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Historical corner

Photosynthesis by isolated chloroplasts: The early work in Berkeley

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It is a privilege to have been invited by the editors of this commemorative volume to recall some of the excitement that prevailed in Arnon's laboratory during the early years of the discovery of photophosphorylation and CO_2 fixation by isolated chloroplasts. What follows are observations by an active participant in a fascinating series of experiments that made a considerable impact on the way we now think of chloroplast metabolism.

I met Dan Arnon in 1948 when he was on sabbatical leave in Keilin's laboratory in Cambridge, England, where he was starting to apply biochemical techniques to his study of inorganic nutrients. Needless to say he already had a distinguished reputation in the field of plant nutrition. At this time I was completing a PhD on 'Coenzymes in Plants' under the supervision of Robin Hill. A postdoctoral appointment in Berkeley with Arnon led us to study the Hill reaction in isolated spinach chloroplasts with o-benzoquinone as the acceptor. We paid special attention to the involvement of metal ions and chloride in oxygen evolution. During this period my research education continued and I finally learned about the merits of scientific bookkeeping and realised the importance of detailed day to day discussion of results, as well as the desirability of designing every experiment with a view to possible publication, by paying particular attention to all the relevant controls.

There being no chance of continuing with a research appointment in 1950, I accepted a job in Sydney, Australia and there I learned some of the facts of academic life, becoming immersed in the teaching of biochemistry and accomplishing very little research. The only bright points were the fourth year student research projects in biochemistry, one of which by R.M. Smillie on mitochondrial respiration in leaves was to influence our thinking later.

I returned to Berkeley at the end of 1953 to join D.I. Arnon and M.B. Allen. The latter had previously worked on aspects of photosynthesis with C.S. French and C.B. van Niel. Our discussions became focused on the light reactions of isolated chloroplasts. On the basis of experiments with $^{14}CO_2$, a scheme had been put forward at that time (Bassham et al. 1954) for CO_2 fixation in green plants (the reductive pentose or Calvin cycle) that emphasized the need for ATP and reduced pyridine nucleotide for the reduction of the first product of CO_2 fixation, PGA; Ruben (1943) had earlier indicated the importance of ATP in the reduction of CO_2. Needless to say I found it easy to join enthusiastically into the discussion of where ATP was being synthesized and what part chloroplasts played.

At the time there were clear indications that isolated chloroplasts could photoreduce pyridine nucleotide, although none ever accumulated directly and its reduction could only be detected by adding a coupling enzyme system to the reaction vessel and allowing the accumulation of another reaction product, e.g. malate (Arnon 1951; Vishniac and Ochoa 1951) or lactate (Vishniac and Ochoa 1951). Vishniac and Ochoa (1952) coupled the photoreduction of DPN to its aerobic reoxidation by added animal mitochondria and were able to detect ATP formation as a consequence. This experiment was taken to indicate that the chloroplasts had a function restricted to producing a reductant that could subsequently be used to generate ATP in an oxidative reaction sequence in mitochondria.

In Berkeley we had difficulty in accepting this view because mitochondria are scarce in palisade parenchyma cells of the leaf, although these cells are photosynthetically the most active. We therefore decided

to look for ATP formation in illuminated chloroplast suspensions.

For our first experiments we modified our earlier protocol for isolating chloroplasts for the Hill reaction work (Arnon and Whatley 1949) and used 0.35 M sodium chloride as an osmotic replacement for 0.5 M glucose. This would enable us later to do $^{14}CO_2$ experiments. We used a simple grinding of spinach leaves with sand in a cooled mortar, followed by a speedy separation of the chloroplasts by a simple differential centrifugation. At 200 g × 1 min, the nuclei were sedimented, at 1000 g × 7 min the 'whole' chloroplasts were sedimented, leaving mechanically fragmented chloroplasts and mitochondria still in the supernatant. The 'whole' chloroplasts were quickly washed and resedimented. Although under the microscope they looked intact and had sharp outlines these 'whole' chloroplasts were later found to lack the external membrane of the intact chloroplast and were leaky. They were subsequently to be referred to as 'naked lamellar systems'. It was important for the 'whole' chloroplasts to be leaky since this allowed the substrate that we used as phosphate acceptor, AMP (later shown to be ADP), to come into contact with the thylakoids where phosphorylation was to occur. So we were lucky!

