ACTIVATION OF THE HILL REACTION BY AMINES*

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SUMMARY

1. Unidentified non-metal bases from plant and animal tissues, ammonia, and a number of monofunctional amines increase the rates at which ferricyanide and other oxidants are reduced by illuminated chloroplasts. The relatively polar polyfunctional amines are completely inactive.

2. The amount of activation of the chloroplast reactions is a function of the concentration of the unionized amine. The reciprocal of the rate increase plotted against the reciprocal of the concentration of the amine (methylamine) gives a straight line. Moreover the action of the amine is completely reversible. However there is no evidence that any of the amine is consumed.

3. The electron transport system operative in the absence of amines and the electron transport system activated by amines utilize different pathways. The amine-insensitive system is very stable below pH 7.0, is somewhat labile at pH 7.8, and does not reduce FMN at appreciable rates. The system activated by amines is very labile below pH 7.0, is fairly stable at pH 7.8, readily reduces FMN, and reduces all oxidants at rates which are several times greater than the maximum rate of the amine-insensitive system.

4. The amine-activated system is closely related to the system which is activated by the presence of ADP, orthophosphate and magnesium ions: the two systems disappear simultaneously on storage of the chloroplasts; the activations are not additive if optimal amounts of amine have been used; and the presence of amines prevents the formation of ATP.

5. The stoichiometry of the phosphorylating reaction and the manner in which amines might affect this reaction are discussed.

INTRODUCTION

Several years ago the author[1] investigated the reduction of flavins by illuminated chloroplasts. In the course of these studies it was discovered that the crude nucleotide fraction of yeast extracts stimulated not only the reduction of flavins but also the

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Abbreviations used: AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; FMN, flavin mononucleotide; $FMNH_2$, reduced flavin mononucleotide; TPN^+, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; IDP, inosine diphosphate.

reduction of all the classical HILL reaction oxidants. It was subsequently shown that the catalyst or activator could be adsorbed on cation exchange resins and eluted there from with dilute acids. Moreover, the activating substance was destroyed by boiling the preparation with nitric acid. Consequently, it seemed probable that an amine was involved.

At this point in the investigation we became aware that KROGMANN, JAGENDORF AND AVRON[2] had observed a somewhat similar stimulation of ferricyanide reduction by ammonium sulfate. Suspecting that the ability to stimulate chloroplast reactions might be a general property of non-metal bases, we tested the efficacy of a number of amines. Many of these caused marked increases in the rate of reduction of various oxidants. Since the activation seemed to be quite unspecific, attempts to identify the biologically produced active substance were set aside in favor of an investigation of the mechanism of activation.

In the experiments described below we have attempted to show that there are quite distinct amine-sensitive and amine-insensitive electron transport systems in chloroplasts. We have shown that the amine-sensitive pathway is closely related to the phosphorylation-coupled system described by AVRON *et al.*[3,4] and ARNON *et al.*[5]. Some evidence which may bear on the relative reducing potentials of the amine-sensitive and the amine-insensitive pathways is presented and the nature of the competition between these systems is described. Preliminary experiments measuring the efficiency of ATP formation and the effect of amines on this efficiency are also described.

METHODS

Chloroplasts from greenhouse-grown peas (*Pisum sativum*), variety Alaska, were used in all the experiments described below. Freshly picked pea leaves from young plants were placed in a chilled mortar with approximately isotonic KCl or sucrose buffers and ground for about 1 min with a pestle. The resulting mash was strained through glass-wool into chilled centrifuge tubes and the chloroplast suspension was spun for about 3 min at 3000–4000 $\times g$. The chloroplasts which had been thrown down were re-suspended in ice-cold buffer, spun down again and again suspended in buffer. This final suspension was filtered through glass-wool and stored in a vessel well submerged in an ice bath. Aliquots of the suspension were made up to 80 % acetone and centrifuged, and the chlorophyll concentration was determined from the extinction coefficients of MACKINNEY[6]. A number of different buffers were used for isolation of the chloroplasts and as part of the reaction medium. These are described separately for each experiment.

In most of the experiments the reduction of ferricyanide was followed by measuring the decrease in O.D. of the reaction mixture at 420 mμ. The apparatus is shown diagrammatically in Fig. 1a. The monochromatic beam from a Beckman D.U. spectrophotometer passed through the 16 holes of a disc rotating at 1800 rev./min. The "chopped" beam then passed through the reaction vessel (a standard 1-cm cuvette) through a liquid filter, and finally impinged on a photomultiplier tube. By means of a load resistor and a capacitor the alternating component of the tube's output was fed into the microphone input of a standard audio amplifier. A small capacitor was placed across the input to the amplifier to eliminate most of the predominately high frequency photomultiplier noise. The voltage from the amplifier's

output transformer (500 Ohm tap working into a 1000 Ohm load) was fed to an LC series resonant circuit tuned to 480 cyc./sec. The voltage across the inductance, which was about 5 times the input voltage and very low in noise, was rectified, smoothed and fed through a voltage divider to a Brown recorder. The actinic light consisted of an intense beam from a 300-W projector for 35 mm transparencies and was filtered through a red glass filter which transmitted only wave-lengths longer than 620 mμ. The projector was powered by ripple-free direct current. Light intensities were varied by the use of neutral filters. The liquid filter in the monochromatic beam (F) consisted of a solution made by adding copper sulfate to a concentrated solution of ammonium carbonate. This filter was practically opaque to the red actinic light, but transmitted nearly all of the blue measuring beam. Thus practically none of the actinic light scattered by the chloroplasts could reach the photomultiplier tube, and if any did, it was unmodulated and could give no response through the alternating current amplification system. The intensity of the actinic light was sufficient to saturate even at the highest reaction rates. At the beginning of each run the complete reaction mixture (2.0 ml) was placed in the cuvette and the slit-width of the monochromator was varied until the "optical density" indicated on the recorder equalled the known O.D. of the amount of ferricyanide present. Thus, regardless of the true O.D. of the suspension, the recorder registered at all times throughout the experiment only that part of the O.D. due to ferricyanide. Because of the tremendous cumulative gain of the photomultiplier tube, amplifier and tuned circuit and the low noise level of the overall system, it was possible to use very turbid suspensions and high concentrations of ferricyanide when this was necessary for special purposes. However, the experiments described here were carried out with relatively low concentrations, and hence with fairly transparent reaction mixtures.

