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Minireview

Chloroplasts in envelopes: CO_2 fixation by fully functional intact chloroplasts

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Abstract

Dan Arnon, Bob Whatley, Mary Belle Allen, and their colleagues, were the first to obtain evidence for 'complete photosynthesis by isolated chloroplasts' albeit at rates which were 1% or less of those displayed by the intact leaf. By the 1960s, partly in the hope of confirming full functionality, there was a perceived need to raise these rates to the same order of magnitude as those displayed by the parent tissue. A nominal figure of 100 μmol/mg·chlorophyll/h (CO_2 assimilated or O_2 evolved) became a target much sought after. This article describes the contributions that Dick Jensen and Al Bassham [(1966) Proc Natl Acad Sci USA 56: 1095–1101], and my colleagues and I, made to the achievement of this goal and the way in which it led to a better understanding of the role of inorganic phosphate in its relation to the movement of metabolites across chloroplast envelopes.

Introduction

As the academic year of 1952 came to an end, I had completed the first year of research for a PhD. As was the English custom of the time, I had been given a difficult task and told to get on with it (Walker 1997). Supervision was neither promised, expected nor forthcoming. The department in Newcastle was not well equipped, and the isolation of a then unknown enzyme, from the leaves of a Crassulacean plant, a forbidding prospect. Not too surprisingly, I had made very little progress. The prospect that I might be offered something easier (and, by implication, a project only suitable for someone unsuited to do research) did not lighten my gloom. Then, by happy chance, another Newcastle graduate, who had emigrated to the United States some four years earlier, asked his old department to nominate someone who would wish to join him. Naturally, I leaped at the chance and so, in the summer of 1953 I found myself at Purdue University (as in 'Hail, Hail to Old Purdue') Indiana. There, having joined Harry Beevers, in the comforting surroundings of his tiny but well-equipped laboratory in the 'Peirce Conservatory and Small Animal House' everything suddenly seemed much easier. Given a sack of castor beans and a Warburg apparatus, I found to my surprise (and no doubt to Harry's) that I was able to isolate mitochondria that operated the Kreb's cycle at a hundred times the going rate and worked all manner of wonders (Beevers and Walker 1956; Walker and Beevers 1956). Emboldened by this unlikely outcome, I was able (Walker 1956) to subdue *Kalanchoe crenata* when I returned to Newcastle, a year later, in order to complete my PhD. My external PhD examiner turned out to be no less than Robert (Robin) Hill (Walker 1992, 2002a, b; Bendall 1994). Somehow I survived his fierce interrogation and was rewarded with an invitation to do post-doctoral research with him in the Cambridge Biochemistry School; a building that echoed with famous names, a place where a young man called Frederick Sanger, destined to become a double Nobel Laureate, worked across the corridor. It was by this fortuitous combination of circumstances that I first came to isolate chloroplasts. At this time,

Dan Arnon, Bob Whatley and their colleagues were repeatedly electrifying the world of photosynthesis with a stream of new findings (see e.g., Arnon et al. 1954b, 1958; Arnon 1961). Naturally, therefore, Robin wished me to venture into photophosphorylation (Walker and Hill 1958; Hill and Walker 1959; for a history of photophosphorylation, see Allen 2002 and forthcoming in Part 3 of these history issues; Jagendorf 2002) and to isolate chloroplasts, in the Berkley manner, in Tris-NaCl.

To fields and pastures new

In Cambridge, I met Charles Whittingham who was about to take a chair at Queen Mary College, a modest college of the University of London, just down the road from the 'Blind Beggar' (of ill repute) and another pub called 'The Prospect of Whitby' where Judge Jeffries had once dined while watching the bodies swinging at 'Execution Dock.' Charles Whittingham offered me a lectureship at Queen Mary College and he was also largely responsible for what happened next. If I could isolate active plant mitochondria, he said, why not chloroplasts capable of carbon assimilation? With remarkable faith in his young lecturer he not only secured the financial wherewithal for this new venture but, having done so, turned its prosecution over to me, lock stock and barrel. What is more, he was happy that I should pursue this research at a new 'field centre' (Figure 1) that he created some 20 miles away at Dytchleys, in rural Essex. For good measure, I was given the help, one half day a week, of a then junior technician Carl Baldry. I maintained my contacts (Walker 1997) with my mentor, Robin Hill, as I did for many years (Figure 2), by travelling once a week to Cambridge, to do what Bob Whatley would have called 'Sunday afternoon experiments' and sometimes just to talk, over a quiet beer. It was Robin who suggested that I used 'third molar sugar' as an osmoticum in my quest for chloroplasts capable of rapid carbon assimilation. Having used half molar sugar in my mitochondrial work this seemed eminently good advice but it also illustrated the magnitude of the variables ahead. Let us take a quick look at these.