The reaction included dilute 'tris' buffer, substituting for the phosphate buffer of our Hill reaction experiments, and inorganic phosphate was now added as $K_2H^{32}PO_4$, which would be taken up if ATP was formed. The choice of *dilute* tris buffer was fortunate. It is an NH_4^+ analogue and as such is a weak uncoupler of photophosphorylation. NH_4^+ is a powerful uncoupler (Jagendorf 1958). Mg^{++} was added because ATPases were known generally to require it. Ascorbate was added on the basis of Molisch's silver staining technique, which indicated that $AgNO_3$ stains chloroplasts because they are rich in the reductant, ascorbate. We now suppose that the function of the added ascorbate, which enhanced our initial experiments, was to 'poise' and stabilise the system, rather than to act as a catalytic component.

The reaction was carried out in a Warburg flask at 15 °C under air or nitrogen and illuminated with a bank of incandescent lamps. After a period of illumination the formation of $AT^{32}P$ was determined by precipitating the inorganic ^{32}P as $MgNH_4{}^{32}PO_4$ and determining the radioactivity in the supernatant, which contained any organic phosphate. The identification of the product as ATP was shown by converting it to glucose-6-phosphate with hexokinase in the reaction mix or by adsorbing it on to activated Norite, and showing the release of inorganic phosphate in 7 min on heating at 100 °C in N-HCl. The glucose-6-phosphate formed in the hexokinase reaction was identified chromatographically.

Initial experiments showed that ATP was indeed formed on illumination of isolated chloroplasts and since the mitochondria had been eliminated during the chloroplast isolation procedure we could conclude that mitochondria were not involved in the ATP synthesis (Arnon et al. 1954b).

The 'whole' chloroplasts were also offered $^{14}CO_2$ under the same conditions as resulted in ATP formation to see if they could fix ^{14}C into intermediates of the Calvin cycle; this was successful in the early experiments. Although the ^{14}C uptake was small it continued for an hour and was accompanied by an equivalent evolution of oxygen which was manometrically determined. We could conclude that the Calvin cycle was operating and that we were not simply exchanging ^{14}C into pre-existing intermediates.

The preliminary conclusion then was that 'whole' chloroplasts can make ATP and fix $^{14}CO_2$, that chloroplasts are the unique site of photosynthesis in green plants and that they do not require the participation of other organelles to carry out photosynthesis (Arnon 1955). Although the initial rates of ATP synthesis and CO_2 fixation were very low they could be observed consistently and we had a firm base on which to carry out further research. We were soon successful in increasing the rates of ATP synthesis on a chlorophyll basis to very high levels. Although we were also able to push up the rates of CO_2 fixation on a chlorophyll basis, particularly in a reconstituted system, we never achieved rates comparable to those in the leaf; these rates were later to be accomplished by others using chloroplasts which still retained the outer membrane (Jensen and Bassham 1966; Walker 1971).

In isolated 'whole' chloroplasts the major insoluble product was starch and the soluble products identified by two-dimensional paper chromatography included phosphate esters of fructose, glucose, ribulose, sedoheptulose, dihydroxyacetone and phosphoglycerate, as well as glycine, aspartate and malate. This indicated right from the beginning of our researches that CO_2 fixation occurred in isolated chloroplasts by the same mechanism as in green algae and higher plants (Allen et al. 1955).

By treating 'whole' chloroplasts with dilute NaCl solution we obtained a pale brownish chloroplast extract (CE) and a preparation of 'broken chloroplasts'.

These were used to reconstitute the CO_2 fixing system. Addition of chloroplast extract obtained from an amount of 'whole' chloroplasts containing 2 mg chlorophyll (that used in the preliminary experiments) to only small amounts of broken chloroplasts resulted in good $^{14}CO_2$ fixation rates, which on a chlorophyll basis were perhaps tenfold better in the reconstituted system than in the original experiments. The products of $^{14}CO_2$ uptake were however similar (Whatley et al. 1956). All the enzyme systems needed to catalyse the reactions of the reductive pentose cycle were subsequently shown to be present in chloroplast extracts (Losada et al. 1960; Trebst et al. 1960). This confirmation eventually led to the exciting experiment in which the light and dark phases of photosynthesis were experimentally separated (Trebst et al. 1958).

In the original experiments (Arnon et al. 1954b) the light induced ATP synthesis occurred without net consumption of oxygen and without the need to supply any substrate to serve as an external energy source. The only 'substrate' consumed in photophosphorylation was light.