This photometric equipment is quite versatile, because two independent methods of separating the effects of the actinic light and the measuring light are employed: separation by the use of complementary filters and separation on the basis of modulation. Thus with appropriate changes in the filters other photochemical reactions may be studied. For instance, by using a solution of methylene blue in the liquid filter (F) and a 340 mμ measuring beam, it was possible to follow directly the effect of light on the reduction of TPN^+ by chloroplasts.

All the reactions reported here were conducted with constantly rising temperatures. However, probably because of the short duration of the runs (usually 2 min), there seemed to have been no serious loss of reproducibility. The starting temperature with ice-cold reagents pipetted into a cuvette at room temperature was usually 6 to 8° and the final temperature was from 10 to 14°.

In some experiments oxygen production or consumption was measured instead of oxidant disappearance. For these experiments manometers and a special illuminated bath were used. The determinations were made over a longer time, usually 15 min, and the temperature was held at 8°. Reflector type incandescent lamps provided a saturating or nearly saturating light intensity.

The amount of ATP formed during the reduction of ferricyanide was determined through the almost stoichiometric formation of glucose-6-phosphate and TPNH. The TPNH was measured by its light absorption at 340 mμ, by assuming a molar extinction coefficient of $6.3 \cdot 10^3$. The 2.0-ml reaction mixture contained a tris(hydroxymethyl) aminomethane–phosphate buffer at pH 7.9, AMP, ADP, sodium fluoride, hexokinase,

glucose, magnesium sulfate, potassium ferricyanide and freshly isolated chloroplasts containing 30–60 μg chlorophyll. After the reaction the mixture was transferred to a centrifuge tube and the cuvette was rinsed twice with 1.0 ml $M/15$ phosphate buffer, pH 7.3. The reaction mixture and washings were combined and immediately heated in a boiling water bath for 5 min. After cooling cysteine (2 μmoles in 0.2 ml), TPN^+ (1.0 μmoles in 0.1 ml) and glucose-6-phosphate dehydrogenase (0.15 mg in 0.1 ml) were added and the suspension was centrifuged until it was clear. The O.D. at 340 mμ were read after 6 h at room temperature. Since the O.D. resulting from 1.0 μmoles of TPNH in the total volume of 4.4 ml is 6.3/4.4 or 1.43, the observed O.D. were divided by 1.43 to give the number of μmoles of TPNH formed. The O.D. resulting from the addition of known amounts of ATP indicated a good recovery of TPNH. However the blanks, that is the O.D. of reaction mixtures without added ATP and without illumination, were high and somewhat erratic. For this reason the data on ATP formation must be considered only semi-quantitative until further investigations of the reaction system have been completed.

The adenosine phosphates (ATP, ADP and AMP), the triphosphopyridine nucleotide (TPN^+), and the hexokinase were obtained from Nutritional Biochemical Corporation, Cleveland, Ohio. The glucose-6-phosphate dehydrogenase was obtained from Sigma Chemical Company, St. Louis, Mo. The catalase was prepared from horse liver by the method of KEILIN AND HARTREE[7].

EXPERIMENTAL

Activation of the HILL *reaction by naturally occurring bases*

At the Annual Meeting of the American Society of Plant Physiologists in 1957, the author reported that basic substances capable of increasing HILL reaction rates manyfold occurred in aqueous extracts of spinach leaves, horse liver and baker's yeast. These substances were destroyed by boiling nitric acid. Amines, including ammonia, are often destroyed by hot nitric acid in the presence of organic matter, presumably because some of the nitric acid is reduced to nitrous acid which readily attacks amines. Consequently it was suspected that the unknown active substances were amines.

Figs. 1b, c and d summarize the data presented at the 1957 meeting. The catalytic bases were obtained in the following manner: Tissue was ground, mixed with water and boiled for a few minutes. The filtrate was extracted with phenol. Ether insoluble materials such as nucleotides and other salts were extracted from the phenol into water by the addition of several volumes of water saturated ether. The water phase was then passed alternately through several columns of Amberlite IR_4B (quaternary amine) resin and Amberlite IRC_{50} (carboxylic acid) resin. The active bases were eluted from the carboxylic acid resin with 1.0 N acetic acid and the acetic acid was removed by distillation at reduced pressure. The dry residue was taken up in a small volume of water, neutralized with dilute NaOH and tested in the spectrophotometric apparatus depicted in Fig. 1a. Very small amounts of active material were obtained by this laborious procedure.

Figs. 1b and c show that the reduction of ferricyanide by illuminated chloroplasts was about 6 times as rapid in the presence of these catalytic bases as in their absence. However, from Fig. 1c it can be seen that the ability of the chloroplasts to respond to

the bases was quite labile. After 1.5 h at room temperature addition of the erstwhile activators had no effect. In the same time the uncatalysed rate remained completely unchanged.

