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Discovery of causal mechanisms

Oxidative phosphorylation and the Calvin-Benson cycle

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Abstract We investigate the context of discovery of two significant achievements of 20th century biochemistry: the chemiosmotic mechanism of oxidative phosphorylation (proposed in 1961 by Peter Mitchell) and the dark reaction of photosynthesis (elucidated from 1946 to 1954 by Melvin Calvin and Andrew A. Benson). The pursuit of these problems involved discovery strategies such as the transfer, recombination and reversal of previous causal and mechanistic knowledge in biochemistry. We study the operation and scope of these strategies by careful historical analysis, reaching a number of systematic conclusions: 1) Even basic strategies can illuminate "hard cases" of scientific discovery that go far beyond simple extrapolation or analogy; 2) the causal-mechanical approach to discovery permits a middle course between the extremes of a completely substrate-neutral and a completely domain-specific view of scientific discovery; 3) the existing literature on mechanism discovery underemphasizes the role of combinatorial approaches in defining and exploring search spaces of possible problem solutions; 4) there is a subtle interplay between a fine-grained mechanistic and a more coarse-grained causal level of analysis, and both are needed to make discovery processes intelligible.

Keywords context of discovery · causality · mechanisms · biochemistry · oxidative phosphorylation · photosynthesis · P. D. Mitchell · M. Calvin · A. A. Benson

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1 Introduction

Is the generation of novel scientific hypotheses methodologically tractable? The answer used to be a clear-cut and widely accepted "no". Popper summarized this consensus most felicitously when he wrote that unlike the context of justification, the context of discovery seemed "neither to call for logical analysis nor to be susceptible of it" (Popper 2002, p. 7). In the 1970s, a number of "friends of discovery" criticized the consensus, culminating in two volumes edited by Thomas Nickles (1980b; 1980a). Since that time, conceptual analysis has clarified the distinction between the context of discovery and the context of justification, while the continued analysis of historical cases has shown that systematic insight into discovery may be possible after all.[1]

In recent years, the discovery of biological mechanisms has received particular attention. A new approach to discovery was suggested by Machamer, Darden, and Craver (2000), and this has been developed in particular by Darden and Craver (collected in Darden 2006) as well as by Thagard (2003). In a related tradition, Bechtel and Richardson (1993) and Bechtel (2005) have explored the strategies of decomposition and localization in biology and psychology. At least in outline, we now have an account of biological discovery that promises to be both philosophically insightful and adequate to past and present scientific practice.

Despite this progress, the number of thorough historical case studies of mechanism discovery remains relatively small. Yet such studies are needed in order to test, refine and extend our philosophical tools, even as these same tools help us to write better history. Therefore, we here consider two cases of discovery in biochemistry in detail: oxidative phosphorylation (the major mechanism of ATP synthesis in mitochondria) and the Calvin-Benson cycle (the light-independent reaction of photosynthesis). Oxidative phosphorylation has already received considerable attention in the historical and philosophical literature (Allchin 1992, 1994, 1997; Prebble 1996, 2001; Prebble and Weber 2003; M. Weber 2002, 2005). However, the main focus has been on the mechanism's context of justification (broadly construed). Here we will address its context of discovery in the late 1950s, which has received much less attention (but see B. Weber 1991, and literature cited above). In deference to the existing literature on the development and confirmation of the hypothesis, we will restrict our discussion to the period before the publication of the initial mechanism in 1961. In contrast to oxidative phosphorylation, the discovery of the Calvin-Benson-cycle has only recent been studied in depth (Nickelsen in press). We will therefore discuss at some length not only how hypotheses were generated, but also how conceptual and experimental laboratory work interacted in the development and establishment of the cycle's mechanism.

