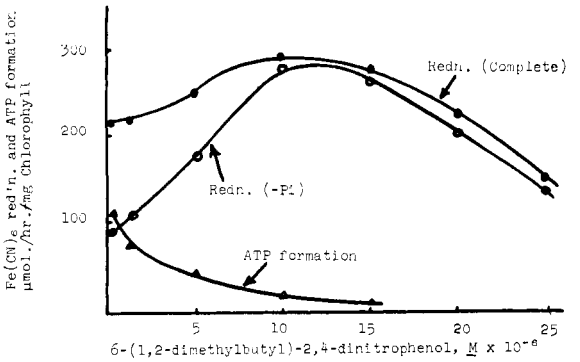

FIGURE 4: Effect of 6-(1,2-dimethylbutyl)-2,4-dinitrophenol on photophosphorylation and ferricyanide reduction at pH 7.6 and saturating light intensity.

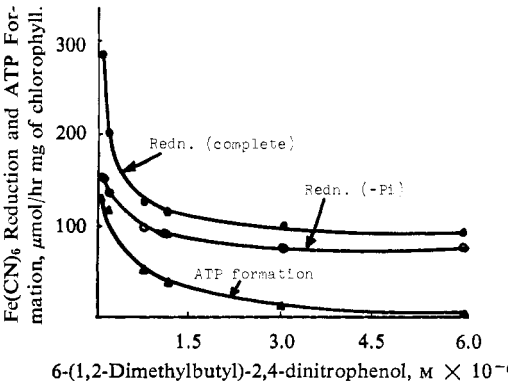

FIGURE 6: Effect of 6-(1,2-dimethylbutyl)-2,4-dinitrophenol on photophosphorylation and ferricyanide reduction at pH 9.0 and saturating light intensity.

phosphate buffer, were shaken for 3 hr at 25° with an equal volume of xylene. The concentration of total phenol in the aqueous layer was estimated spectrophotometrically before and after equilibration and the partition coefficient, Q, was calculated in terms of pQ as follows: pQ = $-\log Q$ = $-\log$ (concentration of undissociated phenol in xylene/concentration of undissociated phenol in water). The pQ's of the compound are summarized in Table I.

Results and Discussion

Figures 1–3 show the effect of 2,4-dinitrophenol on ferricyanide reduction in the presence or absence of simultaneous phosphorylation, and on the amount of ATP formed in the phosphorylating reaction. The result for 2,6-dinitrophenol was essentially the same (rate plots not shown). The two compounds inhibit ATP formation at a similar concentration range and inhibit ferricyanide reduction either in the presence or absence of phosphorylation. At all the pH values tested, inhibition of electron transport was almost as high as that observed during ATP formation in the phosphorylating Hill reaction. These results are consistent with those reported (Krogmann *et al.*, 1959) for 2,4-dinitrophenol at pH 7.8. Thus, if one considers the results of each pH separately, these compounds unquestionably behave as inhibitors of electron transport for chloroplasts at the particular pH values.

Results with two representative alkyl-substituted phenols are shown in Figures 4–11. As may be seen these results appear to be quite different from those with the parent phenols, but in each series, the two derivatives behave in exactly the same manner. In the case of the 2,4 series, the substituted compounds are about 60-fold more effective than the parent compound; at pH 7.6, they completely inhibit ATP formation at a concentration of about 10^{-5} M in either case (Figures 4 and 7), while 2,4-dinitrophenol itself causes an inhibition of

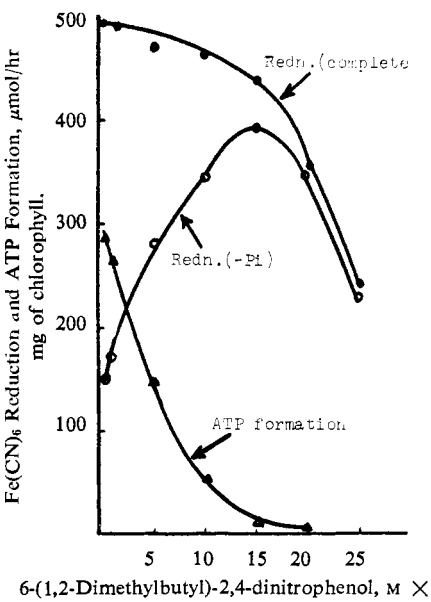

FIGURE 5: Effect of 6-(1,2-dimethylbutyl)-2,4-dinitrophenol on photophosphorylation and ferricyanide reduction at pH 8.3 and saturating light intensity.