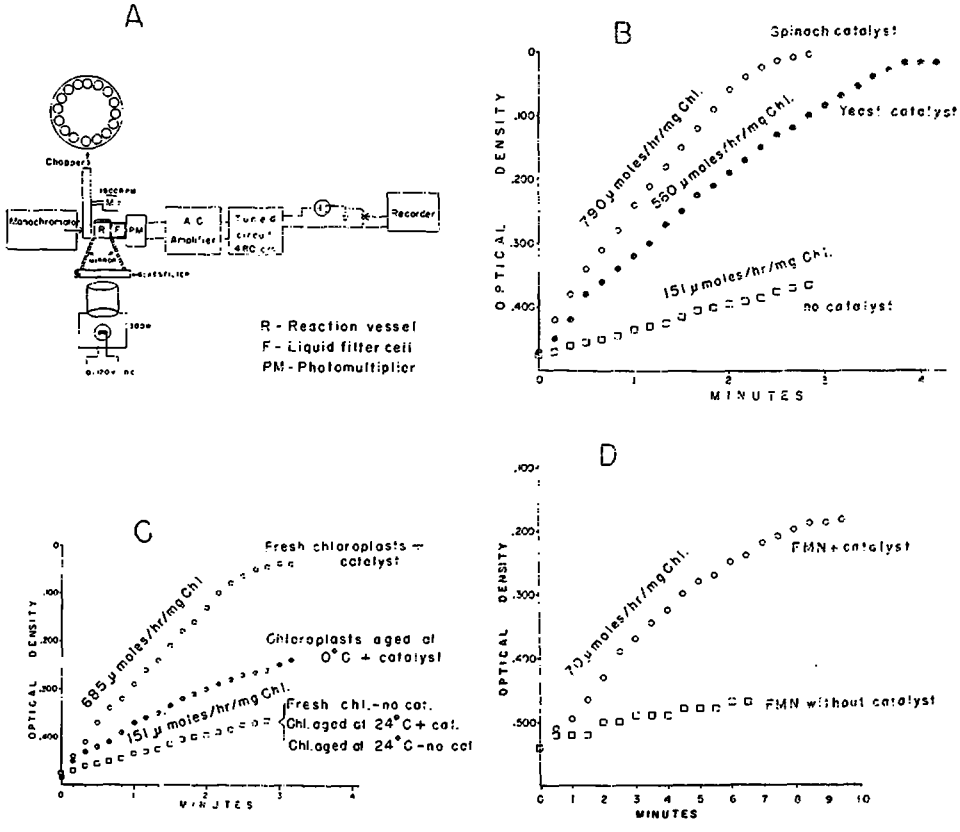

Fig. 1. a. Diagram of the reaction vessel, light sources and associated photometric equipment. The actinic light was filtered through red glass which transmitted only those wave lengths longer than 620 mμ. The complementary liquid filter (F) in the monochromatic measuring beam (420 mμ) consisted of a solution of cupric ammonium carbonate. See METHODS. b. The reduction of ferricyanide by illuminated chloroplasts and the catalytic effects of bases extracted from yeast and spinach leaves. Buffer: 0.033 *M* sodium phosphate, pH 7.0. containing 16 g KCl/l. Potassium ferricyanide 0.89 μmole. Total volume 2.0 ml. c. The effect of ageing chloroplast preparations on the ability of the chloroplasts to respond to the catalytic bases. Reaction system as in Fig. 1b. Bases extracted from spinach leaves as described in text. Chloroplasts aged 20 h at 0° or 1.5 h at 24°. d. The anaerobic reduction of flavin mononucleotide (FMN) by illuminated chloroplasts. Buffer as in Fig. 1a. Catalase in large excess and ethanol to 2 % added. See text for a description of the overall reaction.

The anaerobic reduction of flavin mononucleotide (Fig. 1d) requires special comment. In order to demonstrate the reduction of such autoxidizable substances by chloroplasts, an accessory hydrogen peroxide trapping system is required. Thus

$$FMN + H_2O \xrightarrow[\text{light}]{\text{chloroplasts}} FMNH_2 + \tfrac{1}{2}O_2 \quad (1)$$

$$FMNH_2 + O_2 \longrightarrow FMN + H_2O_2 \quad (2)$$

$$H_2O_2 \longrightarrow H_2O + \tfrac{1}{2}O_2 \quad (3)$$

and consequently there is no net reaction. However, in the presence of ethanol and excess catalase reaction (3) no longer occurs[8]. Thus there is a net uptake of oxygen in aerobic systems and a net reduction of flavin in anaerobic systems. The experiment illustrated by Fig. 1d was therefore conducted in an anaerobic cuvette with added ethanol and catalase. It is clear that the activating bases were necessary for FMN reduction.

The reduction of oxidants in the presence of illuminated chloroplasts is ordinarily interpreted as evidence of the HILL reaction, that is, as evidence of the functioning of one part of the photosynthetic process. However, when we observed these strikingly enhanced rates of ferricyanide reduction, we had some misgivings. Our spectrophotometric data, which represented the reduction of very small amounts of fericyanide, could easily have indicated uninteresting, non-biological photosensitized oxidations. Consequently it seemed imperative that the conclusions drawn from these experiments should be confirmed (a) by showing that the higher rates of reduction could be sustained, and (b) by demonstrating a similar increase in the rate of oxygen production. Therefore we resorted to the use of manometers and measurements were continued over longer periods. The experiment illustrated in Table I successfully established both points; oxygen production was stimulated and the stimulation persisted for at least 15 min.

TABLE I

THE EFFECT OF A CATALYST FROM SPINACH LEAVES ON THE RATE OF OXYGEN EVOLUTION BY ILLUMINATED CHLOROPLASTS

Manometric data. Buffer: 16 g KCl/l, 0.033 *M* sodium phosphate, pH 7.0. Potassium hydroxide (10 %) in center wells. Temperature 8°. Incandescent lights. The light intensity and the chlorophyll content of the chloroplasts were not measured.

Oxidant	*Oxygen evolution (µl/15 min)*	
	No catalyst	*Catalyst added*
Ferricyanide (22 µmoles)	6.8	32
Quinone (10 µmoles)	12.7	24
FMN (5 µmoles) + Catalase + Ethanol	0	−20.2*

* The rate of net oxygen uptake in this system equals the rate of oxygen production by the chloroplasts[1].

Two other facts were established by the data of Table I. Oxygen production with benzoquinone as oxidant was increased by the catalytic bases. Moreover FMN reduction, as measured by oxygen uptake in the presence of the peroxide trapping catalase and ethanol, was not only increased by these catalysts; it depended on them completely.