In studying oxidative phosphorylation and the Calvin-Benson cycle, we pursue four main philosophical goals. First, both qualify as "hard cases" of scientific discov-

[1] For conceptual analysis, see particularly Simon (1973), the essays in Nickles (1980b), and Hoyningen-Huene (1987, 2006). For early historical work, see the case studies in Nickles (1980a), Darden (1991), Bechtel and Richardson (1993) and Schaffner (1994). More recently, scientific discovery was reinvigorated by the volumes by Bechtel (2005), Darden (2006), Schickore and Steinle (2006), Meheus and Nickles (2009) and Craver and Darden (2013). See also references cited therein.

ery: they are achievements of acknowledged originality and importance that are by no measure only extrapolative. If strategies of mechanism discovery can account for the genesis of such hard cases, then they can be expected to be powerful enough to handle a host of less difficult cases as well. Second, we are interested in how the causal-mechanical approach makes intelligible the "units of recombination" of hypothesis generation in the life sciences, and how this allows us to steer a middle course between a substrate-neutral understanding of biological discovery (which is probably illusory) and an entirely domain-specific account (which would make systematic insight into discovery impossible). Third, we place more emphasis than the existing literature on how "search spaces" of possible causal and mechanistic hypotheses are defined and explored. Fourth and finally, we will argue that in addition to the fine-grained mechanistic level of analysis, we need a more coarse-grained causal analysis in order to understand continuity in the medium-term development of scientific theories. Each of these points will be discussed in detail below.

We will begin by briefly situating our work in ongoing debates and by reviewing the strategies of hypothesis generation that we are interested in. We will then discuss our two case studies in turn.

2 Discovery from a causal-mechanical point of view

2.1 Discovery and justification

The distinction between the context of discovery and the context of justification can be understood in a number of different ways (Hoyningen-Huene 1987; Schickore 2014). First, there is a *temporal* distinction, according to which hypothesis generation occurs before hypothesis testing. Second, there is a *methodological* distinction, according to which hypothesis generation is a process different from hypothesis testing, although the two may be temporally interleaved. Third, there is a distinction between *disciplines*, where discovery is seen as an empirical matter (for history, psychology or sociology to explore) whereas justification is seen as a logical matter (for philosophy to explore). A fourth main distinction may have to be introduced as well: between discovery in both the vernacular and the historian's sense of "discovering a new entity" and discovery in the philosopher's sense of "formulating a new hypothesis".

The first, temporal distinction has been successfully dispatched by historical studies showing the temporal interleaving of discovery-type and justification-type processes (see for instance recent contributions by Steinle 2006, and Arabatzis 2006). We reject the third, discipline-based distinction because we believe that both discovery and justification must be approached by a fully naturalistic historical and philosophical approach to science. As to the fourth distinction, we here restrict our usage to "discovery" in the philosopher's sense of generating new hypotheses.

Our main concern is the second distinction: By what processes are novel causal and mechanistic hypotheses generated? We refer to these processes as *discovery strategies*. What we expect of our strategies is not truth-conduciveness, but only that

they can efficiently populate the space of possible causal and mechanistic hypotheses for the explanation of a phenomenon.

2.2 Causal-mechanical discovery strategies

The best known set of discovery strategies for the generation of causal and mechanistic hypotheses was proposed by Darden (2006), who outlined the following types: schema instantiation, modular subassembly, and forward/backward chaining. We will briefly introduce Darden's strategies and then discuss how they relate to the strategies we have found to be particularly important for understanding our historical cases.[2]

In (1) schema instantiation, a mechanism schema is imported from solutions of related phenomena. For instance, evolutionary biologists may explain the origin of a trait as an instance of the general mechanism of natural selection. In (2) modular subassembly, one proceeds from known or suspected components of the mechanism under study and tries to arrange them such that a "plausible candidate mechanism" (p. 361) can produce the phenomenon to be explained. Known functional modules – say, G-protein coupled signaling – may be imported to generate novel overall mechanisms. In (3) forward/backward chaining, we reason based on known entities and activities and ask how they can link up. For instance, Watson and Crick in their paper on the structure of DNA famously noted that it "has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material" (Watson and Crick 1953, p. 737).

In the analysis of our historical cases, a number of strategies have emerged as particularly important. The first is the wholesale *transfer* of existing causal structures from one scientific problem to another. This is related closely to Darden's "schema instantiation". Second, progress is often achieved through an aggressive *combinatorial* approach to a problem. We label a strategy combinatorial if many possible causal interactions are considered systematically (see especially section 3.2.3), or if a new causal model is constructed by recombining parts of previous causal models and integrating it with new elements (4.3). This bears some resemblance to Darden's "modular subassembly", although our notion of combinatorial processes is broader. Third, the *reversal* of known causal processes is key in both our studies. It is often possible to take knowledge about how one gets from A to B, and to hypothesize the