The nature of the problem

Within a leaf, chloroplasts dwell in the film of cytosol that surrounds a large central vacuole. There they are maintained at a favorable osmotic pressure and pH. They are supplied with what they need to function in the way of appropriate ions and metabolites and are no doubt protected by mechanisms which do their best to fend off unwelcome nasties. In order to separate them from this protective cocoon, cells have to be opened in some manner. They will then be immediately exposed to whatever lies in the vacuole. Evidently, there is a need to limit, as far as possible, the rigors that they must endure during isolation and restore them, as quickly as possible, to a friendly artificial environment in which they can work. The list of variables that might be employed, to this end, soon becomes astronomic. Moreover, it is compounded by the obvious need to proceed within the constraints imposed by the availability of apparatus and materials (including the chosen species, age of leaf, etc., etc.). I was reminded then, as so often since, of the man who asked the best road to Dublin and was told 'better not to start from here.' Clearly, there was little hope of an entirely logical and methodical approach. Educated guesswork and hard labor would have to suffice. These combined well enough to eventually allow me (Walker 1964) to report 'improved rates of carbon dioxide fixation by illuminated chloroplasts' in a paper which boasted all of 24 μmol/mg·chlorophyll/h.

How things were in the wider world

Here, I must pause and attempt to put these matters in the context of the early 1960s. First of all, 25 years or more had passed since Hill reported his fabled reaction (Hill 1937) in which isolated chloroplasts evolved oxygen but, seemingly, could not assimilate CO_2. Indeed the very term 'isolated chloroplasts' was not even used with any real precision. There was talk of 'whole,' 'broken' and 'stripped' chloroplasts but no full realization of what these adjectives implied (cf. Lilley et al. 1975). The final elucidation of the Andy Benson–Melvin Calvin cycle was still contemporary science (see Benson 2003; Bassham, 2003). Dan Arnon, Mary Belle Allen and Bob Whatley published their first paper on CO_2 uptake by isolated chloroplasts in Nature in 1954 (Arnon et al. 1954). This was followed by a detailed paper (Allen et al. 1955). Without in any way diminishing the immense importance of this historic observation, it has to be recognized that their rates of carbon assimilation were very low (0.1–2 μmol/mg·chlorophyll/h compared with a nominal 100 μmol/mg·chlorophyll/h ascribed to the parent tis-

Figure 1. The Dytchleys 'Field Centre' round about 1964. *Left:* Shirley (with back to laboratory exterior), Marney and Richard Walker. *Right:* Interior of the 'Centre,' showing centrifuge, notoriously brought from full speed to rest in 60 s, or less, by grasping the spinning rotor between pads of cloth.

sue. Moreover, the associated oxygen evolution was not measured under aerobic conditions and characterized only by bacterial luminescence. Marty Gibbs and Elchanan Bamberger (Gibbs and Bamberger 1962; Bamberger and Gibbs 1963) subsequently raised the going rate to about 5 or 6 μmol/mg·chlorophyll/h but it was not yet unthinkable (e.g., Mary Stiller 1962) to question the extent to which isolated chloroplasts were capable of complete photosynthesis *in vitro*. Much of this may help to explain why I (Walker 1969), and many others, invested so much effort in attempts to isolate what eventually became known as 'fully functional' chloroplasts. The rate of photosynthetic carbon assimilation that they displayed was perhaps a simplistic indicator of function but it was always clear (Walker 1969) that an isolated chloroplast that 'worked' well would be more likely to advance our knowledge (of how it worked) than the one which struggled to do a small fraction of what is achieved within the leaf.

First steps

In my early work at Dytchleys, I used young pea shoots. True spinach (i.e., *Spinacea oleracea*) could not be readily bought in the UK market at that time. In Britain, true spinach (unlike 'perpetual' spinach, Swiss chard, New Zealand spinach and the like, which proved themselves unsatisfactory for my purpose) is very difficult to grow throughout the year (Edwards and Walker 1983; Walker 1987). Spinach demands short days and high light. The first are absent throughout British summers, the second rarely experienced in English winters. So peas were easier in some regards but they introduced yet one more variable. I had no properly controlled growth chambers, the physiological age at which pea shoots were at their best turned out to be short and I was not then aware that pea leaves exhibit remarkably fast and unwelcome post-harvest deterioration.