Activation of the HILL *reaction by amines*

Since the naturally occurring activators of the HILL reaction had the properties of amines, a number of known amines were tested on the ferricyanide illuminated chloroplast system. Table II summarizes some of the results. In other experiments it was found that glycine, choline and aniline were inactive while tryptamine and the ethyl ester of glycine were active. Obviously tris(hydroxymethyl)aminomethane,

which has long been used interchangeably with phosphate as a buffer for chloroplast reactions, is also inactive. A consistent pattern is evident in these data. All "monofunctional" amines, with the exception of the very weakly basic aniline, stimulated ferricyanide reduction while all of the "polyfunctional" amines tested were inactive. The most conspicuous difference in the properties of these two groups of amines is the degree of polarity; the monofunctional amines in their unionized forms are quite lipid soluble, while none of the polyfunctional amines could be expected to have any lipid soluble form at or near neutrality.

TABLE II

THE EFFECT OF VARIOUS AMINES ON THE RATE OF REDUCTION OF FERRICYANIDE BY ILLUMINATED CHLOROPLASTS

Ferricyanide reduction was determined spectrophotometrically. The reaction mixture (total volume 2.0 ml) consisted of buffer (16 g KCl, 0.02 moles tris(hydroxymethyl)aminomethane and 0.02 moles sodium dihydrogenphosphate/l adjusted to pH 7.5), potassium ferricyanide (0.89 μmole), and chloroplasts containing 22.5 μg chlorophyll. The amines were all 0.002 M Reaction time: 2.0 min.

Amine used	*Rate of ferricyanide reduction (moles × 10^{-4}/mg chl./h)*	*Increase due to amine (moles × 10^{-4}/mg chl./h)*
(A) Monofunctional amines		
None	2.9	—
Ammonia	8.1	5.2
Methylamine	5.4	2.5
Ethylamine	5.4	2.5
n-hexylamine	3.9	1.0
Cyclohexylamine	3.9	1.0
Diethylamine	3.9	1.0
Triethylamine	3.6	0.7
(B) Polyfunctional amines		
None	2.7	—
1,2-diaminoethane	2.7	0
1,5-diaminopentane	2.7	0
2-aminoethanol	2.7	0
Lysine	2.7	0
Arginine	2.7	0
2-amino-1,2,4-triazole	2.7	0
Hydroxylamine	1.8	—0.9 (inhibitory)

The status of triethylamine is crucial if one is to postulate mechanisms by which the activation occurs; tertiary amines enter into very few of the reactions characteristic of primary and secondary amines. Therefore the triethylamine was carefully purified, first by incubation with benzoylchloride to remove any reactive primary and secondary amines and then by crystallization of the hydrochloride from a mixture of benzene and alcohol. The purified triethylamine still stimulated ferricyanide reduction several-fold when large amounts were used.

Table III shows that, as in the case of the unknown naturally occurring activator, the stimulation of ferricyanide reduction by ammonia or methylamine was accompanied by the expected stimulation of oxygen production. Again oxygen production with benzoquinone as oxidant was similarly accelerated and again the reduction of FMN depended on the presence of the activator.

TABLE III

THE EFFECT OF METHYLAMINE AND AMMONIA ON THE RATE OF OXYGEN EVOLUTION BY ILLUMINATED CHLOROPLASTS

Manometric data. Buffer: 0.25 *M* sucrose, 0.01 *M* NaCl, 0.035 *M* sodium phosphate; pH 7.2. Potassium hydroxide (10%) in the center wells. Temperature 8°. Incandescent lights. The light intensity and chlorophyll content of the chloroplasts were not measured

Oxidant	*Oxygen evolution (μl/15 min)*		
	No catalyst	*Methylamine (0.03 M)*	*Ammonia (0.002 M)*
Ferricyanide (15 μmoles)	8	12	16
Quinone (6.5 μmoles)	12	27	40
FMN (1.25 μmoles) + Catalase + Ethanol	0	−10*	−13*

* The rate of net oxygen uptake in this system equals the rate of oxygen production by the chloroplasts[1].

Table IV shows that the stimulation by methylamine is a function of the concentration of the unionized amine. It shows also that the mechanism of stimulation is not otherwise dependent on the hydrogen ion concentration of the medium. This dependence on the unionized molecule is consistent with the earlier observation that the polar amines are inactive since all the ionized molcules also would be polar. When allowance is made for the different p*K*'s, most of the differences between the activities of the low molecular weight amines and ammonia disappear. However, the larger amines, such as tryptamine and triethylamine, are definitely less active even on the basis of the concentration of unionized molecules.

The reciprocal of the activation plotted against the reciprocal of the amine concentration gives a straight line (Fig. 2). This suggests that the amine somehow functions as a reactant in the system catalysed. The stimulation by methylamine is

TABLE IV

DEPENDENCE OF THE METHYLAMINE STIMULATION OF THE AMOUNT OF UNIONIZED AMINE

Ferricyanide reduction was determined spectrophotometrically. The reaction mixture consisted of buffer, potassium ferricyanide (0.89 μmole), methylamine in the amounts specified, and chloroplasts containing 27.5 μg chlorophyll. The three buffers contained 0.25 *M* sucrose, 0.01 *M* NaCl, 0.033 *M* sodium phosphate, and 0.016 *M* tris(hydroxymethyl)aminomethane. They were adjusted to pH's 6.8, 7.3 and 7.8 with HCl or NaOH. The total volume of the system was 2.0 ml and the duration of each experiment was 2 min.