[2] Our focus on causal-mechanical reasoning strategies is not meant to be exclusionary: We believe that our approach is one of many useful points of view. First, we deal with our case studies at a much less fine-grained resolution than analyses of laboratory notebooks (see especially Holmes et al 2003; Holmes 2004). Like Holmes, we believe that multiple scales of temporal "graininess" can each reveal important aspects of the context of discovery (2004, chapter 2; 2009, pp. 77–78). Second, our study is not a contribution to cognitive science because we do not analyse the process of hypothesis generation in terms of the immediate mental operations performed by scientists (cf. Holmes 1991, Holyoak and Thagard 1996, Nersessian 2008, and see references therein). For the same reason, moreover, we do not hold that the strategies we describe could be implemented computationally so as to automate scientific discovery: we do not know if this will ever be possible, but at this stage of the project it certainly is not. Finally, we do not use Rheinberger's (1997) "experimental systems" approach because we have a different focus: like Weber (2005, chapter 5) we take a methodological analysis of hypothesis generation to be a necessary complement to the study of the capacities of experimental systems.

existence of a reverse process leading from B to A. This can be seen as a subtype of Darden's forward/backward chaining.

We also see scientific discovery more broadly in terms of what Kärin Nickelsen (2009) has called the "building block strategy", which refers to the transfer of problem solutions from (1) the store of standard contemporary knowledge, (2) one's own past achievements (possibly in different scientific disciplines), and (3) highly successful problem solutions used by others in one's own or related fields (p. 74). Nickelsen successfully applies the building block strategy to explain Otto Warburg's revolutionary work on photosynthesis between 1919 and 1923. While Darden's strategies are specific to the elucidation of mechanisms, Nickelsen refers more generally to the construction of causal models. Here we will steer a middle course: We will focus on strategies for the generation of causal hypotheses which may, but need not be resolved at a sufficiently fine-grained level to qualify as mechanisms.[3] In line with the literature already cited, we take a causal structure to be resolved at the mechanistic level if it is understood in terms of a continuous chain of entities and activities that a field accepts as fundamental.

Our accounts are intended descriptively: as empirical hypotheses about the strategies actually used in the generation of the hypotheses under consideration. However, we do not claim that the historical record unambiguously settles the matter in all cases (see also Darden 1991, especially pp. 15–17). Following Lipton (2004, chapter 1), we believe that the philosophy of science ultimately includes a normative task, but that we are at too early a stage of the descriptive task to attach much normative weight to the strategies we describe. It remains for further studies to investigate how much work these and similar strategies can do and have already done.

3 The discovery of Mitchell's chemiosmotic theory

Peter Dennis Mitchell (1920–1992) was a British biochemist. He was educated and worked at Cambridge University until 1955, when he moved to the University of Edinburgh to lead a new research group. In 1961, Mitchell proposed a mechanism for the oxygen-dependent generation of ATP (the "energetic currency" of cells) in mitochondria – oxidative phosphorylation or "OxPhos" for short. His mechanism was remarkable for the fact that it dispensed with the then-usual biochemistry of the homogeneous cytosol and instead gave an integral role to the mitochondrial membrane structures. In the mechanism as it is accepted today (see figure 1), energy from the reduction of oxygen is used by a number of membrane-bound proteins (the so-called electron transfer chain) to pump protons across the inner mitochondrial membrane. The resulting proton gradient is then used by another membrane-bound protein, the ATP synthase, to synthesize ATP from ADP and phosphate.[4] For the proposal and

[3] We do not commit to any particular philosophical account of causation. We expect that our epistemological conclusions will be compatible with whatever the best metaphysical analysis of causation turns out to be – be it a probabilistic theory, a counterfactual theory, a process theory, or something else.

