tion at pH 5.5 resulted in preferential destruction of IC photoreduction ability.

4. Addition of zinc ion preferentially inhibited the photooxidation of ascorbic acid.

5. Treatment of chloroplasts with digitonin resulted in a drastic loss of ability to catalyze the Hill reaction and IC photoreduction, yet yielded a preparation which was over 6 times as active in terms of ascorbic acid photooxidation.

6. These results are briefly discussed in terms of reaction mechanisms for these 3 photochemical reactions.

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UNCOUPLERS OF SPINACH CHLOROPLAST PHOTOSYNTHETIC PHOSPHORYLATION [1, 2, 3]

DAVID W. KROGMANN [4], ANDRE T. JAGENDORF AND MORDHAY AVRON [5]

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

It was recently observed by Arnon et al (3) that the formation of ATP[6] could occur during the course of the Hill reaction, with either ferricyanide or TPN as the Hill oxidant. The formation of ATP was stoichiometric with and dependent on the transfer of electrons to the Hill oxidant. The converse obtained to some extent also; that is, the Hill reaction with ferricyanide as oxidant proceeded approximately twice as fast if the phosphorylating reagents were present. We were able to repeat these observations of Arnon et al, including both aspects—ATP formation dependent on simultaneous electron flow, and electron flow stimulated by simultaneous phosphorylation (4). By using somewhat different conditions we found the stimulated rate of ferricyanide reduction to be from 3 to 3.5 times that of the control rate (7).

In investigating the mechanism by which this coupling occurs it may be useful to find reagents or physical treatments that lead to a disruption of the mechanism—i.e., uncouplers. A compound that is an uncoupler in this system, we will define as one that permits electron flow to proceed at a rapid rate in the absence of phosphorylation. Specifically it should meet the following criteria: a) in the phosphorylating Hill reaction, the reagent will inhibit the formation

[1] Received revised manuscript September 18, 1958.

[2] Supported in part by research grant no. RG 3923 from the National Institutes of Health, Research Grants Division.

[3] Contribution no. 259 from the McCollum-Pratt Institute.

[4] Present address: Biochemistry Department, University of Chicago, Chicago, Illinois.

[5] Present address: Biochemistry Section, Weizmann Institute of Science, Rehovoth, Israel.

[6] Abbreviations to be used include: ATP for adenosine triphosphate, ADP for adenosine diphosphate, TPN for triphosphopyridine nucleotide, TRIS for tris (hydroxymethyl) aminomethane, DNP for dinitrophenol, PCP for pentachlorophenol.

of ATP but will not affect the rate of ferricyanide reduction and b) ferricyanide reduction in the absence of simultaneous phosphorylation will be stimulated by the reagent, at least as much as by phosphorylating reagents.

With these criteria now available, we have investigated two compounds that are effective as uncouplers of oxidative phosphorylation by mitochondria: dinitrophenol (11) and pentachlorophenol (15). Treatment of chloroplasts by dilution in sodium chloride at pH 6.0, which was previously found to activate them to a faster rate of ferricyanide reduction (12) is shown here to fulfill the criteria for uncoupling of the plastids. Arsenate was previously shown to replace phosphate in the stimulation of the Hill reaction (5); in the present work we show that arsenate fulfills one of the criteria for an uncoupling reagent. Finally we have found that ammonium ions are very effective uncouplers of chloroplast phosphorylation.

Materials and Methods

Chloroplasts were prepared from grocery spinach as described previously (6). Leaves were ground in 0.4 M sucrose, 0.01 M NaCl buffered at pH 7.8 with 0.05 M TRIS. The chloroplasts were washed once in the same medium. Chlorophyll was determined by the method of Arnon (1). Incorporation of P^{32} into ATP was determined by adsorbing the ATP^{32} on charcoal, washing the charcoal, then removing the labelled phosphate by hydrolysis (10).