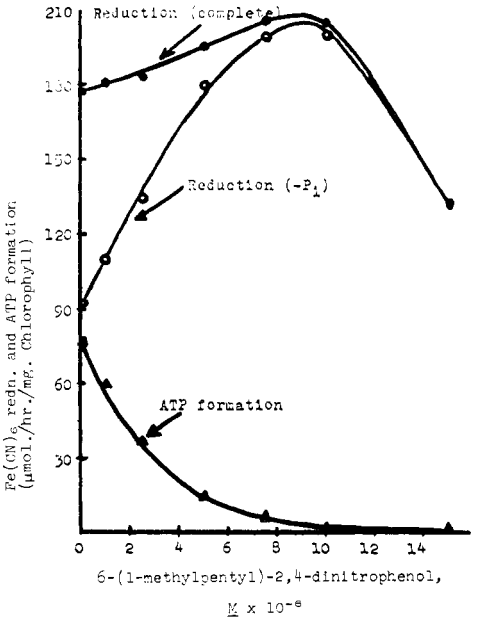

FIGURE 7: Effect of 6-(1-methylpentyl)-2,4-dinitrophenol on photophosphorylation and ferricyanide reduction at pH 7.6 and saturating light intensity.

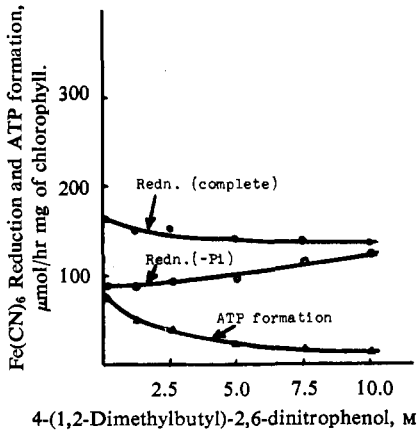

FIGURE 8: Effect of 4-(1,2-dimethylbutyl)-2,6-dinitrophenol on photophosphorylation and ferricyanide reduction at pH 7.6 and saturating light intensity.

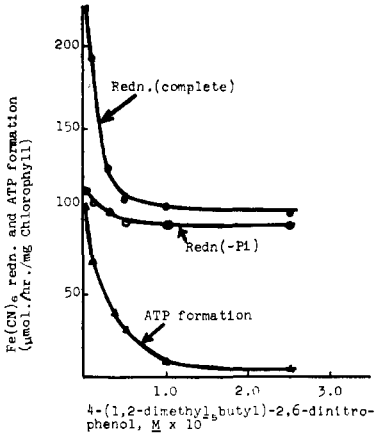

FIGURE 10: Effect of 4-(1,2-dimethylbutyl)-2,6-dinitrophenol on photophosphorylation and ferricyanide reduction at pH 9.0 and saturating light intensity.

phosphorylation by about 40% at a concentration of 3×10^{-4} M (Figure 1). At the same pH (7.6), they also stimulate electron transport in both phosphorylation and nonphosphorylation. At the concentration at which ATP formation is completely inhibited, the maximal stimulation of the nonphosphorylating electron transport is increased to 290% (Figure 4) and 210% (Figure 7) of the control rates for the 6-(1,2-dimethylbutyl) and 6-(1-methylpentyl) derivatives, respectively. Thus, the most crucial test for the definition of uncoupling, simultaneous stimulation of electron transport and inhibition of ATP formation, is fulfilled by the action of these compounds at pH 7.6. Their uncoupling effect, however, is decreasing with increasing pH and at the same time their inhibitory effect becomes more pronounced. Thus at pH 8.3, while the nonphosphorylating electron transport is still stimulated, the phosphorylating electron transport becomes slightly inhibited, while at pH 9.0, both electron transport systems are inhibited. However, even in the latter case, the inhibition of electron transport is not as great as the inhibition of ATP formation.