[4] We present a schema of the mechanism accepted today as a guide for readers who are unfamiliar with the biochemical details. Mitchell's original proposal differed from this schema in several respects. For instance, his proposal imagined the orientation of the membrane transport systems (components 2

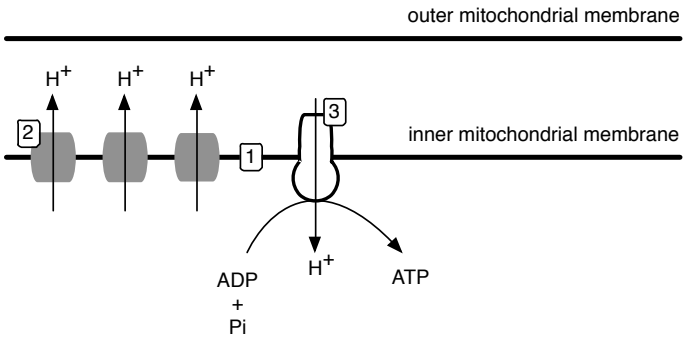

Fig. 1 A schema of the mechanism of oxidative phosphorylation accepted today. The components of the electron transport chain (gray rectangles) transport protons across the inner mitochondrial membrane to create a proton gradient. The proton gradient is then harnessed by the ATP synthase for the synthesis of ATP from ADP and inorganic phosphate. Boxed numbers designate the three principal components of Mitchell's mechanism: (1) the mitochondrial membrane as osmotic barrier; (2) the electron transport chain creating a H^+ gradient; (3) the reverse ATPase / ATP synthase. Note, however, that Mitchell's proposal differed form this schema in several respects, as discussed in footnote 4. Adapted from Alberts et al (2002).

pursuit of this mechanism, which is known as "the chemiosmotic theory", Mitchell was awarded the 1978 Nobel Prize in chemistry.

Two aspects of Mitchell's proposal are usually taken to be extraordinary: first, that it came from an outsider to the oxidative phosphorylation field, and second, that it was exceedingly speculative and yet proved successful. For instance, E. C. Slater wrote in a short biography:

> Up to the appearance of the 1961 *Nature* paper, Mitchell, although well known to those studying transport phenomena, was largely unknown to most of those working on oxidative or photosynthetic phosphorylation [...]. (Slater 1994, p. 293)

Slater goes on to point out the speculative nature of the theory:

> It is a striking fact that, at the time Mitchell proposed his theory, there was not a shred of experimental evidence in its favor. (p. 294)

We should be suspicious of an overall story that falls so conveniently into place: after years of skepticism, an out-of-the-mainstream but visionary scientist is found to have been right all along with his highly speculative ideas. It then only remains to explain why Mitchell of all people was able to propose such an outlandish but ingenious idea – and here John Prebble (2001) offers the view that Mitchell was influenced by tacit but deeply held, quasi-Heraclitean philosophical views. In Prebble's account, Mitchell is moved more by his inner philosophical light than the humdrum empiricism of ordinary biology.

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and 3) to be the reverse of what is actually the case. Moreover, Mitchell included a fourth component: an ion exchange system which would allow H^+/K^+-exchange, reducing the pH differential and replacing it with a potassium membrane potential (also driving H^+ flow). Finally, Mitchell's hypotheses about the mechanisms by which these components operate differed significantly from what is accepted today.

Historians of the episode know that both points need to be reevaluated. First, with the benefit of hindsight Mitchell's outsider status is unsurprising: Given what we now know about oxidative phosphorylation, the relevant expertise was in fact more likely to come from membrane transport than metabolism. Second, while Mitchell's proposed mechanism for oxidative phosphorylation was highly original, its building blocks are firmly anchored in earlier empirical work.

In the following account we will take what is already known about Mitchell's discovery process (see especially Weber 1991; Slater 1994; Prebble and Weber 2003), combine it with new material, and provide a fresh analysis. The starting point of our story is not, however, Mitchell's work. It is instead an earlier attempt to solve the problem of oxidative phosphorylation by E. C. Slater.

3.1 Slater's transfer from glycolysis

By the end of the 1930s, it had been established that two pathways of ATP formation existed: the glycolytic (anaerobic) and the oxidative (aerobic) pathways (Prebble 2010). The glycolytic pathway was already fairly well understood, in large part thanks to work carried out in Otto Meyerhof's laboratory (Bechtel 2005, p. 97–105).