Two kinds of reaction mixtures were employed in the present study. The first (control) contained 40 micromoles (μM) of TRIS buffer at pH 7.8, 70 μM of NaCl, 10 μM of $MgCl_2$, 15 μM of phosphate at pH 7.8, 2.0 μM of potassium ferricyanide, 0.1 μM of ATP and chloroplasts containing 0.030 mg of chlorophyll in a total volume of 3.0 ml. The optical density of the entire reaction mixture was determined at 400 mμ in a Beckman spectrophotometer; the cuvette was exposed to 5000 ft-c of white light from a tungsten lamp for 2 minutes, and then the decreased optical density was measured again. A 10 cm path of water was used as a heat shield, and control experiments had shown no changes with boiled chloroplasts.

The reaction mixture proper contained the same components as the control, and in addition 2.0 μM of ADP and approximately 2×10^5 cpm of P^{32}. Thus the only difference from the control mixture was the presence of ADP, which permitted net phosphorylation to occur. The rate of reduction in the complex control mixture is approximately 90 % of that seen in a much simpler control containing only chloroplasts, ferricyanide, buffer and NaCl (7), and the response to inhibitors was in all cases the same as in the simpler control. The complex control mixture was used in order to approximate as closely as possible the conditions in the phosphorylating reaction, except for the absence of net ATP formation.

After the amount of ferricyanide reduction had been measured, the reaction mixtures in the phosphorylation cuvettes were denatured by adding 0.3 ml of 20 % trichloroacetic acid, the mixture was centrifuged, and an aliquot was removed for ATP^{32} determination. In all experiments a zero time control was included in which all reagents were added directly to trichloroacetic acid; this control value for ATP^{32} was subtracted from all of the experimental measurements. The values shown in the tables represent the average of duplicate or triplicate determinations of both the rate of ferricyanide reduction and of phosphorylation. Duplicate values generally agreed within 10 %. Each experiment shown was repeated at least 4 times.

From the measurements obtained in the phosphorylating cuvettes it is possible to determine a P/2e ratio. This is defined here as micromoles of ATP formed per $1/2 \times$ no. of μ equivalents of Hill oxidant reduced, and is listed in the tables as the observed P/2e ratio. If the amount of ferricyanide reduced in the control cuvette is subtracted from that reduced in the phosphorylation cuvette, one determines the extra ferricyanide reduction due to simultaneous phosphorylation. Using this net value for electron flow a calculated P/2e ratio is obtained, defined as micromoles of ATP formed per $1/2 \times$ net μ equivalents of Hill oxidant reduced in the phosphorylating reaction. These calculated values are also listed in the tables.

With a variable source of leaves, the extent of stimulation of the Hill reaction due to simultaneous phosphorylation has varied between 2 and 3.5-fold. The experiments chosen for the tables were ones in which the degree of stimulation was in the upper range, since presumably these would be the better chloroplast preparations. In these experiments where the net stimulation of electron flow is relatively high, the calculated P/2e ratio is minimal.

The values shown in the tables are μ equivalents of ferricyanide reduced or micromoles of ATP formed in the 3 ml of reaction mixture. Multiplying any of these numbers by 1000 will give the μ equivalents or micromoles formed per mg chlorophyll per hour.

Results

Table I shows the effect of dinitrophenol on ferricyanide reduction in the presence or absence of simultaneous phosphorylation, and on the amount of ATP formed in the phosphorylation. It can be seen that dinitrophenol at 3.3×10^{-4} M, and even more at 1×10^{-3} M, inhibits ATP formation. These same concentrations also inhibit ferricyanide reduction either in the presence or absence of phosphorylation. The extent of the inhibitions of electron flow and of ATP formation are approximately equal in the phosphorylating Hill reaction.

Also shown in this table is the observed P/2e ratio which is close to 1.0. Note that in this experiment the net stimulation is 2.5 times as large as the control rate, and the total stimulated rate is 3.5 times as great as the control. The calculated P/2e ratios range

from 1.3 to 1.7 in this experiment, and in general they vary from 1.3 to 2.0 in our experience.

