Photosynthesis Research **73:** 51–54, 2002.

A tribute

'And whose bright presence' – an appreciation of Robert Hill and his reaction

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Received 15 August 2001; accepted in revised form 14 September 2001

Key words: chloroplasts, electron transport oxygen evolution, Robert Hill, methaemoglobin, oxidants

Abstract

The Hill reaction, its elucidation, and significance is briefly described. Hill oxidants, the role of the methemoglobin reducing factor and its relation to ferredoxin, and the part played by chloroplast envelopes are discussed.

Reputedly the best multiple pun ever (flavored, as it was, with literary allusion) came, on an occasion in the last century, when Lord Maughn gave a gold coin to a boy who had helped him with his luggage. The boy was called Hill. This prompted an erudite bystander to declare 'Hail smiling morn that tips the hills with gold.' There is a later line in this same verse which reads 'and whose bright presence darkness drives away.' As in every sort of science, our understanding of photosynthesis has grown by the combined contributions, large and small, from researchers in every land. It is fair to say, however, that Robert (Robin) Hill's 'bright presence' drove away a deal of darkness in the field of photosynthetic electron transport. His experiments (Hill 1965; Bendall 1994) in photosynthesis, which were to influence our thinking for 60 years or more (see e.g. Rich 1992), started, in prewar Cambridge (Hill 1937, 1939) with what inevitably came to be known as 'the Hill reaction.'

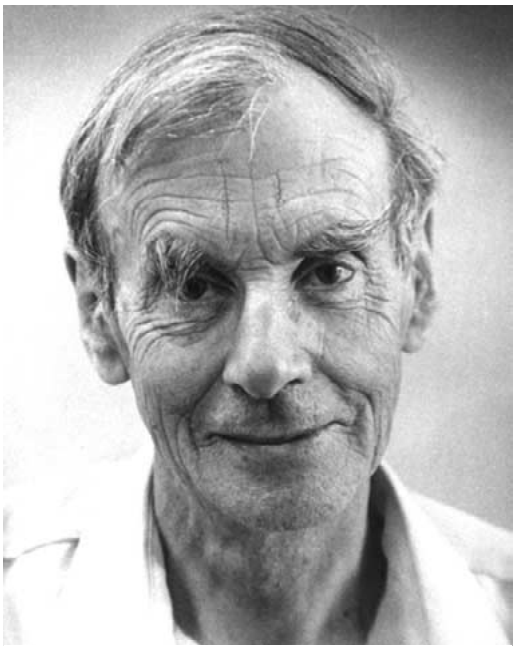

Figure 1. Robert Hill (1899–1991).

What is it?

The Hill reaction occurs when isolated 'chloroplasts' are illuminated in the presence of an electron acceptor 'A.' The acceptor is reduced (to AH_2) and molecular oxygen (O_2) is evolved.

$$2\ H_2O + 2\ A \rightarrow 2\ AH_2 + O_2$$

As we shall see, there are 'artificial' and 'natural' acceptors and the term 'chloroplasts' can mean different things in different contexts. In the 1930s, Hill used the change in the absorption spectrum that occurs as oxygen binds to hemoglobin to form oxyhemoglobin as a measure of oxygen evolution by isolated

chloroplasts. He also reported stimulation of oxygen evolution by the addition of an extract of leaves, and of yeast. Moreover, he found that the addition of ferric potassium oxalate to a suspension of chloroplasts 'caused the evolution of oxygen in a quite startling manner on illumination.'

Necessary as it was at the time (Hill 1939) to seek unequivocal evidence of oxygen evolution with hemoglobin, this approach was immensely complex at a practical level and might well have misled a lesser man. As it was, Hill carried out all manner of control experiments that allowed him to conclude that (a) 'when the oxygen output is measured from illuminated chloroplasts, the effect is not due to some property of the haemoglobin,' (b) the 'ferric oxalate could be regarded simply as a reagent to demonstrate a property of the chloroplast,' and (c) 'there must therefore be some primary substance which is reduced, while at the same time giving oxygen.' Thus, in the equation:

$$2\ H_2O + 2\ A \rightarrow 2\ AH_2 + O_2$$

'A' would represent such a primary substance 'not easily removed from the chloroplasts because great dilution of the suspending fluid did not diminish the rate of reaction with ferric oxalate' and the ferric ion would re-oxidize AH_2 allowing the overall reaction to continue.