Concentration of amino ($M \times 10^{-3}$)	*pH*	*Concentration of unionized amine ($M \times 10^{-6}$)*	*Increase in rate of ferricyanide reduction (μmoles/mg chl/h)*
60	6.8	8.7	530
20	7.3	9.1	424
6	7.8	8.7	500
30	6.8	4.35	320
10	7.3	4.45	275
3	7.8	4.35	344

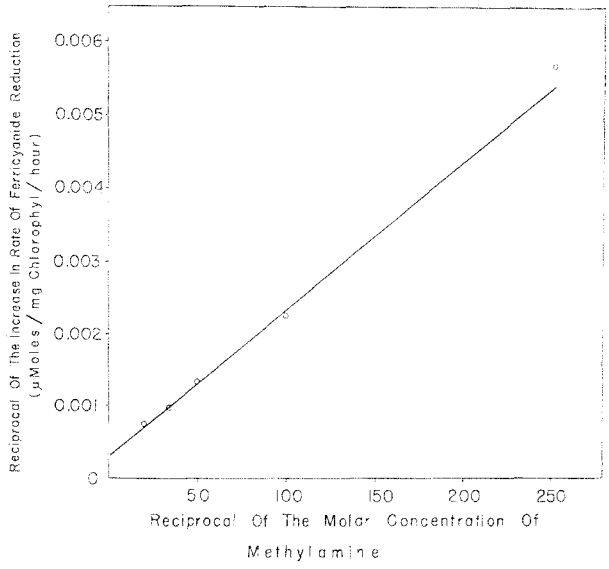

Fig. 2. The increase in rate of ferricyanide reduction by illuminated chloroplasts as a function of the concentration of methylamine. Buffer: 0.25 *M* sucrose, 0.01 *M* NaCl, 0.035 *M* sodium phosphate, pH 7.2. Potassium ferricyanide: 0.89 μmole. Chlorophyll content of chloroplasts: 18 μg. Total volume: 2.0 ml. Reaction time: 2.0 min. Rate without methylamine: 101 μmoles ferricyanide reduced/mg chlorophyll/h.

also completely reversible. Chloroplasts may be treated with very high concentrations of methylamine and then washed free thereof without being changed in any detectable manner; the abilities to reduce ferricyanide in the absence of amines, to be stimulated by amines and to be stimulated by the components of the phosphorylating system are retained unaltered. One is therefore tempted to suppose that the amine is directly involved in some of the reactions associated with electron transport. However, it has not been possible to obtain evidence of amine disappearance. Concentrations of ammonium sulfate which represent much less than one ammonia molecule for each molecule of ferricyanide reduced gave appreciable and persistent stimulations. Moreover after the ferricyanide had been reduced, the amount of ammonia, as measured by Nessler's reagent, had not diminished.

Amines and the phosphorylation of ADP

AVRON, KROGMANN AND JAGENDORF[3] and ARNON *et al.*[5] have shown that the rate of reduction of ferricyanide by illuminated chloroplasts is considerably increased by the presence of the components of a system which phosphorylates ADP. The stimulation requires the simultaneous presence of ADP, orthophosphate and magnesium ion[3]. They therefore concluded that part of the electron transport is obligatorily coupled to ADP phosphorylation. On the other hand, KROGMANN, JAGENDORF AND AVRON[2] have shown that very high rates of ferricyanide reduction may be achieved without concomitant phosphorylation if ammonium sulfate is present. This they have attributed to an "uncoupling" of phosphorylation by the ammonium sulfate. We have been able to confirm all these observations. We have found that the rate of ferricyanide reduction by freshly made pea chloroplasts preparations may be increased approximately

threefold by the presence of ADP (or IDP), phosphate and magnesium ion. The exclusion of ADP, phosphate or magnesium prevented the increase. And, of course, ammonia and many amines activate the process of ferricyanide reduction.

There are thus at least two means by which increased electron transport capacity may be conferred on chloroplasts—the addition of amines or the addition of the components of the phosphorylating system. The series of experiments illustrated by Fig. 3 was designed to establish the similarities and dissimilarities of the two activations and to compare the activated condition with the unstimulated condition. Chloroplasts were isolated and stored at 0° in buffers of different pH. At intervals the rates of ferricyanide reduction were determined (a) in the presence of methylamine, (b) with ADP, phosphate and magnesium ion, (c) with both methylamine and the phosphorylating system and (d) without addenda. Obviously the stimulation by methylamine is akin to the stimulation by the phosphorylating system since the

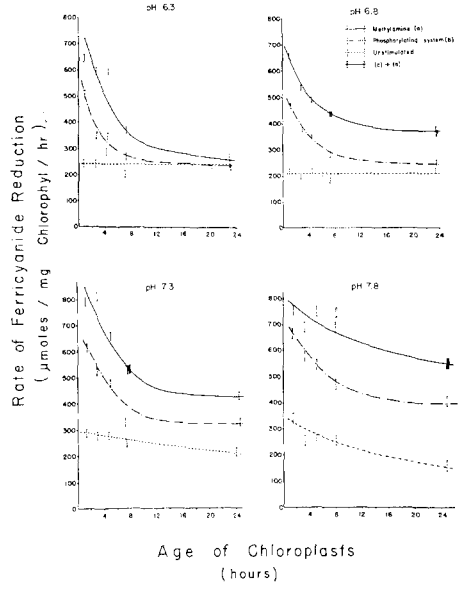

Fig. 3. The rate of ferricyanide reduction by illuminated chloroplasts as a function of the age of the chloroplast preparations and the pH of the storage medium. The chloroplasts were stored at 0° in buffers containing 0.4 *M* sucrose, 0.01 *M* NaCl, 0.01 *M* tris(hydroxymethyl)aminomethane, and 0.01 *M* sodium dihydrogen phosphate. These buffers were adjusted to pH's 6.3, 6.8, 7.3 and 7.8 by addition of HCl or NaOH. All reactions were conducted at pH 7.9 in buffer containing 0.4 *M* sucrose, 0.01 *M* NaCl, 0.05 *M* tris(hydroxymethyl)aminomethane and 0.05 *M* sodium phos phate. Potassium ferricyanide (0.89 μmole) was added to each reaction mixture and its disappearance was determined by following the decrease in O.D. at 420 mμ. Final volume: 2.0 ml. Reaction time: 2.0 min. Methylamine was added to give a final concentration 0.02 molar. The "phosphorylating system" consisted of the following addenda: ATP (2 μmoles), hexokinase (0.4 mg), glucose (40 μmoles), and magnesium sulfate (15 μmoles). The length of the vertical lines indicates the error introduced by reading the recorder charts to the nearest half millimeter.

