Effect of Uncouplers on the Light-induced pH Rise with Spinach Chloroplasts*

ANDRE T. JAGENDORF AND JOSEPH NEUMANN†

From the McCollum-Pratt Institute and Biology Department, The Johns Hopkins University, Baltimore, Maryland 21218

(Received for publication, October 19, 1964)

A readily reversible rise in pH of the medium occurs when unbuffered spinach chloroplasts are illuminated, especially when the initial pH during incubation in the dark is at or below 6.5 (1, 2). This rise in pH had been predicted from an examination of the chemiosmotic mechanisms for phosphorylation detailed by Mitchell (3). It was correlated, in at least a preliminary way, with the existence in the same chloroplasts of a light-induced high energy (nonphosphorylated) state designated X₈, a possible intermediate in the complete photophosphorylation mechanism (1, 4, 5). It is likely that the pH rise is closely related to a light-driven efflux of potassium ions recently described by Dilley (6).

The maximum extent of the pH rise seems to be determined by an equilibrium between two opposing reactions: light-dependent formation and a light-independent decay. The latter can be measured directly during a postillumination period. It is of interest to see whether inhibitory reagents affect the yield by depressing the rate of formation or by increasing the rate of the back reaction. Addition of the inhibitor in a postillumination period gives conclusive evidence as to whether decay is affected or not, but the kinetics of formation in itself provides only indirect evidence as to whether the true formation rate is affected or not.

It was found previously (2) that the initial rate of the pH rise was too rapid for quantitative determination by means of the pH-stat. It will be shown here that the direct recording of the pH can be used for this purpose, with a fair degree of reliability. Within the limitations of the techniques, the various uncouplers have been analyzed as to their mode of action.

MATERIALS AND METHODS

Chloroplasts were prepared from spinach leaves by grinding and washing in buffer containing 0.40 M sucrose, 0.02 M Tris (pH 8), and 0.01 M NaCl, as described previously (2). The chloroplasts were broken before use by suspension in 0.01 M NaCl. For best results with the quantitative analysis of the pH curves, these broken chloroplasts were washed twice in 0.01 M NaCl by centrifugation at 10,000 × g in the Servall centrifuge. After washing, the actual pH shift was greater, sometimes as much as 1.0 pH unit per mg of chlorophyll in 10 ml, and always 0.6 pH unit or more. Titration curves showed that much less buffering action was left after washing, especially at pH 6.7 and above. pH changes were measured with a Radiometer pH meter and recorded continuously, or else they were titrated with a pH-stat. If not otherwise specified, the standard reaction mixture contained 1 mg of chlorophyll in 10 ml of 10 mM NaCl, and 0.05 mM pyocyanine.

Chlorophyll was measured according to Arnon (7). Ferricyanide reduction was measured either by titration with NaOH at a constant pH or by measurement of the residual absorption of deproteinized reaction mixtures at 420 μM (8). ATP formation was assayed with radioactive orthophosphate, with separation accomplished by Avron’s modification (9) of the Nielsen and Lehninger procedure (10).

RESULTS

Quantitative Determination of pH Curves

In the previous work (2), it was assumed that the pH was a logarithmic function of the hydrogen ion concentration and therefore could not be used as a direct linear measure of the protons consumed. That this is not the case is shown by the titration curve of the standard reaction mixture (Fig. 1). In the range employed in these studies, a constant amount of added NaOH causes an almost constant increment in pH. In other titration curves there was sometimes more of an inflection point around pH 6.5; however, in no case did the pH increment due to addition of a constant amount of NaOH vary by a factor of more than 1.5 or 1.6. It is apparent that the buffering action of the chloroplasts themselves, and possibly that of bicarbonate in solution, is a much more prominent factor in the actual change observed than is the theoretical relation between moles of protons and pH in an unbuffered solution. The fact that the curve is so close to linear means that the kinetics of the proton uptake reaction can be estimated directly from the recording of change of pH with time. This estimation will be reasonably accurate, providing measurements are taken over a constant and specified pH range, where the buffering capacity is the same from one sample to the next.