The nature and extent of maceration was important. I abandoned the traditional pestle and mortar, which I had pounded to good purpose on the floor of Robin Hill's laboratory in earlier years, in favor of a Waring Blendor. I settled for about 30 s maceration. I soon recognized the virtues of separating chloroplasts from grinding medium and vacuolar contents as quickly as possible. In the absence of an adequate brake on the only centrifuge at my disposal (and the welcome absence of any one looking over my shoulder), I achieved rapid deceleration by clasping cloths to the side of the rapidly spinning rotor. From commencement of maceration to starting re-suspension took about two minutes. To facilitate re-suspension I used, like Bob Whatley before me, a wisp of cotton at the end of a glass rod in the hope, for which there was to be some future evidence, that damaged chloroplasts might be selectively retained on the cotton.

I used a sugar alcohol (sorbitol) rather than a more metabolically active sugar as the osmoticum. I abandoned 'Tris,' made famous by Dan Arnon (as in Tris-NaCl) because I suspected it might do damage to membranes. Norman Good (cf. Bucke et al. 1967) had yet to invent his gentle and metabolically inert alternatives. Instead, I used inorganic phosphate at pH 6.8 for no better reason than it was the most common buffering agent of the day. Adding an antoxidant seemed sensible. My choice of isoascorbate,

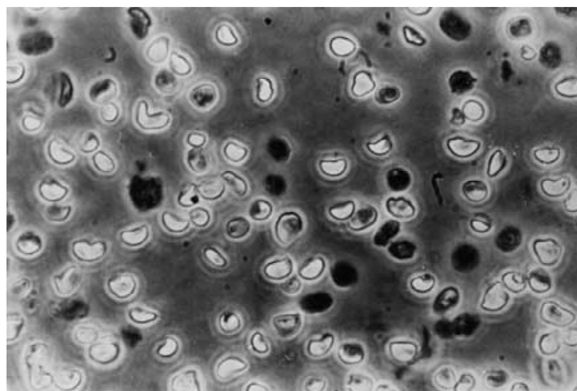
which was to puzzle some future users of my method, was determined by the fact that it, unlike its more famous isomer, was more readily available as the sodium salt in the UK Adding Mg^{++} was a hangover from previous procedures. In the presence of orthophosphate it necessitated the inclusion of ethylene diamine tetra acetic acid (EDTA) as a chelating agent (see e.g., Walker 1971).

Figure 2. The author (DAW) with Robin Hill (right), some 30 years or more after first meeting.

Imperial days

At this stage, not particularly happy with my slow progress towards the chloroplast Grail of 100 μmol/mg·chlorophyll/h, my life and that of my family was to be changed forever by the transfer of Charles Whittingham from Queen Mary College, to Imperial College (also ostensibly part of the University of London). By now I had been promoted to a readership (which doubled my salary) and lived in the house of my dreams in Epping (Essex) which, such are the changes in the UK housing market, could now be scarcely afforded by a bank manager. I was sorely tempted to stay put at Queen Mary College but an interview with the new head of department changed all that. No way, he said, would he have 'his reader' working at Dytchleys (i.e., away from the main campus in London's Mile End Road). That, as my wife Shirley observed (Walker 1997) would be like taking candy from a baby. Moreover, 'my' research grant was in Charles Whittingham's name and, although I might well have got another, I was told that it could not be on the same subject. So I was very pleased to be invited to move to Imperial College. I came to hate the three hours a day commuting that this entailed but my chloroplasts prospered. I also had the advantage, there, of collaboration with Dennis Greenwood, renowned for his electron microscopy but notoriously shy of publication. It was he who confirmed that my isolated pea chloroplasts had retained the intact double envelope detectable in electron micrographs of leaves. Regrettably, I was unable to persuade him to publish this observation in his own right and a simple acknowledgement along with the pictures had to suffice. These electron micrographs, together with light-microscope and phase-contrast pictures of whole and envelope-free chloroplasts (Figure 3), a comparison of the rates of light dependent carbon assimilation that they achieved (by now, at best, 36.9 and 2.7 μmol/mg·chlorophyll/h respectively), a comparison of the ^{14}C products of photosynthesis by pea leaf and pea chloroplasts (cf. Gibbs and Cynkin 1958a) etc., were presented at (what was to be for me, Figure 4) a historic symposium in 1965 at Aberystwyth (Walker 1967). It was at the Aberystwyth meeting that I was able to renew lasting friendships with such notables in my field as Marty Gibbs and Clint Fuller (Figure 5) and to start a new and lifelong friendship with a then young German, Ulrich Heber, who was soon to routinely isolate record breaking chloroplasts from spinach. It was here, at Robin Hill's behest that he, Charles Whittingham, and I (Figure 6) were to seek, and find, *Schistotheca* (a remarkable moss that dwells in holes in Welsh hills and focuses incoming light with lenses on to single chloroplasts in its protonema).