A similar activity is exhibited by the alkylated compounds in the 2,6 series (only figures for pH 7.6 are shown). These compounds, however, are less effective than the corresponding compounds in the 2,4 series, but they are still much more effective than the parent compound. Moreover, even at pH 7.6, they stimulate only the nonphosphorylating but not the phosphorylating electron transport (Figures 8 and 11). 4-Isooctyl-2,6-dinitrophenol has been shown (Baltscheffsky, 1965) to exhibit uncoupling action. The compound at pH 8.0 inhibited 90% of ATP formation but only 50% of electron transport at 10^{-4} M concentration.

Table I summarizes the physical constants (pK and pQ) of the dinitrophenols used. In all cases, the uncoupling effect increases with decreasing pH, which indicates that the active species in uncoupling is the acidic form of the compound over the pH range used. This correlation does not, however, hold over an extended range. Similarly the inhibitory effect, which increases with in-

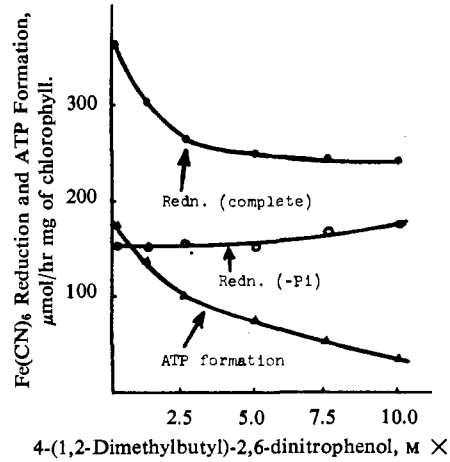

FIGURE 9: Effect of 4-(1,2-dimethylbutyl)-2,6-dinitrophenol on photophosphorylation and ferricyanide reduction at pH 8.3 and saturating light intensity.

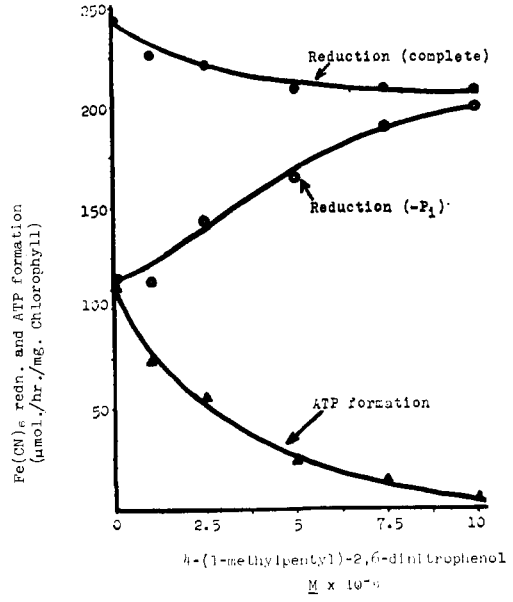

FIGURE 11: Effect of 4-(1-methylpentyl)-2,6-dinitrophenol on photophosphorylation and ferricyanide reduction at pH 7.6 and saturating light intensity.