abilities of the chloroplasts to respond show the same pattern of decay. Both the amine-activated electron transport and the phosphorylation-dependent transport involve pathways which are labile below pH 7.0, but fairly stable at pH 7.8. Furthermore, the rate of ferricyanide reduction in the presence of both methylamine and the phosphorylating system was the same as the rate with methylamine alone. Since the rates of transport by the two activated pathways never seemed to be additive when optimal amounts of amine were used, the pathways must share a rate limiting step. Consequently they must compete for electrons and one would expect an apparent "uncoupling" of phosphorylation and ferricyanide reduction if the amine-activated transport competes on favorable terms with the phosphorylating pathway. Alternatively the amine might actually uncouple the phosphorylation step by modifying the structure or environment of some enzyme in a manner which permitted an electron transfer without the normally associated phosphorylation.

Either mechanism of amine activation—true uncoupling or effective competition

from a process parallel to the phosphorylating process—would decrease the efficiency of phosphorylation, perhaps even to the point of suppressing phosphorylation altogether. In order to determine the amount of phosphorylation in terms of the electron transport and to investigate the effect of amines thereon, experiments were undertaken in which the formation of ATP and the reduction of ferricyanide were measured simultaneously. ATP production was determined through the hexokinase-catalysed formation of glucose-6-phosphate and the subsequent reduction of an equivalent amount of TPN^+ by glucose-6-phosphate dehydrogenase. Fig. 4 shows that ATP added to the complete reaction mixture may be measured practically quantitatively as TPNH. Unfortunately there was a large and rather variable amount of TPN^+ reduction in the absence of added ATP and in the dark. Part of this TPN^+ reduction was traced to a slight contamination of the ADP with ATP. Another part may have been due to the myokinase activity of the preparations; to inhibit this fluoride and AMP were added[9]. A more systematic investigation of these background reactions must be

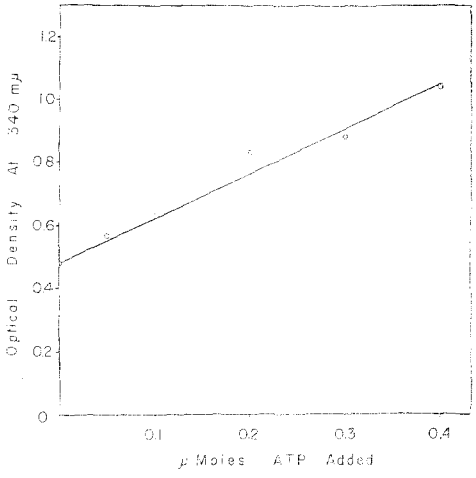

Fig. 4. The formation of reduced triphosphopyridine nucleotide (TPNH) as a function of the amount of added ATP. The reaction mixture consisted of buffer, AMP, ADP, sodium fluoride, hexokinase, glucose, magnesium sulfate, potassium ferricyanide and chloroplasts as described in Table V. ATP was added in the indicated amounts and the mixture was allowed to stand for 10 min. It was then diluted with 2.0 ml of phosphate buffer (pH 7.3), boiled for 5 min and cooled. Cysteine (2 μmoles), TPN (1 μmole) and glucose-6-phosphate dehydrogenase (0.15 mg) were added. The mixture was then centrifuged and the O.D. at 340 mμ was measured after 6 h at room temperature. The points show the values actually obtained, while the line indicates the values to be expected on the assumption that each mole of ATP results in the reduction of one mole of TPN^+.

undertaken before data such as those presented in Tables V and VI should be considered conclusive. Nevertheless the production of ATP as a concomitant of the reduction of ferricyanide—inferred by AVRON *et al.*[3,4] and ARNON *et al.*[5] from the existence of an ADP-dependent phosphate uptake, and measured directly by KROGMANN *et al.*[2]—is again confirmed by the data of Table V. The data of Table V also support the concept of "coupled phosphorylation" by showing a dependence of ATP formation on the presence of an oxidant. As expected methylamine prevented ATP formation.

Enough data were accumulated to permit a tentative estimate of the quantitative relation of ATP formation to electron transport, and hence a reasonable prediction of the stoichiometry of the phosphorylation reaction. However, the estimate is very much complicated by the known presence of a system which operates in the absence of phosphorylation—the amine-insensitive, ADP-independent "basic" HILL reaction. Obviously an evaluation of the competition for electrons between the phosphorylating system and the "basic" system is necessary before the efficiency of the phosphorylating reaction can be determined even approximately. Such an evaluation may be approached by comparing ATP yields at different light intensities.

TABLE V

THE INFLUENCE OF FERRICYANIDE AND METHYLAMINE ON THE FORMATION OF ATP BY ILLUMINATED CHLOROPLASTS

ATP was measured through the formation of glucose-6-phosphate and the subsequent reduction of an equivalent amount of TPN by glucose-6-phosphate dehydrogenase. The complete system consisted of buffer, AMP (6 μmoles), ADP (6 μmoles), sodium fluoride (20 μmoles), hexokinase (0.3 mg), glucose (40 μmoles), magnesium sulfate (20 μmoles), potassium ferricyanide (0.89 μmole) and chloroplasts containing 34 μg chlorophyll. The total volume was 2.0 ml. 28 μmoles of methylamine were used. The buffer consisted of 0.4 *M* sucrose, 0.05 *M* tris(hydroxymethyl)aminomethane, 0.01 *M* NaCl, 0.25 *M* KH_2PO_4. The pH was adjusted to 7.9.