As one more preoccupied with function than structure I confess that I was not, in 1965, aware of the evolutionary implications of the double envelopes in Dennis Greenwood's pictures. By now, I am intrigued by the widely accepted notion that, sometime in the late Proterozoic or early Cambrian, the outer envelope might have been derived from a bacterial membrane which wrapped around the invading cyanobacterium that became the first ever chloroplast. I cannot remember whether it was me or Dennis who first used the term 'envelopes' to describe the bounding membranes of isolated chloroplast but it made a nice title for my inaugural lecture when I eventually escaped the rigors of London commuting by moving to the University of Sheffield in 1970.



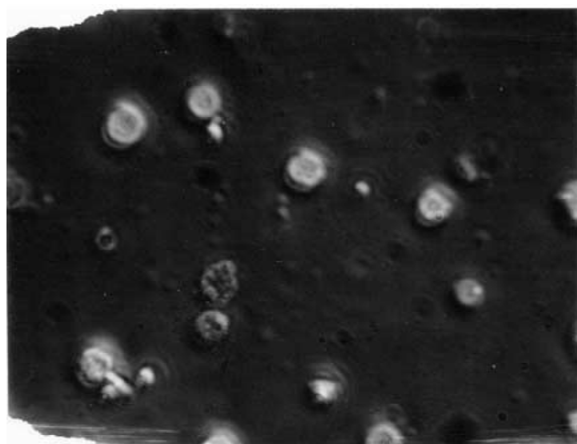


Figure 3. 'Whole' (bright) and 'broken' (dark) chloroplasts. *Top*: by phase-contrast microscopy. *Bottom*: by conventional light microscopy. About 1964.

Back in the swinging 1960s (Figures 7 and 8) and joined by Carl Baldry and Chris Bucke, we were able (using ^{32}P) to show that isolated chloroplasts incorporated externally supplied phosphate, as well as ^{14}C, into Benson–Calvin cycle intermediates (Baldry et al. 1966a). Moreover, rates of CO_2 fixation as high as 67μmol/mg·chlorophyll/h seemed to bring the magic 100 μmol/mg·chlorophyll/h within reach (Baldry et al. 1966b). Indeed we were soon to get there (Bucke et al. 1966). Undoubtedly, by then, an important factor was the availability of spinach either grown for us in the famous Chelsea Physic Garden or bought by the crate from the equally famous Covent Garden market. Then came the dramatic news from the United States that Dick Jensen (then Al Bassham's PhD student) had got there first (Jensen and Bassham 1966). Headlines in *The New York Times*, no less. With his unfailing and commendable generosity Dick acknowledged that he had followed all but one of our procedures to the

Figure 4. The author (DAW) addressing the NATO Advance Study Institute on the Biochemistry of the Chloroplast, Aberystwyth, 1965.

Figure 5. Notables in author's field, Aberystwyth 1965. Clint Fuller, Tony San Pietro and Marty Gibbs.

Figure 6. The author (DAW) with Charles Whittingham (left) in search of *Schistotheca*, Aberystwyth, 1965.

letter, checking their utility at every step. However, his chloroplasts, unlike ours, did not need to be coaxed into action by 'catalytic' quantities of Benson–Calvin cycle intermediates. At Imperial College, we should have been opening champagne to celebrate Dick's achievement but, human nature being what it is, I confess that our feelings were more akin to those of Scott when he arrived at the South Pole only to find that Amundsen had got there three weeks before him. The chagrin was short lived. Everything soon started to become clearer. The only substantive change in procedure that Dick Jensen had reported in his 1966 paper (Jensen and Bassham 1966) was that he had used inorganic pyrophosphate where we used orthophosphate. The significance of this was yet to follow. By 1965 (Walker 1965, 1967), I had already found a clear correlation between rates of fixation and chloroplast integrity By then rates for 'intact' chloroplasts were approaching 40 μmol/mg·chlorophyll/h whereas osmotically shocked chloroplasts struggled to reach 3 (μmol/mg·chlorophyll/h). We (Baldry et al. 1966) had frequently confirmed the earlier findings by Marty Gibbs and Elchanan Bamberger (1962, 1963) that an initial lag in photosynthetic incorporation of $^{14}CO_2$ by isolated spinach chloroplasts could be shortened by the addition of sugar phosphates.