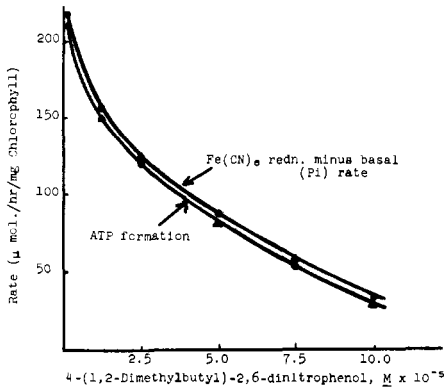

FIGURE 12: Correspondence of ATP formation to excess ferricyanide reduction (over basal, nonphosphorylating process) at various concentrations of 4-(1,2-dimethylbutyl)-2,6-dinitrophenol.

creasing pH, is also due to its dissociated form within the pH range used. One may deduce for each compound a particular pH at which the acidic form and the dissociated form of the compound exist in such a ratio that the uncoupling effect and the inhibitory effect are in equilibrium, *i.e.*, a point at which neither stimulation nor inhibition of basal electron transport will occur. This pH point may be referred to as the null point. A point like this was found to exist in all cases in the present study and is concentration independent. For 2,4- and 2,6-dinitrophenol, this point may be found around pH 7.6, and for all substituted phenols, a sharp transition from uncoupling to inhibition occurs between pH 8.3 and 9.0.

The uncoupling effect of dinitrophenol is enhanced by the addition of an alkyl group to the phenyl group of the compound. The addition of an alkyl group results in modifying the property of the compound in two ways. It reduces the acidity of the compound and also increases the lipid solubility of the acidic form of the compound. It should be noted that even though 2,4-dinitrophenol has a p*K* close to those of the 4-alkyl-2,6-dinitrophenols, it behaves as an inhibitor at the pH values at which the substituted phenols are actually uncouplers. These facts indicate that it is not only p*K*, but

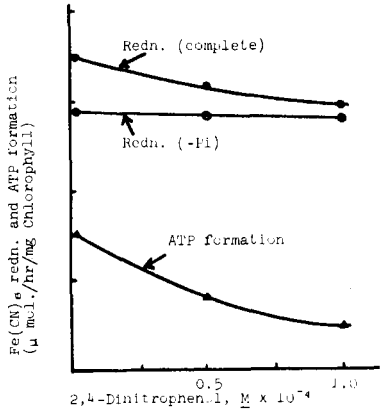

FIGURE 13: Effect of 2,4-dinitrophenol on photophosphorylation and ferricyanide reduction at low light intensity and pH 8.3.

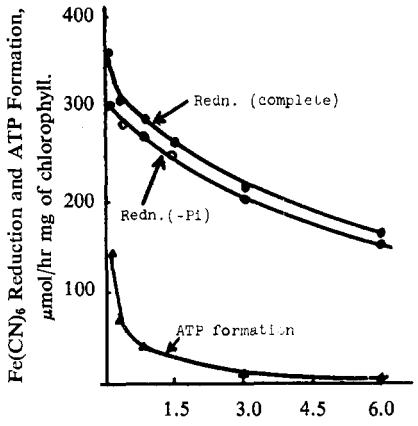

FIGURE 14: Effect of 6-(1,2-dimethylbutyl)-2,4-dinitrophenol on photophosphorylation and ferricyanide reduction at low intensity and pH 8.3.

also the partitioning characteristics of these compounds that determine whether or not a compound will show a clear uncoupling action at lower pH values. The stronger stimulation of mitochondrial ATPase (Hemker, 1962) of substituted nitrophenols at lower pH values was interpreted as due to greater solubility of the undissociated molecule in the mitochondrial membranes. This indicates that the above consideration for chloroplast systems is quite reasonable.

The Stoichiometry of Photophosphorylation. The basic assumption in the evaluation of the stoichiometry was if phosphorylation and ferricyanide reduction were the consequence of a single process, then the stoichiometry should be constant regardless of the over-all rate of reaction. In all cases, it was found that the rate of ATP formation was directly related to electron transport if the nonphosphorylating rate is subtracted from the rate obtained in a phosphorylating rate system. This is evident in that in all figures shown (Figures 1–11) the area covered between the two curves of ferricyanide reduction (complete and $-P_i$) is approximately equal to