The formation curve (“light on” reaction) shows about a 2- or 3 sec lag of instrumental origin. After this, the initial rates are approximately linear for at least the first one-third of the
rise. This initial linear portion of the formation curve could be used in comparing control and inhibited chloroplasts. The dark decay ("light off") reaction has a similar linear initial rate. Unfortunately it could not be used for comparison of control and inhibited reactions, because with the inhibitor present the extent of the pH rise was smaller, and the linear part of the curve came at a different (lower) absolute pH, than for the controls. The rate of decrease of pH is certainly a function of the buffering capacity, and it is most probably a function of the amount of the reacting internal component (or components); both of these would vary with the actual pH achieved at the onset of the dark period.

With these considerations in mind, a standard set of conditions was established. (a) The initial pH of the reaction mixture was adjusted to 6.1 ± 0.05 in all cases, before the light was turned on; (b) only chloroplast preparations giving a total pH change of 0.50 or greater were used; (c) the concentration of each inhibitor was adjusted to give, as close as possible, 40% inhibition (range achieved was 34 to 47%), so that the net yield with inhibitor present was at least 0.30 pH unit; (d) the "on" reaction rate was taken from the time required to rise from 0.05 to 0.15 pH unit above the starting base-line; and (e) the decay reaction rate was taken from the time required to drop from 0.25 to 0.15 pH unit above the initial pH.

To assess the reliability of these measurements, 20 successive runs were made, first without, and then with, 1 mM ammonium chloride. Table I shows the measurements of total yield, rates of the "on" and "off" reactions, and relative rates as indicated above, together with their standard deviations. As can be seen, the measurement of initial rate of the pH rise is somewhat more reliable than that of the "off" reaction. The day-to-day variation of the control values was fairly large; therefore each observation is reported as the effect of a given reagent relative to that of the pH rise. This initial linear portion of the formation curve could be used in comparing control and inhibited chloroplasts. The dark decay ("light off") reaction has a similar linear initial rate. However, at all pH values the inhibition of phosphorylation is more severe than that of electron flow. At pH 7.0, in particular, the P/2e ratio drops 94% (with 8 × 10^{-5} M Dicumarol) while the rate of electron flow drops only 31%. Dicumarol can clearly be put in the class of "inhibitory uncouplers" (16) and is very similar to dinitrophenol in its action. As with other reagents, the inhibitory and uncoupling actions of Dicumarol, it was added to phosphorylating reaction mixtures with ferricyanide as the Hill acceptor. Table II shows that Dicumarol has a dual activity: it inhibits both phosphorylation and electron flow. However, at all pH values the inhibition of phosphorylation is more severe than that of electron flow. At pH 7.0, in particular, the P/2e ratio drops 94% (with 8 × 10^{-5} M Dicumarol) while the rate of electron flow drops only 31%. Dicumarol can clearly be put in the class of "inhibitory uncouplers" (16) and is very similar to dinitrophenol in its action. As with other reagents, the inhibition of electron flow is greatest at the higher pH values, and indeed a small stimulation can be seen at pH 6.6.

Some Notes on New Uncouplers

Dinitrophenol was earlier said to inhibit electron flow and phosphorylation to equal extents in chloroplasts (11). We recently found, however, that the inhibition of electron flow is minimized at pH 7 and below, and that a true uncoupling action can be demonstrated in this range (12).

Correlated with uncoupling by dinitrophenol is its interference with the light-induced pH rise. When the reduction of ferri cyanide at pH 6 is measured by acid production, for instance, there is a lag for the first 45 sec as protons are taken up by the chloroplasts equivalent to the amount of ferricyanide reduced (2). Dinitrophenol at 3 or 6 × 10^{-4} M abolishes this lag.

Dicumarol is known as an uncoupler of oxidative phosphorylation, which inhibits electron flow at higher concentrations (13) as well. Wessels (14) showed that this compound inhibits the Hill reaction with dichlorophenolindophenol, and Whatley, Allen, and Arnon (15) found an inhibition of phosphorylation supported by vitamin K_I. In order to assess the relative inhibitory and uncoupling actions of Dicumarol, it was added to phosphorylating reaction mixtures with ferricyanide as the Hill acceptor. Table II shows that Dicumarol has a dual activity: it inhibits both phosphorylation and electron flow. However, at all pH values the inhibition of phosphorylation is more severe than that of electron flow. At pH 7.0, in particular, the P/2e ratio drops 94% (with 8 × 10^{-5} M Dicumarol) while the rate of electron flow drops only 31%. Dicumarol can clearly be put in the class of "inhibitory uncouplers" (16) and is very similar to dinitrophenol in its action. As with other reagents, the inhibition of electron flow is greatest at the higher pH values, and indeed a small stimulation can be seen at pH 6.6.