Experiment	*O.D. at 340 mμ*	*TPNH equivalents (μmoles)*	*ATP formed due to light (μmoles)*
Complete system (dark)	0.220	0.154	—
Complete system (light)	0.660	0.460	0.306
Complete system + Methylamine (light)	0.255	0.178	0.024
System less ferricyanide (light)	0.295	0.206	0.052

TABLE VI

THE EFFICIENCY OF ATP FORMATION

Reaction system as in Table V. In the presence of optimal amounts of methylamine the limiting light intensity gave 44 % of the rate with saturating light. The saturating light was eight times more intense than the limiting light. Chloroplasts containing 45 μg chlorophyll were used and 0.89 μmole of ferricyanide were reduced. Values represent the average of six determinations.

Experiment	*O.D. at 340 mμ*	*TPNH equivalents (μmoles)*	*ATP due to light (μmoles)*	*Moles ferricyanide reduced/ mole ATP formed*
Dark controls	0.394	0.275	—	—
Limiting light	0.842	0.592	0.315	2.8
Saturating light	0.552	0.386	0.111	8.0

We suspect that the combined phosphorylating and non-phosphorylating systems are rate-limiting at high light intensities, because amines acting at or near the phosphorylating electron transfer cause a considerable additional activation (Fig. 2). Consequently at high light intensities both processes probably operate without serious competition and near capacity. Therefore the contribution of each may be deduced from the rates with and without the presence of the components of the phosphorylating system. For instance, if at high light intensity the rate in the presence of ADP, phosphate and magnesium ion is twice the "basic" rate, it is probably safe to assume that one half of the electrons are transferred by the phosphorylation-coupled pathway, and that the efficiency of the phosphorylation-coupled pathway. may be taken as twice the overall efficiency. KROGMANN *et al.*[2] have used this method. On the other hand, the two processes of electron transfer must compete at very low light intensities, although the nature of this competition cannot be predicted *a priori*. Actually, we find that the overall efficiency of phosphorylation with severe competition (*i.e.*, low light intensity) is similar to the calculated value obtained in the manner described above for conditions of no competition (high light intensity). This can only

mean that the phosphorylating pathway competes for electrons (or hydrogens) very favorably with the ADP-independent pathway. Our preliminary observations (Table VI) indicate that the transfer of somewhat less than 3 electrons was required for the formation of one ATP. This value probably approaches the value for the coupled system alone, and agrees reasonably well with the values reported by ARNON *et al.*[5], KROGMANN *et al.*[2] and AVRON *et al.*[4]. The determination by AVRON *et al.* seems also to have been made under conditions which would produce competition between the two systems. From their data a rate of 200 μmoles ferricyanide reduced/mg chlorophyll/h may be calculated; this probably represents considerably less than 50 % of the maximum attainable reaction velocity and therefore suggests the use of rather low light intensities. ARNON *et al.* gave no information from which rates or light intensities could be deduced.

A simple mechanism of phosphorylation—the creation of a high energy bond by the oxidation or reduction of an organic substance—would in all probability require the transfer of two electrons (or two hydrogens) for each ATP formed. The experimental value of 2.8 in Table VI, perhaps adjusted slightly downward to allow for some competition from the uncoupled system and also to allow for less than perfect recovery of the ATP formed, certainly points to the theoretical value of 2.0. However, we must beware of accepting too uncritically the implications of this convergence of the data from several laboratories on the gratifying value of 2.0; evaluation of the competition between the phosphorylating and the non-phosphorylating systems is still so uncertain and estimates of efficiencies are consequently so ambiguous that the postulated stoichiometry of the phosphorylation reaction could be quite wrong.

DISCUSSION

It has long been apparent that photosynthesis must consist, basically, of two processes, (a) the fixation of carbon dioxide in some organic substance, presumably to form a carboxyl group, and (b) the reduction of this carboxyl to the aldehyde level by a reductant formed in the photochemical dissociation of water. Unfortunately the direct reduction of carboxyl groups by any known biological reductant is a thermodynamic impossibility; when carboxyls are reduced in respiratory systems the combined action of pyridine nucleotides and ATP is required. It was necessary to suppose, therefore, either that the photosynthetic reductant formed *in vivo* by illuminated chloroplasts was an unknown substance of exceptional reducing potential or that the carboxyl reduction was accomplished in a coupled reaction analogous to the reactions in the respiratory system. When radioactive carbon dioxide was used to trace the "path of carbon" in photosynthesis, it was found[10] that the photosynthetic intermediates were, by and large, the intermediates of respiration. The participation of reduced pyridine nucleotides accordingly became more and more probable. Meanwhile a number of workers were able to show that illuminated chloroplasts could in fact reduce pyridine nucleotides, especially TPN^+ [11–13]. SAN PIETRO AND LANG[13] were even able to obtain a protein from chloroplasts which catalysed the reduction, photosynthetic pyridine nucleotide reductase. Since the involvement of the pyridine nucleotides in photosynthesis seemed very probable, the question of the origin of the associated ATP was raised. At least one author[14] suggested that the ATP might be

formed by the phosphorylation reactions of respiration using the primary photosynthetic reductant as electron donor. However, such a mechanism should result in an appreciable increase in oxygen uptake, an increase which does not seem to occur[15]. The problem was resolved independently by ARNON *et al.*[16] and FRENKEL[17] when they found a light-induced, oxygen-independent formation of ATP in *in vitro* preparations from photosynthetic organisms. This process has come to be called "photosynthetic phosphorylation" or "photophosphorylation".