Results with pentachlorophenol are shown in table II. As with dinitrophenol, both ATP formation and ferricyanide reduction are inhibited in the phosphorylation cuvette and at the same concentrations of pentachlorophenol. The control rate, however, is hardly affected except for a slight stimulation at 1×10^{-5} M.

Although the observed P/2e ratios drop from 0.84 to 0.42 when phosphorylation still occurs, the calculated P/2e ratio remains between 1.25 and 1.55. The difference is due to the failure of pentachlorophenol to inhibit the control rate.

Since the basal rate is stimulated 20 % by 1×10^{-5} M pentachlorophenol, it was thought that the reagent might have some uncoupling ability which could not show up due to the brief (2 minute) exposure time. However, even after a 60 minute preincubation of chloroplasts with 1×10^{-4} M pentachlorophenol no further stimulation of the basal rate was seen (final concentration in the reaction mixture after preincubation was 1×10^{-5} M). If there is any uncoupling effect it is only a minor one, and it is completely masked by the inhibiting action.

Chloroplasts were diluted, to a final concentration of 0.001 mg chlorophyll per ml, in 0.35 M NaCl at pH 6.0 and in agreement with previous results (12) their ability to reduce ferricyanide in the absence of phosphorylation more than tripled (table III). However, the rate of ferricyanide reduction in the untreated chloroplasts was stimulated to about the same level by phosphorylation, and the treated chloroplasts show almost no response to phosphorylating reagents. As might be expected, therefore, these treated chloroplasts make almost no ATP when provided with the phosphorylating reagents. The P/2e ratio, either observed or calculated, falls to a negligible value.

Arsenate (table IV) inhibits ATP formation by virtue of competition with phosphate (5). However ferricyanide reduction is quite unaffected, even when ATP formation is inhibited 65 %. The P/2e ratios, both observed and calculated, drop steadily. The control rate is not stimulated by arsenate because ADP is absent. The stimulation by arsenate requires the presence of ADP (5).

It was observed accidentally that ammonium sulfate could induce a large increase in the rate of ferricyanide reduction by fresh chloroplasts, entirely in the absence of phosphorylating reagents. This is shown in table V, as is a 95 % inhibition of ATP formation by NH_3 without any inhibition of ferricyanide reduction in the phosphorylating reaction. Actually

TABLE I

EFFECT OF DINITROPHENOL ON HILL REACTION PHOSPHORYLATION

DNP Concentration, M	Ferricyanide reduced −ADP	Ferricyanide reduced +ADP	ATP formed	P/2e Observed	P/2e Calculated*
0	0.24*	0.83*	0.39	0.94	1.4
1×10^{-4}	0.24	0.74	0.32	0.86	1.3
3.3×10^{-4}	0.19	0.45	0.22	0.98	1.7
1×10^{-3}	0.07	0.10	0.0	...	...

* Micromoles in 2 minutes. Reaction conditions described in Materials and Methods section.

TABLE II

EFFECT OF PENTACHLOROPHENOL ON HILL REACTION PHOSPHORYLATION

Pentachlorophenol concentration M	Ferricyanide reduced −ADP	Ferricyanide reduced +ADP	ATP formed	P/2e Observed	P/2e Calculated
0	0.26	0.76	0.32	0.84	1.3
1×10^{-6}	0.25	0.75	0.31	0.83	1.2
3.3×10^{-6}	0.29	0.51	0.17	0.67	1.6
1×10^{-5}	0.31	0.43	0.09	0.42	1.5
3.3×10^{-5}	0.25	0.27	0.0	...	...

TABLE III

HILL REACTION PHOSPHORYLATION BY CHLOROPLASTS DILUTED IN NaCl AT pH 6.0

Chloroplasts	Ferricyanide reduced −ADP	Ferricyanide reduced +ADP	ATP formed	P/2e Observed	P/2e Calculated
Fresh	0.17	0.62	0.32	1.03	1.4
Diluted	0.49	0.53	0.004	...	...

the reduction of ferricyanide is slightly stimulated as ATP formation disappears.