Pentachlorophenol was shown to cause a decrease in the P/2e ratio of chloroplasts even at pH 8 (11). As with dinitrophenol, we have recently been able to show effective stimulation of the "basal" Hill reaction with ferricyanide, in the pH range from 6.0 to 7.0.

In view of the theoretical considerations which led to the discovery of the pH rise (3), it was of interest to find that detergents inhibit this phenomenon. Under appropriate conditions, Triton X-100 and the other detergents listed in Table III are found to uncouple electron flow from photophosphorylation.\(^1\) Un-

\(^1\) Data to be reported elsewhere.
observed. Levels of EDTA inhibition, stimulation of dark decay was not to the dark control; ATP formation was assayed according to Avron (9).

Following 2 min of light at 2,000 foot-candles, ferricyanide reduction was measured by the loss of absorbance at 420 nm compared to the dark control; ATP formation was assayed according to Avron (9).

Effect of Dicumarol on ferricyanide reduction and accompanying action of one of these (linoleic acid) is noted in Table III.

Dicumarol, and the fatty acids (e.g. linoleic acid), which have relatively minor effects on dark decay, compared to their inhibition of the formation rate. In this case it is important to note that all the concentrations used have been those which will cause at least some stimulation of the Hill reaction at pH 6.2; therefore the effect cannot be due to inhibition of electron flow. However it should be emphasized that the small effect on dark decay is found only at the particular level of the reagent used. Dinitrophenol, for instance, when inhibiting the yield by 60% rather than only 40%, caused an increase in the relative dark decay rate up to 1.8.

Other uncouplers have relatively strong effects on the dark decay process. These include carbonyl cyanide m-chlorophenylhydrazone, ammonia, atebrin, chlorpromazine, the detergents, and trichlorophenolindophenol. The latter dye has been found in several laboratories to be an uncoupling reagent when present in the oxidized form, in the range of 5 × 10⁻⁴ M employed here (17-19). With this dye, measurements in the light stage proper saturated fatty acids were also found to be uncouplers, and the action of one of these (linoleic acid) is noted in Table III.

### TABLE III

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration (mM)</th>
<th>Yield (relative rate)</th>
<th>Decay (relative rate)</th>
<th>Postillumination addition, relative rate of decay</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Chlorophenyl-1,1-dimethylurea</td>
<td>0.05</td>
<td>23</td>
<td>0.45</td>
<td>0.72</td>
</tr>
<tr>
<td>EDTA-treated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dinitrophenol</td>
<td>1.0</td>
<td>38</td>
<td>0.46</td>
<td>1.3</td>
</tr>
<tr>
<td>Dicumarol</td>
<td>0.05</td>
<td>30</td>
<td>0.81</td>
<td>1.3</td>
</tr>
<tr>
<td>Linoleic</td>
<td>0.10</td>
<td>37</td>
<td>0.45</td>
<td>1.4</td>
</tr>
<tr>
<td>Pentachlorophenol</td>
<td>0.015</td>
<td>43</td>
<td>0.55</td>
<td>2.1</td>
</tr>
<tr>
<td>Carbonyl cyanide m-chlorophenylhydrazone</td>
<td>0.004</td>
<td>37</td>
<td>0.45</td>
<td>2.2</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>1.0</td>
<td>38</td>
<td>0.65</td>
<td>4.5</td>
</tr>
<tr>
<td>Atebrin</td>
<td>0.25</td>
<td>38</td>
<td>0.32</td>
<td>3.1</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>0.11</td>
<td>40</td>
<td>1.11</td>
<td>2.6</td>
</tr>
<tr>
<td>Triton</td>
<td>0.005%</td>
<td>22</td>
<td>1.30</td>
<td>1.7</td>
</tr>
<tr>
<td>Triton</td>
<td>0.010%</td>
<td>42</td>
<td>0.86</td>
<td>1.9</td>
</tr>
<tr>
<td>Cetylpyridinium bromide</td>
<td>0.055%</td>
<td>40</td>
<td>0.86</td>
<td>2.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>400.0</td>
<td>21</td>
<td>1.0</td>
<td>1.4</td>
</tr>
<tr>
<td>NaCl</td>
<td>700.0</td>
<td>28</td>
<td>0.86</td>
<td>1.8</td>
</tr>
</tbody>
</table>