The phosphate enigma resolved

Importantly at this time, Robin Hill had introduced me to the oxygen electrode (Walker 1987), and it was with a newly purchased vibrating glass electrode, at Imperial College, that I had the privilege of making the first measurements of CO_2-dependent oxygen evolution under aerobic conditions (Walker and Hill 1967). This, almost overnight, freed me of the tyranny of endless single sampling of ^{14}C and experiments in which time courses and the effects of additions to reaction mixtures had to wait until the day's work was finished. Now, when necessary, we could follow both O_2 evolution and CO_2 uptake simultaneously (Walker et al. 1968). For most purposes, we could rely on O_2 evolution alone for evaluating kinetics. At a time when computers were still as big as buses, pen-recorders became the center of our daily work. Tom Delieu and I remolded the oxygen electrode of the day nearer to the heart's desire (Delieu and Walker 1972). Carl Baldry, Bill Cockburn and I were then able to use simultaneous measurements of O_2 evolution, in twin vessels (Walker et al. 1968; Walker 1987) to good advantage. We were already well aware (Cockburn et al. 1967b, c) that the initial lag, or induction period, (in both CO_2 fixation and O_2 evolution) could be lengthened by increasing the inorganic phosphate (Pi). We knew that this lag extension could be overcome by sugar phosphates (Walker et al. 1967; Cockburn et al. 1968a). What we were to discover (Cockburn et al. 1967a, 1968b; Walker 1974, 1997; see also Walker and Herold 1977; Walker and Robinson 1978) was that inorganic pyrophosphate (PPi) could reverse this lag extension. Luckily, and most fortuitously, enough inorganic pyrophosphatase was released from the few damaged chloroplasts in our reaction mixtures to sup-

Figure 7.Left: DAW at the bench, Imperial College (about 1968). *Right:* 'The Old Firm.' From left to right: DAW, Chris Bucke, Bill Cockburn (disregarding unknown person with back to camera).

ply Pi in amounts that favored rapid rates. No longer, given the presence of PPi, did we need to add catalytic amounts of sugar phosphates to achieve rates in excess of the once elusive 100 μmol/mg·chlorophyll/h. An experiment which illustrates some of these complex inter-relationships, and gave us great pleasure and satisfaction at the time, is illustrated in Figure 9. Here, in reaction mixtures containing very little Pi, the initial lag was short but the rate soon fell as Pi was consumed. Then it could be restored by either Pi or Ppi but, with the latter, not until after $MgCl_2$ had been added to activate the pyrophosphatase released from damaged chloroplasts. In equimolar amounts, the stoichiometries of O_2 evolution in response to these additions were approximately 1 to 3 for Pi and 1 to 6 for Ppi, values consistent with the formation of one molecule of triose phosphate for each molecule of O_2 evolved and phosphate consumed. Put all of this together, i.e. the need for external Pi (or a source of Pi), the need to add sugar phosphates to avoid long initial lags or to reverse inhibition by high Pi (unless PPi was present in amounts sufficient to reverse Pi inhibition), it was possible to arrive at bold conclusions.

Thus, in a contemporary review (Walker and Crofts 1970), we wrote

> If sugar phosphates are exported from the chloroplast there must be a corresponding import of phosphate (in some form) if steady state photosynthesis is to be maintainedþA direct obligatory exchange between orthophosphate (outside) and sugar phosphate (inside) could account for the inhibition of photosynthesis by orthophosphate and it reversal by sugar phosphates.

This, indeed, was the basis of what was to become known as the ‘phosphate translocator’ (Heldt and Rapley 1970).