These effects are shared by ammonium chloride, ammonium formate and ammonium acetate, and so are due to the cation and not the anion. Sodium, potassium, magnesium or calcium ions do not replace the ammonium ions. The effect of a given concentration of ammonium salt is not increased at all by preincubation of the chloroplasts with the salt. The concentration of ammonium ions needed for 50 % inhibition of ATP formation varies between 6×10^{-4} and $4 \times^{-3}$ M in our experience.

An unusual aspect of uncoupling by ammonium ions is that it seems to be freely reversible once the ammonium salt is removed by washing (table VI). Control chloroplasts either before or after a wash have a low rate of ferricyanide reduction, which is stimulated by the addition of phosphorylating reagents. Chloroplasts stored in 5×10^{-2} M ammonium chloride carry with them into the reaction mixture enough ammonium chloride to make the final concentration 1.67 $\times 10^{-3}$ M. This is enough so that they are about 65 % uncoupled in this experiment: the rate of reduction is high, and it is stimulated only slightly by phosphorylating reagents. Also this preparation makes only 31 % as much ATP as the control chloroplasts when provided with the appropriate reagents. These same chloroplasts, after 1 centrifugation and resuspension in the absence of ammonium chloride show an almost unimpaired coupled phosphorylation. The control rate of ferricyanide is back to a low value, and is stimulated by phosphorylating reagents. ATP formation is 85 % of that in untreated chloroplasts.

There is an extra complication to the effect of ammonium salts on chloroplasts, in that an excess is inhibitory to ferricyanide reduction. The inhibition is observed at alkaline pH only; while severe at 7.8 it is apparently negligible at 7.2. The sensitivity to inhibition by excess ammonia has varied with different chloroplast preparations; between 6×10^{-3} M and 2×10^{-2} M has been required for inhibition to appear.

TABLE IV

EFFECT OF ARSENATE ON HILL REACTION PHOSPHORYLATION

ARSENATE CONCENTRATION M	FERRICYANIDE REDUCED −ADP	FERRICYANIDE REDUCED +ADP	ATP FORMED	P/2e OBSERVED	P/2e CALCULATED
0	0.19	0.42	0.21	1.00	1.8
1.0×10^{-2}	0.20	0.44	0.19	0.86	1.6
1.5×10^{-2}	0.22	0.46	0.14	0.61	1.2
3.3×10^{-2}	0.22	0.48	0.07	0.29	0 5

TABLE V

EFFECT OF AMMONIUM SULFATE ON HILL REACTION PHOSPHORYLATION

NH_4 ION CONCENTRATION* M	FERRICYANIDE REDUCED −ADP	FERRICYANIDE REDUCED +ADP	ATP FORMED	P/2e OBSERVED	P/2e CALCULATED
0	0.21	0.59	0.36	1.22	1.9
2×10^{-4}	0.30	0.62	0.32	1.03	2.0
6.6×10^{-4}	0.62	0.74	0.11	0.30	1.8
2×10^{-3}	0.67	0.71	0.016	0.05	[0.8]
4.7×10^{-3}	0.53	0.53	0.00	...	...

* Concentration of the ammonium ion is shown; concentrations of ammonium sulfate were half those shown.

TABLE VI

REVERSIBILITY OF UNCOUPLING BY AMMONIUM CHLORIDE

CHLOROPLASTS	NH_4Cl* CONCENTRATION M	FERRICYANIDE REDUCED −ADP	FERRICYANIDE REDUCED +ADP	ATP FORMED	P/2e OBSERVED	P/2e CALCULATED
Control	0	0.19	0.62	0.34	1.10	1.6
Control after wash	0	0.21	0.65	0.32	0.99	1.5
Stored in NH_4Cl	1.67×10^{-3}	0.73	0.78	0.10	0.27	(4.0)
Stored in NH_4Cl after wash	0	0.23	0.47	0.27	1.15	(2.3)

* Chloroplasts were stored in 5×10^{-2} M ammonium chloride; on transfer to the reaction mixture they carried enough with them to make the final concentration equal to that shown.