Although photophosphorylation when first discovered depended on the presence of air it soon became apparent that oxygen was needed only as a catalyst or a 'poising' agent. When FMN (flavine mononucleotide) and vitamin K_3 were found to act as catalysts for the ATP synthesis the need for oxygen disappeared at optimal concentration of either of these cofactors and the synthesis of ATP proceeded in the light in an atmosphere of nitrogen or argon (Arnon et al. 1955; Whatley et al. 1955). These observations were exciting as showing photophosphorylation as an anaerobic process, particularly in view of the demonstration by Frenkel (1954) that chromatophores (the bacterial equivalent of thylakoids) of *Rhodospirillum rubrum* can carry out an active light-dependent synthesis of ATP under strictly anaerobic conditions. As an example of a possible scientific interaction it might be mentioned that Albert Frenkel was studying effects of micronutrients on photoreduction in *Scenedesmus* while briefly visiting on sabbatical leave in Arnon's laboratory during the latter part of 1953, when the preliminary experiments on photophosphorylation in chloroplasts were being discussed. The suggestion to test chromatophores for their ability to photophosphorylate apparently came from Lipmann, in whose laboratory Frenkel spent the second part of his leave. Other investigators subsequently showed photophosphorylation in cell-free preparations of the obligately anaerobic photosynthetic bacterium, *Chromatium*. A common anaerobic mechanism for photophosphorylation thus appeared to be shared by photosynthetic bacteria and green plants.

Unfortunately the literature contains a number of reviews incorrectly according priority for the demonstration of photophosphorylation to Frenkel's work on chromatophores. Synthesis of ATP, as well as fixation of carbon dioxide by isolated chloroplasts, significantly predated the bacterial work by several months. This work was presented by D.I. Arnon at the 8th Botanical Congress in Paris, July 2–14, 1954 (Arnon et al. 1954a), the resumé being submitted on March 5th. The work on photophosphorylation in chloroplasts became more generally known from the paper by Arnon et al. published in Nature on August 28, 1954 (submitted May 19th) and less officially from a *New York Times* article of July 6, 1954. Frenkel's work on bacterial photophosphorylation was published in the Journal of the American Chemical Society on November 5, 1954 (submitted September 13) and refers to the earlier contribution from Arnon's laboratory.

For bacterial chromatophores to make ATP in the light the addition of an optimal amount of a reducing substance, such as $DPNH_2$, succinate or ascorbate was necessary under certain conditions. These additives are not used up during the photophosphorylation and appear only to affect the redox balance of the particles. Freshly prepared chromatophores of *Rhodospirillum rubrum*, which had been isolated under anaerobic conditions, did not require any of these factors to catalyse a high rate of photophosphorylation (Nozaki et al. 1963). On aging of the particles the rate of ATP synthesis decreased, but the addition of ascorbate or another reductant restored the original rate; the restored activity was antimycin-sensitive, like that of the freshly isolated chromatophores. The importance of the reductant is to 'poise' the system and bring the electron transport intermediates into a suitable redox condition to allow a cyclic electron flow to occur. The recognition of the basic similarity between isolated chloroplast fragments and the chromatophores of photosynthetic bacteria was an important advance. At one time it was envisaged that the only activity catalysed by chromatophores might be light-dependent ATP synthesis, with the formation of reduced pyridine nucleotide being brought about by a back reaction from reduced flavin nucleotide driven by ATP, as has been visualised for mitochondria under certain conditions.

The earliest formulation of a mechanism (a working hypothesis) for light-dependent ATP synthesis involved the photolysis of water, yielding reduced

([H]) and oxidised ([OH]) moieties, followed by recombination of these moieties through a series of electron carriers to release the energy for ATP synthesis. The energy source was light and there was no net consumption of an external electron donor or oxidant. But the work on chromatophores suggested that there was no need to postulate a photolysis of water, either for chromatophores or chloroplasts. Instead it was proposed that the phosphorylating particle operates as a closed system. During the primary photochemical act, one component of this closed system, a chlorophyll protein complex, becomes excited by the absorption of a photon and in the presence of an acceptor expels an electron that has been raised to a higher energy level, i.e. the excited electron becomes the electron donor in the production of the primary reductant. This is a critical point in energy conservation because it involves moving from *physics*, when a photon is absorbed and the 'excited' electron reverts to the ground state by photon emission in fluorescence, to *chemistry*, when the 'activated' electron becomes a 'reducing' electron when it is accepted by a chemical substance – identified at the time as a cofactor, such as FMN, vitamin K or a related physiological equivalent. When the electron is lost from the chlorophyll protein complex the chlorophyll assumes an oxidised state (electron deficient) and can serve as the electron acceptor in photosynthetic phosphorylation. The electron donor and oxidant are formed simultaneously in the photoreaction. The 'reducing' electron initially attached to the cofactor returns in a stepwise fashion through an electron transport chain to the oxidised chlorophyll protein complex, which returns to its normal ground state when the returning electron is accepted. On the way the electron releases free energy as it is passed through a series of carriers, such as the cofactors and the cytochromes, and its passage through these intermediate electron carriers is coupled to ATP synthesis. The return of the electron from the initial acceptor via cytochrome to oxidised chlorophyll constitutes a 'dark phase', which may be coupled to ATP formation and is analogous to part of the oxidative phosphorylation sequence in mitochondria. However, in the mitochondria all the reactions occur in the dark and a reductant (electron donor) and an oxidant (electron acceptor) must be supplied; the electron participates in a linear flow. In photophosphorylation in chloroplasts and chromatophores both reductant and oxidant originate in the initial photoreaction that drives the ATP synthesis. Because of the cyclic pathway followed by the electron in this photosynthetic phosphorylation this type of phosphorylation was termed *cyclic photophosphorylation* (Arnon 1959).