**Effects of Uncouplers on pH Shift**

Uncouplers, added to unbuffered chloroplasts, diminish the extent of the light-induced pH shift. Some time courses for this effect were noted in an earlier publication (see Figs. 11 and 12 in Reference 2). Table III shows the relative effects of a series of uncouplers on the decay reaction (with the inhibitor added either before or after illumination) and on the apparent formation rate under the more closely controlled conditions described above.

The first reagent shown is p-chlorophenyl-1,1-dimethylurea, an inhibitor of electron transport. It would not be expected to accelerate dark decay of the pH shift, and on close examination it seems to retard the "off" reaction somewhat.

Chloroplasts may be uncoupled by exposure to EDTA at low ionic strength (8). The degree of uncoupling can be controlled by the presence of varying amounts of NaCl (or other salts). Similarly, the degree of inhibition of the pH shift by exposure to EDTA is controlled by the simultaneous presence of NaCl or other salts. The EDTA treatment shown in Table III, for instance, consisted of 1 mM EDTA together with 3 mM NaCl, giving 34% inhibition. Without the NaCl the inhibition was over 90%. EDTA treatment appears to be unique (Table III) in causing essentially no stimulation of the dark decay rate, while inhibiting formation and the total yield. Even at higher levels of EDTA inhibition, stimulation of dark decay was not observed.

No other uncoupler tested has failed to affect the dark decay rate. However, there is a group, consisting of dinitrophenol, \( R. McCarty, in preparation. \)
were not attempted because of complications resulting from its reduction in the Hill reaction (see Reference 2).

Among those uncouplers causing marked stimulation of dark decay, two fairly distinct subgroups can be distinguished. Ammonia and atebrin appear to have a greater effect when added in the postillumination period than if present during the light stage as well. It is possible that either the reagent is photolabile, or the chloroplasts are altered by exposure to light to make them more resistant to the action of the uncoupler. Although the reason for this behavior is obscure, similar phenomena are noted for \( X_E \) in the accompanying paper (20).

Chloropromazine and Triton, possibly together with cetylpyridinium bromide, comprise a second subgroup. The rate of formation is distinctively stimulated by these even at a concentration at which the decay reaction is faster and the net yield is inhibited. Although the detergents do not show actual stimulation at the point of 40\% inhibition of net yield, they do show the stimulation at slightly lower concentrations, where the net yield is maintained in spite of faster decay because the formation rate has increased. This faster formation is not seen with the other uncouplers, even at minimally active concentrations.

Finally, the effect of high ionic strength is noteworthy because of its contrast with the other phosphorylation-associated reactions (20). Even 1.4 ionic strength NaCl seems unable to cause uncouplers, even at minimally active concentrations.

After the light is turned off, however, the pH continues to rise for about 5 sec, returning to its original value only after 20 to 30 sec. The extent of the overshoot is at most 0.1 pH unit, in a reaction mixture capable of showing a total change of 0.7 pH unit in the light. The overshoot is most prominent after 3 or 6 sec of illumination.

**Overshoot of pH after Flash of Light**

For comparative reasons, it was of interest to observe the behavior of the pH after a brief illumination only. Fig. 2 shows that a 3-sec flash of light in itself causes only a slight pH rise. After the light is turned off, however, the pH continues to rise for about 5 sec, returning to its original value only after 20 to 30 sec. The extent of the overshoot is at most 0.1 pH unit, in a reaction mixture capable of showing a total change of 0.7 pH unit in the light. The overshoot is most prominent after 3 or 6 sec of illumination.