Discussion

Prior to the discovery by Arnon et al (3) of ATP formation coupled to ferricyanide reduction, it was not possible to measure the rate of electron flow while ATP was being formed by chloroplasts. Consequently it was impossible to determine whether any particular inhibitor of phosphorylation was actually an uncoupler, or whether it was inhibiting electron transport or photolysis primarily and phosphorylation secondarily. Thus dinitrophenol was found to inhibit phosphorylation (2) and by analogy with its effect in mitochondria, would be presumed to be an uncoupler of chloroplast phosphorylation. However with the critical analysis possible now that phosphorylation and electron flow can be measured in the same reaction, we see quite clearly that dinitrophenol is an inhibitor of both reactions and so cannot be called an uncoupler. Similarly pentachlorophenol, which works as an uncoupler of oxidative phosphorylation at concentrations below those effective for dinitrophenol (15) inhibits both ferricyanide reduction and ATP formation by chloroplasts, and is therefore an inhibitor but not an uncoupler.

It is widely considered (8, 11) that the effects of dinitrophenol on oxidative phosphorylation are due to interference with the basic mechanism for the formation of ATP. Since DNP is not an uncoupler of photosynthetic phosphorylation, this may be an indication that the mechanism of ATP formation is different in chloroplasts than in mitochondria. Alternatively of course the mechanism might be very similar, but the specificity for uncouplers might be different due to dissimilar enzymes involved. Entirely different evidence has been presented elsewhere (5) which indicates that the mechanism for ATP formation in mitochondria is different from that in chloroplasts.

Arsenate was previously shown (5) to stimulate ferricyanide reduction providing ADP and magnesium were present. Arsenate is an uncoupler in mitochondria (10) but there is only a partial requirement for ADP in uncoupling oxidative phosphorylation. We see here that arsenate meets one of the criteria for an uncoupler, in that electron flow remains high even when ATP formation is inhibited seriously. The basal rate is not stimulated, however, due to the absence of ADP.

The procedure of diluting chloroplasts in 0.35 M NaCl at pH 6.0 was first developed as a method to permit them to reduce ferricyanide more rapidly (12). It was observed that this treatment also made them unable to phosphorylate in a cyclic system. However the complete proof that the dilution treatment results in uncoupling had to wait for the present experimental procedure, in which phosphorylation and electron flow are measured simultaneously.

The discovery that ammonium salts are uncouplers of the Hill reaction phosphorylation at 10^{-3} M or below was quite unexpected. Nevertheless it is clear that they meet both criteria for uncoupling quite well. The mechanism for this effect is still entirely unknown. However it is possible to rule out any irreversible alteration in chloroplast structure, since the uncoupling action is reversible by washing out the ammonium salt. Whatever ammonium does, it must be present continuously to exert its effect. The action of ammonium ions on photosynthetic phosphorylation might be a partial explanation for some of the well known toxic effects of ammonia in plant tissues (9, 14). Inhibition of cyclic phosphorylation by ammonium ions was observed previously by Ohmura (13).

There are at least two and probably more than two basic ways in which uncoupling can occur (see (7)). The experimental procedures we have used here to determine the existence of uncoupling are not adequate to determine what the mechanism is for a given reagent or treatment.