We now know that the two pathways are mechanistically very different. However, in the early 1950s it was eminently reasonable to ask whether the glycolytic pathway could serve as a template for aerobic ATP generation. This was pursued by E. C. Slater (1953), who wrote:

> In the present state of our knowledge, it seems worth while to see if the same general type of mechanism [as in the glycolytic pathway] can explain respiratory chain phosphorylation. (p. 976)

Slater summarized his proposal in three reaction equations:

$$A\mathrm{H}_2 + B + C \rightleftharpoons A \sim C + B\mathrm{H}_2 \tag{1}$$

$$A \sim C + ADP + \mathrm{H}_3\mathrm{PO}_4 \rightleftharpoons A + C + ATP \tag{2}$$

$$A \sim C + \mathrm{H}_2\mathrm{O} \rightarrow A + C \tag{3}$$

Slater imagined A and B to be components of the mitochondrial respiratory chain and C to be an additional component necessary for the reaction. In reaction (1), A is dehydrogenated and enters into a high-energy compound with C, while B is hydrogenated. The high-energy compound is indicated by the tilde in $A \sim C$, a notation introduced by Fritz Lipmann in the 1940s and somtimes called "Lipmann's squiggle". A can be liberated from C by one of two reactions. In reaction (2), the energy contained in $A \sim C$ is used to phosphorylate ADP. In reaction (3), $A \sim C$ is hydrolysed without ATP synthesis. Slater speculated that similar reactions would occur repeatedly throughout the respiratory chain, ending in the reduction of oxygen.

Slater's proposal offered a possible explanation of the "uncoupling" of respiration and phosphorylation by agents such as dinitrophenol (p. 976–977). Dinitrophenol (DNP) was widely used as a diagnostic tool to test whether processes depended on oxidative phosphorylation, and so proposals for the mechanism of oxidative phosphorylation needed to account for DNP's effects. On Slater's proposal, reaction (3)

is slow in physiological conditions but increases when uncouplers are added. The consequent depletion of $A \sim C$ prevents its use for ATP synthesis.

Thus, the first attempt to explain the mechanism of oxidative phosphorylation consisted in a wholesale transfer of the schema of glycolytic phosphorylation: In the glycolytic pathway, ATP synthesis in the final steps depends on a high-energy intermediate known as phosphoenolpyruvate (PEP); in Slater's schema, $A \sim C$ takes the place of PEP. Although Slater's chemical hypothesis led to fruitful empirical research, its high-energy intermediate $A \sim C$ could not be isolated (Mitchell 1961c, p. 148; Allchin 1994).

3.2 Mitchell's building blocks

The three principal components of Mitchell's mechanism are shown in figure 1. The mitochondrial membrane as osmotic barrier emerges from Mitchell's earlier research and will be discussed in section 3.2.1. Mitchell's theory of specific transport across membranes is discussed in section 3.2.2. Charge separation by the electron transport chain belongs to the biochemical context of the time, as we will discuss in section 3.2.3. The ATP synthase emerges on the one hand from Mitchell's theory of membrane transport and on the other hand from the biochemical context, as discussed in section 3.2.4.

3.2.1 The osmotic barrier

We know biological membranes to have specific permeabilities due to passive and active transport systems. From today's vantage point, the role of the mitochondrial membrane as an osmotic barrier maintaining a H^+ gradient is therefore easy to accept. However, in the 1950s the permeation properties of biological membranes were a relatively new area of research. Mitchell was involved in this research from the time of his Cambridge Ph.D. thesis with the membrane biologist James Danielli; the thesis concerned the mechanism of penicillin's effects on bacterial cell wall synthesis. Mitchell's subsequent research in the 1950s was in part a systematic investigation of the permeability of bacterial membranes. Such research had potential practical implications, for instance in pharmacology (Mitchell 1958).

Mitchell had introduced the term "osmotic barrier" in 1949 to describe the impermeability of bacterial membranes to solutes of low molecular weight (Mitchell 1949). This was not universally accepted since the vigorous metabolism of bacteria seemed to suggest that the starting materials and products of metabolism could diffuse freely into the surrounding medium. Mitchell and his long-time collaborator Jennifer Moyle thus investigated the permeation properties of various bacteria to salts, amino acids, electrolytes, monosaccharides, and so on (Mitchell and Moyle 1956a,b,c, 1957, 1959). Their investigations supported the specific permeability of membranes. Since straightforward diffusion could thus not explain the transfer of substances across bacterial membranes, membrane transport mechanisms became a key concern.