The mechanism of photosynthetic phosphorylation has been the subject of much speculation. ARNON[18] originally suggested that the process was similar, in its essentials, to the process of oxidative phosphorylation in respiration, perhaps differing therefrom only in that precursors of the photosynthetically produced oxygen replaced molecular oxygen as the ultimate electron acceptor. He was led to this conclusion by the fact that some substances involved in the respiratory chain, for example FMN, seemed to be necessary for photosynthetic phosphorylation. However, there were objections to the concept. The requirement for FMN could be satisfied by other substances, such as phenazine methosulfate, which certainly are not intermediates in respiration. Moreover the mechanism proposed represented a somewhat uneconomic back-reaction which would necessarily lower the overall quantum efficiency of photosynthesis below the efficiency of the HILL reaction. Since the data of WAYRYNEN[19] and unpublished experiments by the author have shown that the quantum efficiency of photosynthesis and the HILL reaction are actually very nearly the same, it seems improbable that large amounts of ATP could be produced by this means. Whether we accept the conservative American estimates of quantum yields[20–22] or the more optimistic estimates of WARBURG AND NEGELEIN[23], photosynthesis is so efficient that we must look upon any mechanism which is inherently wasteful as of doubtful importance.

More recently ARNON and his colleagues[5] have produced evidence which suggests that 1 mole of ATP is formed during the reduction of 1 mole of TPN^+ (or 2 moles of ferricyanide). These observations have been confirmed by AVRON *et al.*[4]. If one assumes that each quantum of the red light absorbed by chlorophyll results in the photolysis of one molecule of water, the two quanta required for the reduction of a molecule of TPN^+ should yield more than enough usable chemical energy to form simultaneously a molecule of ATP. Consequently the new notion that the reduction of substances by chloroplasts may be directly linked with the phosphorylation of ADP seems more plausible in terms of efficient energy utilization than does the earlier concept of a phosphorylation-linked back-reaction of the products of the photolysis of water—the "cyclic phosphorylation" of ARNON. The discovery[3] that a large part of the electron transport in the HILL reaction depends on simultaneous phosphorylation and the observation of the converse dependence of phosphorylation on oxidant reduction both support the emerging picture of a phosphorylation-coupled HILL reaction so strongly that one is inclined to wonder if the phenomenon of "cyclic phosphorylation" is not an artifact caused by the spontaneous reoxidation of autoxidizable intermediates such as $FMNH_2$. In any case, it is no longer possible to doubt that chloroplasts can phosphorylate ADP in the course of reducing oxidants in general. The fact that the phosphorylation-independent pathway for the reduction of ferricyanide competes unfavorably with the phosphorylating pathway, and the fact that there is apparently no phosphorylation-independent pathway for the reduction of flavins, suggest that this "basic" system also may be unimportant *in vivo*. Perhaps it

too represents some artifact arising from the use of such powerful oxidants as ferricyanide or such effective protein denaturants as quinones.

The manner in which amines permit electron transfer while by-passing the associated phosphorylation can only be guessed. Lipid solubility of the amine seems to be a requisite for activity, since the more polar polyfunctional amines and the ionized forms of the monofunctional amines are all inactive. Consequently the site of action of the amines is probably enclosed in and permeated by lipoprotein—a not surprising observation when we consider the known composition of chloroplasts[24]. Primary, secondary and tertiary amines are all "uncouplers" or activators, but they do not seem to be consumed in whatever reactions they activate. Any reactions entered into by the amines therefore must be reversible. However, the only reversible reaction which is common to these three classes of amines is probably the process of taking on a proton to become a positive ion. It would seem therefore that the function of amines must be related to this ability to ionize. The author would like to suggest, very tentatively, that the amines may simply be acting as bases which can penetrate to sites not available to highly polar bases, thereby causing hydroxyl ion catalyzed cleavage of high energy bonds which would otherwise transfer their energy to the pyrophosphate bond of ATP.

Let us suppose that phosphorylation is achieved by the following mechanism, which incidentally predicts the transfer of two electrons for each ATP formed:
overall:

$$X + H_2O \xrightarrow[\text{chloroplasts}]{\text{light}} XH_2 + \tfrac{1}{2}O_2$$

$$XH_2 + \text{enzyme} \longrightarrow XH_2\text{-enzyme}$$

$$XH_2\text{-enzyme} + \text{oxidant} \longrightarrow X\sim\text{enzyme} + \text{reduced oxidant}$$

$$X\sim\text{enzyme} + \text{orthophosphate} \longrightarrow X\sim\text{phosphate} + \text{enzyme}$$

$$X\sim\text{phosphate} + \text{ADP} \longrightarrow X + \text{ATP}$$

$$\text{oxidant} + H_2O + \text{ADP} + \text{orthophosphate} \xrightarrow[\text{chloroplasts}]{\text{light}} \text{reduced oxidant} + \text{ATP} + \tfrac{1}{2}O_2$$

In this reaction-sequence the omission of either ADP or phosphate would prevent the reduction of oxidant and the formation of oxygen. However, the high energy bond in X ~ enzyme very probably would be labile, since a "high energy bond" is by definition a bond which can be broken with the expenditure of little energy. Consequently it is not unreasonable to suppose that hydroxyl ions associated with amines might catalyse the hydrolysis of such a bond, in which case the dependence of the system on phosphorylation would disappear. On the other hand, the direct reaction of strong oxidants with XH_2 might result in a phosphorylation-independent, amine-insensitive electron transfer, the "basic" system which, heretofore, has been the object of most HILL reaction studies.

The phosphorylation mechanism outlined above is analogous to the substrate level phosphorylations of respiration. It is, in fact, taken directly from the postulated mechanism of triosephosphate dehydrogenation. There are several reasons for relating the HILL reaction phosphorylations to substrate phosphorylation rather than to respiratory chain phosphorylations: (a) The apparent stoichiometry of two electrons transferred for each ATP formed coincides with the stoichiometry of the single stage phosphorylation-coupled exidation of substrates and not with the multistage oxidations through the respiratory chain. (b) HILL reaction phosphorylations may be

associated with the reduction of pyridine nucleotides. Consequently XH_2 must be an electron donor of reducing potential comparable to triosephosphate or α-ketoglutarate. (c) HILL reaction phosphorylations, like substrate phosphorylations, are not uncoupled from the associated electron transfers by 2,4-dinitrophenol. However, this analogy has limits; the author is not aware of any evidence which suggests that ammonia or amines affect the phosphorylations associated with the oxidation of α-ketoglutarate or triosephosphate.

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