Postscript

As this story unfolded, it became increasingly clear that if isolated chloroplasts were to function as well as they do in their parent leaves it would not only be necessary to isolate them in such a way that they retained much of their structure but also then to try and recreate an *in vitro* environment which would allow them to exhibit best function. The mechanical separation of chloroplasts (Walker 1987) has played its part in adding to our understanding of photosynthesis, but it remains a crude procedure, which really only

Figure 8. DAW with his research student, Judy Emmett (Emmett and Walker 1969), Imperial College (about 1969).

works well with spinach. Apply the same procedures to leaves of *Mercurialis perennis* and you get nice intact but ‘dead’ chloroplasts. Add a little of the *Mercurialis* brei to a spinach preparation and that too fails to function. Happily, isolation via protoplasts (Robinson and Walker 1979; Robinson et al. 1979, 1983), as pioneered by Gerry Edwards (e.g., Edwards et al. 1978), offers a route into other species. There is also a certain irony in the fact that although so much effort went into preserving intact envelopes they are neither necessary nor desirable if the aim is to investigate what goes on within the chloroplast. The illuminated reconstituted chloroplast system (in essence thylakoids plus stroma) will generate Dan Arnon’s beloved ‘assimilatory power’ (Arnon et al. 1958; and see Walker 2000). Simultaneously it will use this newly formed ATP and NADPH to support photosynthetic carbon assimilation (i.e., the reactions of the Benson–Calvin cycle) in its entirety (Walker et al. 1971). Here, the intact envelope, because of its selective permeability, is a barrier to investigation rather than something to be strived for. I was never able to convince grant-awarding agencies of what might be learned from total reconstitution with purified enzymes but I still believe that this approach has much to offer. Perhaps someone out there will suggest it as a promising PhD topic?

Acknowledgments

I am most grateful to Govindjee without whose continuing help and encouragement this article would not have been written. Because the wheel continues to be regularly re-invented, I am also glad to have had opportunity to list, in one place, some of the major contributions made to this history (and to the pleasure

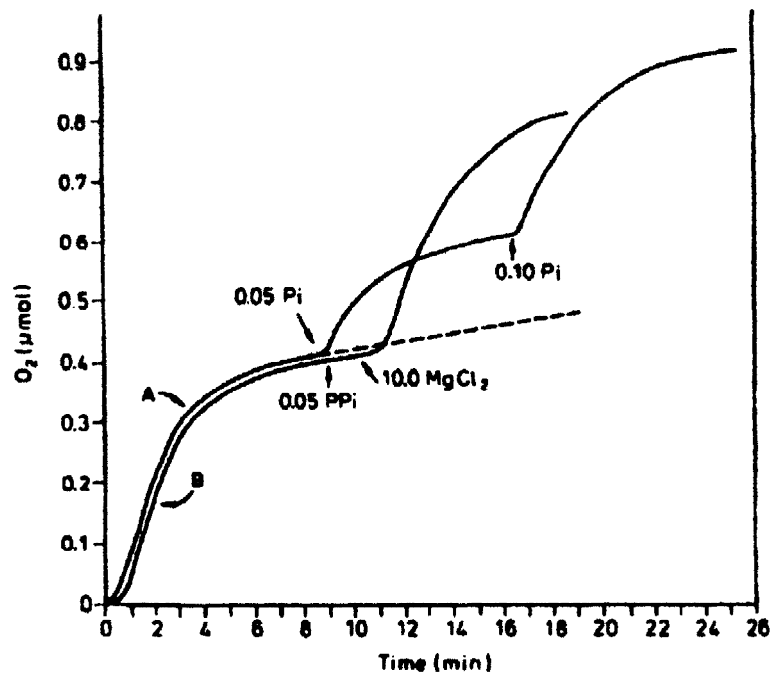


Figure 9. Time course of photosynthetic oxygen evolution by isolated spinach chloroplasts, the requirement for added orthophosphate (Pi) or inorganic pyrophosphate (PPi). Traces of orthophosphate present in the chloroplast preparation allow photosynthesis to start after a brief induction period. Thereafter, the rate falls as the Pi is consumed but can be restored (trace A) by the addition of Pi with a stoichiometry (of approximately 1 to 3) consistent with the formation of triose phosphate. Trace B (recorded simultaneously) as for trace A until the addition of PPi. This was without effect until the further addition of mg^{++} activates the inorganic pyrophosphatase present in the chloroplast preparation. Thereafter, the response to added PPi is the same as to added Pi except that the stoichiometry is approximately 1 to 6 (consistent with external hydrolysis of PPi to Pi and internal consumption of Pi to triose phosphate). For experimental details, see Cockburn et al. 1967c; Walker 1969. Reproduced from Walker (1977).

that I found in working at the bench) by Carl Baldry, Chris Bucke and Bill Cockburn.

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