![Fig. 2. Postillumination overshoot of pH. Reaction conditions were those described in "Materials and Methods." Illumination was started at the small arrow, and terminated as shown by the vertical line. It can be seen that illumination for 3 sec leads to a large overshoot, amounting to 14\% of the total pH change possible. Less overshoot is found after 12 sec, and none after 60 sec of illumination.](image)

The present experiments add weight to the correlations between the reversible, light-induced pH rise and the mechanism for phosphorylation. In particular, very few exceptions were found to the correlation between reagents that inhibit phosphorylation, or the formation of the high energy nonphosphorylated intermediate \( X_E \) (18), and those that inhibit the pH shift.

The exact nature of the relation between \( X_E \) and the pH shift is a matter which the present experiments bear on indirectly. If the two are related at all, there are three major possibilities. (a) The conversion of \( X \) to its energetic form \( X_E \) is a reaction (such as formation of an acyl anhydride; see Reference 21) in which hydroxyl ion is formed. The rise in pH would therefore be another aspect of this specific chemical event. (b) The high energy "intermediate" \( X_E \) might actually represent energy stored as a pH gradient across the grana disk membranes (1-3). In that case the observed pH rise would be a sign of the occurrence of proton transport, leading to the high energy state. (c) The pH shift could be entirely secondary, possibly representing some sort of ion transport driven by means of the energy otherwise stored in \( X_E \).

In the first possibility, one would expect a perfect correspondence between the kinetics of the pH shift and the kinetics of \( X_E \), as well as some small number stoichiometry between the two. In the second possibility the stoichiometry is an open question, and one might expect threshold effects or other complications in the relative kinetics of the two. In the third case the correspondence in behavior would be the smallest of all. It is therefore of interest to look carefully for discrepancies between the two, to see if any of these possibilities can be ruled out.

**Stoichiometry**—As noted earlier (2) the pH shift can amount to 1 proton for every mole of chlorophyll, while measured \( X_E \) yields have never gone above 1 mole of ATP per 8 moles of chlorophyll (1). However, it was recently suggested that the total amount of \( X_E \) could be as high as 1 mole for every 4 or 4.5 moles of chlorophyll (22) after the loss of \( X_E \) during the phosphorylation stage at pH 8 had been accounted for. This number is reasonably close to the amount of proton uptake detected. It also seems significant that both \( X_E \) formation (22) and proton uptake (2) keep pace with the course of ferricyanide reduction during the first 30 sec at pH 6.

**Kinetics**—The pH shift has a linear early portion in both formation and decay (2), whereas \( X_E \) is apparently first order in both directions (1). The pH overshoot after 3 or 6 sec of light (Fig. 2) is not duplicated in the kinetics of \( X_E \).

**pH Response and Inhibitors**—Although the pH curves for the two phenomena are roughly similar, that for the pH rise shows a marked diminution on going from pH 6.2 down to 5.5 (2), whereas \( X_E \) shows no decrease over that range (4). Superficially it would appear that the pH shift is inhibited 70\%, with no effect on \( X_E \). However, in order to show \( X_E \) formation at pH 5.5, it was essential to work very rapidly, keeping the chloroplasts for only 5 sec or less in this acid medium before exposure to light (4). The preillumination adjustment of chloroplast pH to 5.5, in the present system without the use of buffers took considerably longer, up to several minutes; hence the drop in pH shift yield at pH 5.5 is likely to reflect acid denaturation, rather than a real difference from \( X_E \).

High ionic strength is relatively ineffective in inhibiting the pH shift (Table III), with 0.4 M NaCl causing only a 22\% inhibi-
tion. This concentration of NaCl inhibits the \( X_\mathrm{p} \) yield over 94%, and inhibits phosphorylation with pyocyanine approximately 65% (18).

As noted in Table III, fatty acids appear to have relatively little effect on dark decay of the pH shift. The most extreme case was that of oleic acid, in the presence of which the dark decay rate was only 10% faster; nevertheless the same concentration was able to cause 4-fold stimulations of the dark decay of \( X_\mathrm{p} \).