After the discovery of the artificial cofactors for photophosphorylation in chloroplasts it became of interest to look for other ways of increasing the experimentally obtainable rates. This was done by using ADP as the phosphate acceptor and by increasing the pH to 8.3 from pH 7.2, that in the earliest experiments was optimal for CO_2 fixation. The AMP used in the early experiments was just as effective as ADP as phosphate acceptor, but as the rates increased ADP was soon identified as the true acceptor. However, the presence of variable amounts of myokinase in our chloroplast preparations meant that it was not easy to rule out AMP as a possible phosphate acceptor. Until we realised what the problem was we spent some considerable time establishing ADP as the true acceptor.

The direct photoreduction of pyridine nucleotide (DPN or TPN) and its accumulation in the reduced form could be accomplished in the presence of large amounts of chloroplasts – e.g. 2 mg chlorophyll equivalent or more per reaction vessel (San Pietro and Lang 1956). A pyridine nucleotide reducing factor required for this activity was identified. In Arnon's laboratory this was of great interest, especially since there was experimental evidence to hand that TPN, but not DPN, could under certain conditions double the rate of phosphorylation catalysed by FMN and vitamin K_3 (Arnon et al. 1957). The photoreduction of TPN, but not of DPN, in the presence of small amounts of chloroplasts supplemented by a specific TPN-reducing factor present in chloroplast extract (the chloroplast extract used in reconstitution of the $^{14}CO_2$ fixing experiments) was quickly shown. The factor was purified (San Pietro and Lang 1958), becoming specific for TPN in the process, and was termed photosynthetic pyridine nucleotide reductase (PPNR). The obvious question for us in Arnon's laboratory was to check if the electron transport between water and TPN was accompanied by oxygen evolution and the formation of ATP, since the expected electron flow would probably have several steps in common with cyclic photophosphorylation. Evolution of oxygen was observed manometrically, TPN reduction spectrophotometrically and $AT^{32}P$ formation from ADP and ^{32}Pi radioactively. The stoichiometry is represented by the equation:

$$2ADP + 2Pi + 2TPN + 4H_2O \xrightarrow[chloroplasts + CE]{light} 2ATP + O_2 + 2TPNH_2 + 2H_2O$$

This reaction was termed *non-cyclic photophosphorylation.* The two components of the 'assimilatory power' needed for CO_2 fixation are thus produced simultaneously in a single light reaction sequence (Arnon et al. 1958). We could regard this reaction as the physiological equivalent of the Hill reaction, i.e. the evolution of oxygen in the presence of an added electron acceptor. In making a non-physiological variant of the reaction we used ferricyanide in place of TPN. This reaction showed the photoreduction of ferricyanide and stoichiometric evolution of oxygen, and synthesis of ATP was again observed. In this case no TPN reducing factor was required. Even more exciting was the observation that in the absence of a phosphate acceptor system (ADP + Pi) the rate of ferricyanide reduction was lower in many cases, i.e. the simultaneous conservation of energy in pyrophosphate bonds led to a stimulation of the rate of electron transport. This suggested that the chloroplasts were under phosphate control, analogous to metabolic control seen in mitochondria. We did not observe this stimulation on adding ADP + Pi with TPN, presumably because the rate of photoreduction was slower and probably limited by the TPN-reducing factor in the added chloroplast extract. If we added FMN or vitamin K_3 to the system photoreducing TPN, the accumulation of $TPNH_2$ was partly or fully suppressed and the rate of photophosphorylation was increased. This increased ATP synthesis occurred by way of cyclic photophosphorylation.