A further contribution by Mitchell and Moyle from the same period would prove important. In 1956, Mitchell and Moyle published the finding that the cytochrome system in bacteria was located in the bacterial membrane (Mitchell and Moyle 1956d). As Slater (1994, p. 287) notes, this showed the similarity between bacterial and mitochondrial membranes. Coming a decade before the modern revival of the endosymbiosis theory (Sagan 1967), the finding gave an experimental foundation for Mitchell's later transfer of causal mechanisms from bacterial to mitochondrial membranes.

We do not claim that the transfer from Mitchell's earlier work on membrane permeability to oxidative phosphorylation was straightforward: H^+ ions are small compared to other ions, and whether membranes were equally impermeable to H^+ became one of the early empirical questions that needed to be addressed (Weber 1991, p. 583). Nevertheless, the mitochondrial membrane as osmotic barrier is firmly anchored in Mitchell's theoretical and experimental work in the 1940s and 1950s.

3.2.2 The Mitchell-Moyle-theory of membrane transport

By the middle of the 1950s, several hypotheses were on offer to explain specific membrane transport. It was suggested that membranes might contain "shuttle" components that travel from one side to the other; that some membrane components could rotate; or that contractile pores existed.[5]

In two papers published in *Nature*, Mitchell and Moyle suggested their own hypothesis of membrane transport (Mitchell 1957; Mitchell and Moyle 1958). The theory's main distinguishing feature is its attempted theoretical unification. It resisted the notion that membrane transport is accomplished by special (perhaps rotating or contractile) proteins that remained to be discovered. Instead, it asked how ordinary biochemical enzymes could serve the purpose, with "translocation being a normal enzymic attribute when the enzyme forms part of the membrane" (Mitchell 1957, p. 135). This would have aligned nicely with Mitchell and Moyle's finding that aspects of membrane transport seemed to follow enzyme kinetics (Mitchell 1954; Mitchell and Moyle 1956c).

Mitchell and Moyle imagined an enzyme involved in a group-transfer reaction embedded in a membrane (figure 2). In a first step, the starting materials bind to the enzyme: in this case, ATP on the left-hand side and a substrate S on the right-hand side. In the second step, the enzyme performs its ordinary function of transferring a phosphoryl group from the ATP to the substrate. In the third step, ADP and the phosphorylated substrate dissociate from the enzyme. In this way, an ordinary enzyme of a group-transfer reaction could accomplish transport of phosphate "in orthodox biochemical terms" (Mitchell and Moyle 1958, p. 372).

Mitchell and Moyle's mechanism of membrane transport is itself an illustration of our strategies of causal hypothesis generation. First, known enzymatic mechanisms are transferred wholesale. Then follows a combinatorial step: we ask what would happen if an ordinary enzyme occurred not in solution but in a lipid membrane. The

[5] For a contemporary overview of some of these hypotheses, see Danielli (1954).

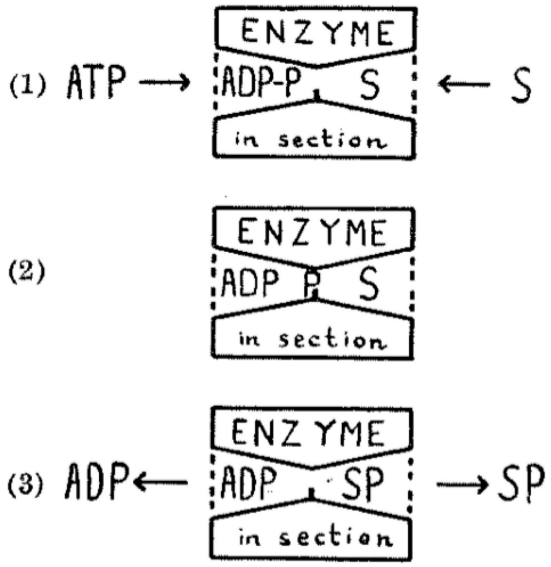

Fig. 2 Mitchell and Moyle's "orthodox biochemical" hypothesis of membrane transport. An enzyme of a reaction involving group transfer is embedded in a membrane. In (1), ATP binds on the left-hand side of the membrane (ADP-P after binding), while the substrate to be phosphorylated binds on the right-hand side of the membrane. (2) The enzyme transfers the phosphoryl group to the substrate. (3) ADP and the phosphorylated substrate dissociate from the enzyme. In this hypothetical scenario, enzymatic group transfer results in membrane transport of phosphate. From Mitchell and Moyle (1958), S. 373.

result is a novel hypothesis which is all the more appealing because its causal building blocks are already well established.[6].