A full evaluation of the above list of discrepancies will not be possible until conditions can be arranged to determine both \( X_\mathrm{p} \) formation and proton uptake in the very same reaction mixture. Nevertheless it seems highly unlikely that the pH shift is a direct measurement of a chemical change which is the conversion of \( X \) to \( X_\mathrm{p} \). Alternatively, if \( X_\mathrm{p} \) is a trans-membrane pH gradient, we have to postulate some additional step between creation of the gradient and measurement of pH in the external medium. This is a possibility, if the significant gradient is that between protons bound to the two sides of the membrane. Equilibration from the medium to a binding site on the membrane might then be a slower process and could account for phenomena such as the 5-sec overshoot in pH but not in \( X_\mathrm{p} \), etc. Even granted this further step to account for kinetic discrepancies, however, it will be difficult to explain the inhibition of \( X_\mathrm{p} \) but not that of the pH shift by high NaCl.

The discovery of potassium ion efflux corresponding in amount to proton uptake (6) does make it appear more likely that the pH rise is basically due to an ionic shift. Even so, the thermodynamic consequences of a pH gradient are not eliminated; the corresponding potassium efflux simply saves one from the embarrassment of failing to maintain electrical neutrality.

We are left with a striking over-all similarity in \( X_\mathrm{p} \) formation and in the pH shift with respect to the pattern of inhibitions. Except for high salt concentrations, and possibly oleic acid, all other inhibitors work on both systems. Furthermore, for both of these, the chemical uncouplers speed up dark decay and EDTA treatment inhibits formation.

It is of additional interest that the number of protons moving is sufficient to satisfy the quantitative aspect of Mitchell's theory (3, 23). Chloroplasts containing 1.0 mg of chlorophyll, achieving ordinarily the uptake of 0.06 \( \mu \)equiv of protons in the light, packed down to a volume of 0.4 ml after centrifugation. On the assumption that half of the packed volume is internal space, the proton concentration was calculated to be 3.2 mM or the equivalent of a pH of 2.5, whereas the outside pH was 6.5. It would be possible, therefore, for internal buffers to neutralize up to 90% of the protons taken in and still maintain a pH differential of 3.0 unite, the required gradient for a chemiosmotic potential sufficient to drive the phosphorylation of ADP (3) under the present conditions.

If the pH shift represents storage of protons on the inside of double membranes, then any reagent or condition enabling protons to leak out of the enclosed inner space should inhibit ATP formation. The action of most of the uncouplers could be rationalized easily on that basis, and the figure showing the sudden drop in pH due to addition of Triton (2) looks like a dramatic instance of protons pouring out of the double membrane enclosures.

Several patterns of inhibition have emerged from this study of uncouplers on the pH shift. These range from the EDTA-uncoupled, with the only effect being inhibition of the formation rate; through dinitrophenol and fatty acids with a weak effect in stimulating dark decay; to ammonium, atebrin, and carbonyl cyanide m-chlorophenylhydrazone with stronger stimulation of dark decay but still an inhibition of formation; and finally to chlorop-promazine and the lower concentrations of Triton which stimulate dark decay and the formation rate at the same time. Since the chemical meaning of the pH shift is not known, the significance of these differences is not clear. Nevertheless, the different patterns of inhibition are likely to be related to differences in the basic mode of action of the different uncouplers, and the classification achieved should be of some value in selecting reagents for further study.

**SUMMARY**

Known uncouplers of photosynthetic phosphorylation inhibit the reversible, light-induced pH rise by spinach chloroplasts. Dicumarol and pentachlorophenol are shown to decrease the P/2e ratio, and at the right pH they stimulate electron transport; hence these are added to the list of uncouplers.

It is shown that the kinetics of proton uptake can be measured from the rate of change of pH with time, under standard conditions. Of the uncouplers tested, only ethylenediaminetetraacetate failed to stimulate the rate of dark decay of pH. Stimulation of dark decay was relatively small for some substituted phenols and unsaturated fatty acids. Chlorop-promazine and Triton are unique in causing a faster formation rate at the same low concentration, where they speed up the dark decay reaction.

After illumination for only 5 sec, the pH continues to rise for a few seconds. On the basis of this and other data presented here, the possible relationship between the pH shift and the high energy state (\( X_\mathrm{p} \)) is defined further.

**REFERENCES**