$$AH_2 + 4\ Fe^{+++} \rightarrow 4\ Fe^{++} + 4\ H^{+}$$

Hill then went on to suggest the existence of a 'mechanism' (which we might now call the 'photochemical apparatus') within the chloroplast, 'the activity of which can be measured apart from the living cell which, under illumination, simultaneously evolves oxygen and reduces some unknown substance which is not carbon dioxide.'

Hill oxidants

What might Hill's 'unknown substance' be? Implicit in this question is the notion of 'artificial' and 'natural' electron acceptors or 'Hill oxidants.' Clearly Hill himself, reporting his findings in the 1930s (Hill 1937, 1939), regarded 'A' as a component of the chloroplast. Conversely, in future years, a 'Hill oxidant' or 'Hill reagent' most often came to be regarded by many as some artificial additive to a reaction mixture which would do the same job as ferric iron in the above equation. For example, if we wish to demonstrate the Hill reaction in a test tube, we might well add a little of the blue dye 2,6-dichlorphenolindophenol. This conveniently accepts electrons from some site within the photochemical apparatus and, in so doing, is reduced to a colorless form. By now, it is clear that different oxidants react with different sites. Carbon dioxide is the ultimate recipient of electrons from water but carbon assimilation is, in some regards, a thing apart from the photochemical apparatus mostly residing, as it does, in the stroma rather than in the thylakoid membranes that contain the chlorophylls and other components of Hill's 'mechanism.' In all of this, it should be remembered that the concept of a chloroplast as an entity contained within limiting envelopes and comprising several compartments was still a thing of the future, a concept that owed much to Hill's pioneering experiments. The impact of his work was such that, even as early as 1956, the second volume of Eugene Rabinowitch's monumental treatise on 'Photosynthesis and Related Topics' (Rabinowitch 1956 contained no less than 200 references to 'Hill's reaction.' Later (Walker and Hill 1967), when it had finally become clear that carbon assimilation (and its associated oxygen evolution) at rates as fast as the parent leaf was a function of 'intact' chloroplasts, there was a certain irony in the fact that ferricyanide was used as a measure of envelope intactness. Unable to penetrate the limiting envelopes of sound and fully functional chloroplasts, external ferricyanide cannot reoxidize AH_2. A comparison of the rates of oxygen evolution in the presence of ferricyanide by nominally intact chloroplasts and those rendered envelope-free by osmotic shock therefore provided a convenient indicator of integrity. Preparations in which a large majority of isolated chloroplasts remain intact exhibit very little Hill reaction activity with ferricyanide as the Hill oxidant. On the other hand, they display fast rates of CO_2-dependent oxygen evolution because carbon dioxide, rather than ferric ion, brings about the re-oxidation of AH_2.

Methemoglobin reducing factor and chloroplasts in envelopes

Such was the impact of the Hill reaction on research and teaching in the field of photosynthesis that the use of ferricyanide, 2,6-dichlorphenolindophenol, etc. for this purpose became commonplace and, with it, the implication that these were substitutes for some part of the photosynthetic electron transport system somehow lost during chloroplast isolation. Indeed this eventu-

ally turned out to be the case. In search of compounds in leaves that could be extracted and added back to chloroplasts, Hill discovered a 'methaemoglobin reducing factor.' Methemoglobin (oxidized hemoglobin) was the sort of natural agent with an appropriate oxidation/reduction potential which could be readily obtained and examined using a hand spectroscope in the Cambridge Biochemistry Department of its day. It 'served both as the ultimate electron acceptor and, after its reduction and reoxygenation, a measure of the oxygen evolved' (Bendall 1994). In the hands of Tony San Pietro (Fry and San Pietro 1963) the 'met factor' became 'photosynthetic pyridine nucleotide reductase' and finally Bob Buchanan and Dan Arnon's ferredoxin (Buchanan 1991). Here I am deliberately avoiding exact terminology (for which see Buchanan 1991; Forti 1999) because it would now be both rash and pointless to speculate about the precise components of the earliest 'factors' beyond the conclusion that they all had ferredoxin in common. What is not in any doubt is that we are discussing some mixture of soluble components located at the 'top' of the Z-scheme (Hill and Bendall 1960) which, *in situ*, accept electrons from carriers in the thylakoid membranes and bring about the reduction of NADP and ultimately CO_2.