In our experiments on $^{14}CO_2$ fixation in the reconstituted system we observed that the addition of cofactors of photophosphorylation in other than trivial amounts were inhibitory. This effect was tentatively explained as due to the diversion of electrons from the reduction of TPN and concomitant ATP production (the formation of assimilatory power in non-cyclic photophosphorylation) to cyclic photophosphorylation, in which the production of $TPNH_2$ was suppressed in favor of increased ATP synthesis, which could not drive CO_2 fixation alone (Trebst et al. 1959).

Subsequently in Arnon's laboratory, the light and dark phases in photosynthesis were separated by Trebst et al. (1958) in a very important experiment. Chloroplast fragments in the presence of chloroplast extract were used to carry out the *light reaction,* to accumulate the components of 'assimilatory power', ATP and $TPNH_2$. The chloroplasts were then removed by centrifugation and $NaH^{14}CO_3$ added *in the dark.* $^{14}CO_2$ was fixed in this system in amounts similar to the amounts fixed in a comparable system in which $^{14}CO_2$ was added at the beginning of the light phase. The ^{14}C-labelled intermediates resulting from both light and dark sequences (simultaneous or sequential) were very similar and consistent with the operation of the reductive pentose cycle. If the components of 'assimilatory power' from external chemical sources, were added in the dark to chloroplast extract without there being a prior light phase, then $^{14}CO_2$ fixation occurred just as when the ATP and $TPNH_2$ were accumulated in a preliminary light phase and the ^{14}C-labelled intermediates were the same. The $TPNH_2$ could also be regenerated enzymatically in the dark from an added substrate and appropriate enzyme in the presence of only catalytic amounts of TPN. Three enzymes used were glucose-6-phosphate, 6-phosphogluconate and isocitrate dehydrogenases. The $^{14}CO_2$ fixation products again included the expected intermediates of the reductive pentose cycle. These observations showed that the fixation of CO_2 in chloroplast extracts is in effect a 'chemosynthesis' that occurs in the dark at the expense of ATP and $TPNH_2$ generated in a light reaction. Under normal conditions of course the light and dark phases go on at the same time and there is no need to accumulate 'substrate' amounts of $TPNH_2$ and ATP. Catalytic amounts constantly recycled are just as effective.

The demonstration of a requirement for a TPN reducing factor (photosynthetic pyridine nucleotide reductase, PPNR) in the light-dependent reduction and accumulation of $TPNH_2$ reminded us of the earlier work of Davenport et al. (1951) on the methaemoglobin (haem protein) reducing factor, a small protein that functioned as if it were the initial electron acceptor in photosynthesis. This protein was highly purified (Davenport and Hill 1960) and shown (Davenport 1959, 1963) to be identical to PPNR. At about this time a small protein was isolated from an anaerobic bacterium and named ferredoxin, because it contains Fe and acts as redox carrier between hydrogen gas and various electron donors and acceptors (Mortensen et al. 1962).

This substance turned out to be the first in a new class of electron carrying proteins of low redox potential and having a wide distribution in all classes of organism. The TPN-reducing factor of spinach chloroplasts was found to be very similar to the bacterial ferredoxin and was termed spinach ferredoxin (Tagawa and Arnon 1962). At the time this water soluble ferredoxin was the most reducing electron carrier found in cellular metabolism, having a redox potential very close to that of hydrogen. Spinach ferredoxin when reduced by hydrogen gas (in the presence of a suitable

hydrogenase) is rapidly reoxidised by oxygen or (in the presence of a flavin enzyme that can be readily extracted from chloroplasts by acetone treatment) by TPN. Normally the enzyme is bound to chloroplast fragments and need not be added for TPN to be reduced. The flavin enzyme has been highly purified and crystallised and is called ferredoxin-TPN reductase (Shin et al. 1963). It forms a spectroscopically recognisable complex with ferredoxin and TPN (Shin and San Pietro 1968). The photoreduction of TPN is a two-step process, in which ferredoxin is first reduced by illuminated chloroplast fragments and subsequently reoxidised by TPN (Whatley et al. 1963). Alternatives to TPN as acceptor of electrons include H^+ ions (Paneque and Arnon 1962), leading to photoevolution of H_2 gas when hydrogenase is added, or oxygen (Arnon et al. 1961). In all three cases the electron transport is accompanied by ATP formation. Cyclic photophosphorylation catalysed by ferredoxin was also examined in a system in which oxygen evolution was inhibited (Tagawa et al. 1963). The importance of 'poising' in this system was examined by Grant and Whatley (1967). An alternative pseudocyclic phosphorylation proceeds in air in the absence of TPN, in which the reduced ferredoxin is re-oxidised by air and no nett oxygen evolution is seen. It involves a variant of non-cyclic electron flow.