The observed P/2e ratios under the present conditions vary between 0.8 and 1.2, in the phosphorylating reactions with no inhibitory agent added. These ratios agree with those observed by Arnon et al (3). However, a basal rate of ferricyanide reduction always occurs in the entire absence of phosphorylation. It seems entirely conceivable that this amount of electron flow might continue to occur, entirely unassociated with the production of ATP, even when the additional electron flow does result in ATP formation as ADP, phosphate and magnesium are provided. If that is true then the only electron flow actually concerned with making ATP is that which comprises the net stimulation of the Hill reaction. The efficiency of the reactions actually concerned would then be better expressed by the calculated P/2e ratio than by the observed P/2e ratio. We have shown this calculated ratio in all of the experiments listed, and it varies from 1.3 to 2.0 or higher. While no great accuracy can be ascribed to the resulting numbers, they do indicate the possibility of a P/2e ratio greater than 1, and presumably approaching 2 as a limit.

If this calculation is valid, the resulting P/2e ratios suggest that there are 2 phosphorylating steps on the way from the first reduced product of photolysis to ferricyanide. The evidence that we have is very definitely inadequate to decide on the validity of the assumptions, however. A decision would be possible if chloroplasts were found in which electron transport was completely coupled to phosphorylation, so that subtraction of the control rate would be a negligible factor.

Summary

The criteria are indicated which can distinguish whether a compound uncouples electron transport from phosphorylation in the phosphorylating Hill reaction, or whether it is an inhibitor of both electron flow and ATP formation.

By these criteria, dinitrophenol and pentachlorophenol do not act as uncouplers in chloroplasts, but only as inhibitors. Dilution of chloroplasts in sodium chloride solution at pH 6.0 results in uncoupling. Arsenate acts as an uncoupler, but only in the presence of ADP and magnesium. Ammonium ions are very

effective uncouplers of photosynthetic phosphorylation. The action of ammonium salts are reversible by simply washing the chloroplasts.

It is suggested tentatively that a P/2e ratio of more than 1.0 might be possible for ATP formation coupled to ferricyanide reduction.

This work was greatly expedited by the assistance of Mrs. M. Evans.

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COMPARISON OF FERRICYANIDE AND 2,3',6-TRICHLOROPHENOL INDOPHENOL AS HILL REACTION OXIDANTS [1, 2, 3]

DAVID W. KROGMANN [4] AND ANDRE T. JAGENDORF

The McCollum-Pratt Institute and Biology Department of The Johns Hopkins University, Baltimore 18, Maryland

With the development of a spectrophotometric assay of the Hill reaction with ferricyanide (9), a comparison between the rates of reduction of ferricyanide and the dye, 2,3',6-trichlorophenol indophenol by intact chloroplast appeared desirable. Previous attempts at such a comparison were hampered by the different methods used for the two oxidants. These differences are minimized by following the reduction of both oxidants spectrophotometrically. Under comparable circumstances, one observes that intact chloroplasts reduce ferricyanide at a much lower rate than that at which they reduce indophenol dye. This difference has been studied and a procedure has been devised which permits chloroplasts to reduce both acceptors at the same rate.

[1] Received September 18, 1958.

[2] Contribution no. 258 from the McCollum-Pratt Institute.

[3] This work was supported in part by Grant no. RG 3923 from the National Institutes of Health, Public Health Service, and by Grant NSF 1298 from the National Science Foundation.

[4] Predoctoral Fellow of the National Cancer Institute, Public Health Service. This work was submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biology at Johns Hopkins University, Baltimore, Maryland. Present address: Biochemistry Department, University of Chicago, Chicago, Illinois.

Materials and Methods

Reagents: 8-Hydroxyquinoline and *a,a'*-dipyridyl were purchased from the Baker Chemical Company and Fisher Scientific Company, respectively. Triphosphopyridine nucleotide (TPN) was obtained from the Pabst Laboratories. 2,3',6-Trichlorophenol indophenol was a product of Eastmen Organic Chemicals.

Preparation of Chloroplasts: Fresh spinach was obtained at the local market, and whole chloroplasts were prepared from it either by the method of Jagendorf (7) or of Arnon et al (2). When sonicated chloroplasts were used, sonication was performed in