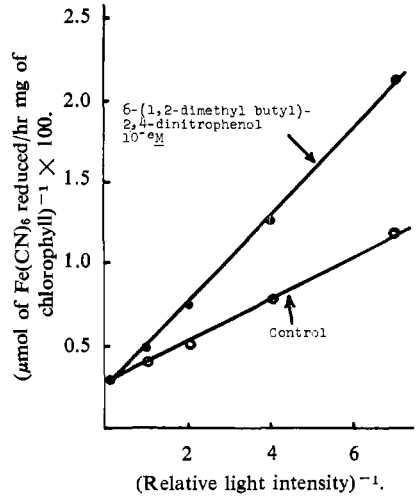

FIGURE 15: Reciprocal plot of relative rate of ferricyanide reduction *vs.* light intensity with and without 6-(1,2-dimethylbutyl)-2,4-dinitrophenol.

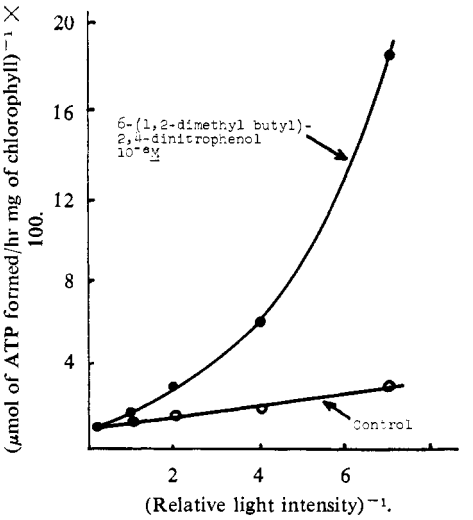

FIGURE 16: Reciprocal plot of relative rate of photophosphorylation *vs.* light intensity with and without 6-(1,2-dimethylbutyl)-2,4-dinitrophenol.

that under the curve of ATP formation. Figure 12 (replotted from Figure 9) more clearly shows that the curve of ATP formation and that of ferricyanide reduction "corrected" for the basal, nonphosphorylating reaction are almost identical. Thus the number of moles of ATP formed and that of ferricyanide reduced in excess of the basal reaction coincide almost exactly regardless of the rate of reaction. These results in conjunction with the above basic assumption suggest that the phosphorylation reaction is exclusively associated with that part of electron transport stimulated by the presence of phosphorylating reagents. If this is the case, the stoichiometry (P/2e ratio) of the phosphorylation process will be 2.0, *i.e.*, an average of one ATP molecule formed for each electron transferred, and confirms the stoichiometry reported by Good and Izawa (1967) and earlier by Good (1960).

It is important to note that the method of correcting for the nonphosphorylating electron transport is valid only under the conditions that the rates of electron transport are limited by the phosphorylating part of the electron-transport chain. As may be seen in Figures 13 and 14, which show the results of experiments at low light intensity, when light or possibly other factors involved in the supply of electrons become limiting, there will be a competition for electrons between the phosphorylating and nonphosphorylating part of the electron-transport chain, and as a result, the over-all electron transport will no longer be simply the sum of the two transport systems. In such cases, the corrected method is not valid, and it is not surprising that the P/2e ratio so calculated will become unreasonable.

The Relation of Light Intensity and Effect of Dinitrophenols. The effects of dinitrophenols on photophosphorylation has been found to be a function of light intensity. As may be seen by comparison of Figures 2 and 13, 2,4-dinitrophenol at a concentration of 10^{-4} M inhibits ATP formation by about 20% at high light (saturating) intensity, whereas it causes about 65% inhibition at low light intensity. Similarly, inhibition by 6-(1,2-dimethylbutyl)-2,4-dinitrophenol (Figures 5 and 14) is also more severe as the light intensity is lowered. Figures 15 and 16 show that this compound at 10^{-6} M concentration inhibits phosphorylating electron transport (Figure 15) and its accompanied ATP formation (Figure 16) as a function of light intensity in a manner which leads to the the same V_{max} as that of the control, but with a much greater slope. These results thus suggest that inhibition by dinitrophenols is at or very close to the light reaction step.

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