Hill and Bendall (1960) put forward a scheme for the involvement of two light reactions in photosynthesis and this suggested to us the possibility of separating the reaction sequence experimentally into two parts by using a dye, dichlorophenolindophenol (DCPIP), that in the oxidised form could accept electrons from Photosystem 2 (the oxygen-evolving system) and in the reduced form (maintained by ascorbate) could donate electrons to Photosystem 1, resulting in TPN reduction and ATP synthesis but no oxygen evolution. This working model appeared to work well, though it was not subsequently favored by Arnon as a full representation of electron flow in chloroplasts. We analysed the effect of monochromatic light on the two partial reactions (Arnon et al. 1961). Photosystem 1 (which can lead to cyclic phosphorylation *or* the reduction of TPN at the expense of ascorbate) operated in far red light, $\lambda = 704$ mμ, whereas Photosystems 1 and 2 were both needed for TPN reduction at the expense of water, and did not function at $\lambda = 704$ mμ (Photosystem 1 alone operating), but only at shorter wavelengths, e.g. $\lambda = 644$ mμ.

Chloride ions were shown to be needed for oxygen evolution in the Hill reaction (Arnon and Whatley

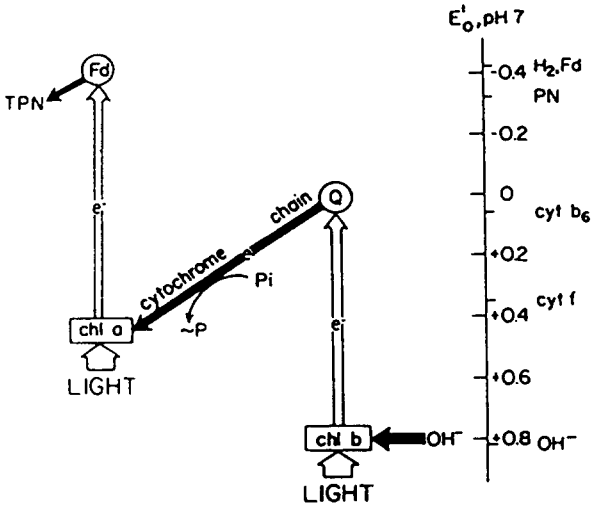

Fig. 1. A scheme to represent non-cyclic electron flow in isolated chloroplasts. Two light reactions are shown, with associated dark reactions. Standard redox potentials of some of the intermediates are indicated on the adjoining redox scale (Whatley et al. 1963).

1949; Bove et al. 1963). When chloride is absent, oxygen evolution is prevented but cyclic phosphorylation can still take place and the photoreduction of TPN with an alternative electron donor (ascorbate/DCPIP) can still occur. In the absence of chloride, cyclic photophosphorylation in chloroplast fragments is very sensitive to the addition of small amounts of ferricyanide. The inhibition is relieved by adding ascorbate to reduce the ferricyanide chemically. This effect is also seen with chromatophores of *Chromatium*. When chloride is absent the chloroplast fragments behave as bacterial chromatophores. The addition of very small amounts of ferricyanide over-oxidises some key catalytic intermediates and inhibits the system by affecting its redox 'poise'. With chloroplast fragments the addition of chloride to the ferricyanide-inhibited system restores their ability to photoreduce ferricyanide at the expense of H_2O and cyclic photophosphorylation is soon reinstated. Since water cannot be used to donate electrons for the reduction of ferricyanide by chromatophores the addition of chloride does not reverse ferricyanide-inhibited cyclic phosphorylation in the bacterial system.

The ability to carry out the Hill reaction with indophenol dyes is lost when lyophilised chloroplasts are extracted with petroleum ether and restored by adding back plastoquinone (Bishop 1959). It has been possible to identify the site of action of the plastoquinone more closely by making use of the two partial reactions previously identified that led to TPN reduc-

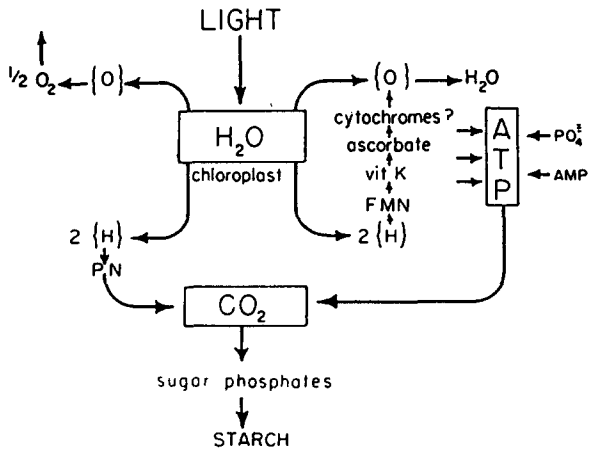

Fig. 2. Scheme for photosynthesis by isolated chloroplasts. Photolysis of water (center) leading either to ATP synthesis and the reconstitution of water (right) or to CO_2 reduction (below) linked to oxygen evolution (left) (Arnon et al. 1956).