That the definitive identification of met factor (as ferredoxin) was never undertaken may relate, as Derek Bendall suggests (Bendall 1994), to the possibility that Robin had a little NADP in his possession during this period but considered it too precious to use. Certainly Robin's laboratory never lost the air of careful frugality which prompted me, while working there on photophosphorylation, to decrease 3 ml reaction mixtures to a more modest 0.3 ml (Hill and Walker 1959). I have already written before about those exciting days (Walker 1992) and noted that 'the word from Berkley was of more and more co-factors.' Robin suggested trying 'spit, urine and floor-sweepings.' We shrank from the first two and felt that the third, given Robin's lab, would have been a bit of a foregone conclusion. Despite the electrifying fast rates of photophosphorylation catalyzed by pyocyanine, Robin hankered after more biologically important molecules. The plan was to try methemoglobin reducing factor, but somehow he never found time to prepare it again, as he had done so often in the past. Methemoglobin reducing factor became ferredoxin and an opportunity was lost.

With the benefit of hindsight, given the nature of the medium that Hill used for chloroplast isolation, it seems very likely that his early preparations would have contained a significant proportion of intact chloroplasts in addition to free thylakoids. The barriers both to fuller function and understanding were the chloroplast envelopes. While still intact, the limiting envelopes constitute 'the skin that keeps the rest in.' They not only prevent the loss of the enzymes of carbon assimilation but also other essential soluble components such as NADP and ferredoxin. Moreover, they also constitute a barrier to the interaction between thylakoids within intact chloroplasts and components released to media in which photosynthetic function of various sorts could be assayed. Conversely, if intact envelopes are deliberately ruptured by osmotic shock, and ferredoxin, NADP, etc. are added back at appropriate concentration, the resulting 'reconstituted chloroplast system' (Walker et al. 1971; Lilley and Walker 1979) will support CO_2-dependent oxygen evolution at rates comparable to the parent leaf.

Significance

I have read, on the Internet, plaintive demands from students wishing to know the significance of the Hill reaction. Clearly Nobel laureate George Porter's view (Porter 1979) of this matter deserves a wider audience than it had at the time that it was written.

> Known universally today, except by its discoverer, as the Hill reaction, this provided the all important route to the study of photosynthesis, if not '*in vitro*' at least without the complications of the whole living organism. The production of oxygen from water, without the associated carbon dioxide reduction, is the essential energy storage reaction of photosynthesis and the way was now open for the elucidation of this process at the molecular level. It was Hill who identified some of the principal performers in this play of electrons; cytochromes *f* and *b* and the 'methaemoglobin reducing factor' which was, in fact, ferredoxin, the most powerful reducing agent known in nature. After Emerson's discovery of the 'red drop'[1] [see note 1] and its interpretation in terms of two photosystems, Hill and Bendall, in 1960, proposed their 'Z-scheme' of photosynthetic electron transport. This provided, and still provides today, the chart by which nearly all explorers of photosynthesis navigate through the reefs of photosynthetic units, light harvesting antennae, electron transport

chains and the reaction centres of Photosystems I and II.

Robin Hill wrote a nice little book, with C. Whittingham, in 1953. It is of historical importance.

Acknowledgments

I am most grateful to Govindjee for giving me the opportunity to write this article, for his help, comments and criticisms and to Derek Bendall whose comprehensive biography of Hill made its writing so much easier to undertake. See also Robert Hill Archive http://www.bath.ac.uk/Centres/NCUACS/html_rh.htm#top for further information.

Note

[1] In his 1965 paper, Hill details work such as the Emerson enhancement effect (Emerson et al. 1957; Emerson and Rabinowitch 1960) that led to the concept of two light reactions.

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