tion viz. (1) photooxidation of water leading to oxygen evolution (2) the subsequent photoreduction of TPN, usually coupled with ATP formation. Plastoquinone is needed for the photooxidation of water but the subsequent photoreduction of TPN can occur without plastoquinone when an alternative electron donor (ascorbate/DCPIP) is provided. Plastoquinone appears to act as an early acceptor of electrons of Photosystem 2 (Arnon and Horton 1963). Plastoquinone is also needed for cyclic photophosphorylation; the simplest hypothesis is to suppose that it operates at the same site where it functions in non-cyclic electron flow (Whatley and Horton 1963). One interesting observation that has no valid explanation is that plastoquinone that has been shorn of most of its isoprenoid side chain (PQ_1 - PQ_3) can restore electron flow from water to TPN reduction, but for ATP synthesis to accompany the restored non-cyclic electron flow only plastoquinones with longer isoprenoid side-chains (e.g. the natural PQ_{10}) are effective. This suggests a dual function for plastoquinone that we have so far failed to accommodate in our electron flow schemes.

As a historical touch I include Fig. 1, a shorthand representation of our thoughts in 1963 on non-cyclic photophosphorylation in terms of the redox potentials of some intermediates. This naive minimal working model incorporated dark arrows representing thermochemical reactions proceeding in the dark, and open arrows representing the intake of light energy by chlorophyll and the elevation of an electron to a more reducing potential, where it is accepted by an appropriate electron acceptor. The addition of a connection between Fd (ferredoxin) and Q (plastoquinone) would incorporate cyclic phosphorylation into the model with vitamin K and FMN as possible intermediates and a further connection between Fd and oxygen would represent pseudocyclic phosphorylation.

I find it interesting to compare this late representation (Fig. 1) with one of the earliest figures we published, summarising our views on photosynthesis by isolated chloroplasts for the International Biochemical Congress in Brussels in 1955 (Fig. 2). The evidence to support many of the proposals in Fig. 2 was of course reinforced later in the laboratory and appropriate modifications were introduced. But Fig. 2 was not bad as a beginning!

Whilst the general principles of electron flow in chloroplasts in these models remain acceptable there was no doubt in 1963 that many detailed modifications of thoughts about photosynthesis in plants and bacteria would follow. This has indeed proved to be the case.

My description of progress in the discovery of photosynthesis by isolated chloroplasts includes various consistent strands in the early work in Arnon's laboratory up to the beginning of the 1960's. These strands are summarised in what follows, as an illustration of the guiding principles under which Dan Arnon operated in science. I think it proper to draw attention to the many general and review articles published in Nature and Science by Arnon, sometimes with a few colleagues, during the course of this early work. They were particularly valuable in continually updating our position, whilst giving proper consideration to the history of photosynthesis research and full acknowledgement to other workers' contributions. New concepts were introduced in this way, leaving the detailed experimental results obtained in the laboratory to be published later in other journals by the research associates in the laboratory. All the papers, review or otherwise, were always widely discussed within the laboratory and represented consensus views at the time.

The principal research strands are summarized under the headings 1–6 below.

1. *Chloride.* Our interest in chloride was of long standing. In 1949 we had confirmed Warburg's discovery that chloride was needed for oxygen evolution in the Hill reaction, but were unwilling at that time to agree that it was a cofactor because it was not known to be an essential nutrient required by plants: provisionally we ascribed to it a protective role. But the status of chloride as an essential nutrient was confirmed in Berkeley in 1954, so the objection was no longer valid.

Later (1963) we returned to study the site of action of chloride and, making use of the new experimental availability of the partial reactions of non-cyclic electron flow, were able to confirm the involvement of chloride directly in the oxygen-evolving step, Photosystem 2. It is not needed for Photosystem 1, so cyclic photophosphorylation is possible in the absence of chloride, provided the system is adequately 'poised'.

2. *Photosynthesis in isolated chloroplasts.* I have tried to point out earlier the reasons why we looked for ATP synthesis and CO_2 fixation in chloroplasts. Their restriction to chloroplasts was a major and exciting advance and emphasised that mitochondria or other cellular organelles are not involved. It was some time before this concept was fully accepted.

3. *ATP synthesis.* The discovery of ATP synthesis in chloroplasts without the help of mitochondria was most satisfactory, even though it was not unexpected to us. It is a direct expression of the conversion of light into chemical energy. The initial rates were modest with chloroplast fragments; increasing the pH of the reaction medium to 8.3, using ADP as the phosphate acceptor (use of AMP depends on the presence of myokinase) and introducing a number of cofactors gave photophosphorylation rates that on a protein-N basis were greater than rates of oxidative phosphorylation with mitochondria.

4. *Rates of CO_2 fixation.* Our development of a reconstituted system, in which smaller amounts of chloroplasts were combined with chloroplast extract, was an important tool in the analysis of CO_2 fixation. The chloroplast extract was shown to contain all the enzymes for the reductive pentose cycle to operate. The rates of CO_2 fixation were initially very low but improved on a chlorophyll basis in the reconstituted system although it was left to others to obtain intact chloroplasts that are capable of high rates of CO_2 fixation, by modifying the medium in which the chloroplast isolation was carried out (Jensen and Bassham 1966; Walker 1971).

5. *Pyridine nucleotide reduction.* In 1951 Arnon had published a paper on the accumulation of malate from pyruvate plus CO_2 at the expense of $TPNH_2$ generated by illuminated chloroplast fragments. In this paper he announced his intention to use only enzymes from leaves in constructing model systems for CO_2 fixation since this was more likely to represent what went on in the leaf. This intention was followed out in the subsequent work.

Although it was later found that TPN added to illuminated chloroplasts had no effect on CO_2 fixation, it did have a stimulating effect on the reconstituted system of chloroplast fragments + chloroplast extract (1955). TPN, but not DPN, also increased the rate of ATP formation in the presence of limiting amounts of cofactors of cyclic phosphorylation (1957). The actual accumulation of $TPNH_2$ was also shown to depend on a pyridine nucleotide reductase present in chloroplast extract. The accumulation of $TPNH_2$ was found to be accompanied by ATP synthesis (non-cyclic phosphorylation). Another exciting discovery was that when ferricyanide replaced TPN in this Hill reaction, phosphate control was observed and the rate of reduction of ferricyanide was increased when pyrophosphate bonds were being generated (1958).

6. *Ferredoxins.* The reductase was soon (1962) recognised as a ferredoxin, one of an exciting new class of low potential non-haem iron sulfur proteins, the first of which was isolated from *Clostridium* and shown to act as an electron carrier in the metabolism of hydrogen gas. The plant ferredoxins were shown to be photoreduced by illuminated chloroplasts and reoxidised by TPN in a subsequent dark reaction catalysed by a flavin enzyme, ferredoxin-TPN reductase. The photoreduction of spinach ferredoxin was accompanied by ATP synthesis. Under carefully controlled conditions plant ferredoxins catalysed anaerobically an active cyclic phosphorylation, or in the presence of oxygen an active pseudocyclic phosphorylation.

The identification of TPN as preferred hydrogen acceptor was particularly satisfactory to me because I had earlier suggested in my PhD thesis (1948) that TPN, not DPN, was the hydrogen carrier in photosynthesis. The identification of the methaemoglobin reducing factor with ferredoxin was also of special interest, because I had played a minor part in the original discovery of the factor (Davenport et al. 1951).

TPN and $TPNH_2$ are used throughout this article for NAD^+ and NADH respectively. A conscious decision was made in Arnon's laboratory to use this nomenclature for didactic reasons, because it illustrated most clearly the fact that the reduction of TPN is a two-electron step process. In most of the literature quoted this 'convention' is also followed.

No attempt has been made to document fully the statements involved in this article, but the papers cited contain full references. I hope to have been able to indicate that several lines of continuing research have given rise to significant contributions towards our views on photosynthesis research. It is a matter of some pride that the experimental results published from Arnon's laboratory have always been entirely

reproducible. Perhaps the interpretation may sometimes have been questioned but by and large chloroplasts have acquired a new status from our efforts. It was an enormous privilege to be able to contribute to this advance in Berkeley during some exciting years of photosynthesis research, 1948–1964.

It is also a pleasure to report that in 1964 I was able to start a research group in London, England, with my Berkeley colleagues, D.O. Hall and M.C.W. Evans, in which physical techniques were applied to ferredoxins from a variety of algae and higher plants. When I moved to Oxford in 1971 I left behind me a group that continued to flourish in this area of photosynthesis.

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