3.2.3 The Robertson review: defining a search space

It is now time to see how Mitchell's work on membrane permeability and transport connects to energy metabolism. A key document for this purpose is a review by R. N. Robertson (1960) titled "Ion Transport and Respiration", which played a recognized but under-explored role in the genesis of the chemiosmotic theory.

Robertson discussed the connection between charge separation and ATP synthesis with regard to such processes as gastric acid secretion and ion accumulation in plants. We will argue that his review defined a combinatorial search space for possible hypotheses about transport and metabolism. It allows us to reconstruct at least one possible and plausible path towards Mitchell's chemiosmotic mechanism. Moreover,

[6] It is telling that Mitchell and Moyle argued for their theory of membrane transport by highlighting not its originality but its orthodoxy (Mitchell and Moyle 1958, p. 373). Although the Popperians are no longer on the scene, the prejudice still prevails that scientists generally aim to propose revolutionary hypotheses (cf. Chalmers 1973). But while iconoclasm is certainly a desirable long-term outcome, conservatism seems to be more highly prized in everyday hypothesis generation (trying to explain oxidative phosphorylation by analogy to glycolysis, or membrane transport in terms of enzyme reactions). We will have more such instances in our second case study in section 4.

Robertson's review helps us to contextualize Mitchell's innovations within late 1950s biochemistry.

The importance of Robertson's review has not gone unnoticed in the literature. Slater (1994) wrote with a note of reprobation:

> It is surprising that Mitchell, who referred to the 'excellent' review of Robertson, did not acknowledge the important step that the latter had taken. (p. 290)

However, this neglects Mitchell's brief summary of his theory in the *Proceedings of the Biochemical Society* (Mitchell 1961b) which appeared a month before his July 1961 *Nature* paper. Here Mitchell wrote of his theory that "this type of conception is latent in work on ion transport and respiration (see Robertson, 1960)" (p. 23P). It would thus be unjust to say that Mitchell failed to give proper credit to Robertson – it is quite a strong claim to say that one idea is latent in another. But for our philosophical purposes this raises a key question: What precisely is the relationship between the Robertson review and the chemiosmotic theory?

The point of departure for Robertson is the observation that secretion processes can be blocked by DNP (compare section 3.1). This indicates that they are dependent in some way on oxidative phosphorylation. Robertson approaches the problem combinatorially. Given that A and B are both blocked by D, the following causal structures are possible in general:

1. A causes B (D blocks A and therefore B).
2. A and B are independent (but blocked by the same substance D).
3. A and B have a common cause C (D blocks C and therefore A and B).
4. B causes A (D blocks B and therefore A).

Robertson explores all of these possibilities except structure (4) – which is the one that Mitchell then developed further. We will argue for the following interpretation of this fact: Robertson confined his exploration of the space of combinatorial possibilities to causal structures for which he could provide plausible underlying mechanisms. Mitchell could explore the fourth causal structure because he had additional mechanistic hypotheses, as we will explain below.

Robertson presents the causal structures (1) to (3) in a key figure, which is here reprinted as figure 3. The first hypothesis is that ATP synthesis depends on oxidation reduction (labeled (b) in the figure), while secretion depends on ATP (c). Since DNP was already known *not* to affect oxidation reduction, DNP can be assumed to block (c) by blocking (b).[7] The second hypothesis is that secretion and ATP synthesis both depend directly on oxidation reduction – and that DNP just happens to block both of these processes but not O_2 uptake. The third hypothesis is that both secretion and ATP synthesis depend on the mechanism of charge separation, which is blocked by DNP. Robertson's last hypothesis is a variant of the third, in which secretion and ATP synthesis are alternative outcomes of the same process.

Robertson favored the common cause structures (3). In some respects, this is similar to Mitchell's proposal. Most importantly, the separation of positive and negative charge (that is, secretion) is accomplished by redox reactions in the respiratory chain.

[7] Robertson distinguishes between charge separation as a *process* occurring in the membrane-bound cytochrome system and secretion as one *result* of charge separation.

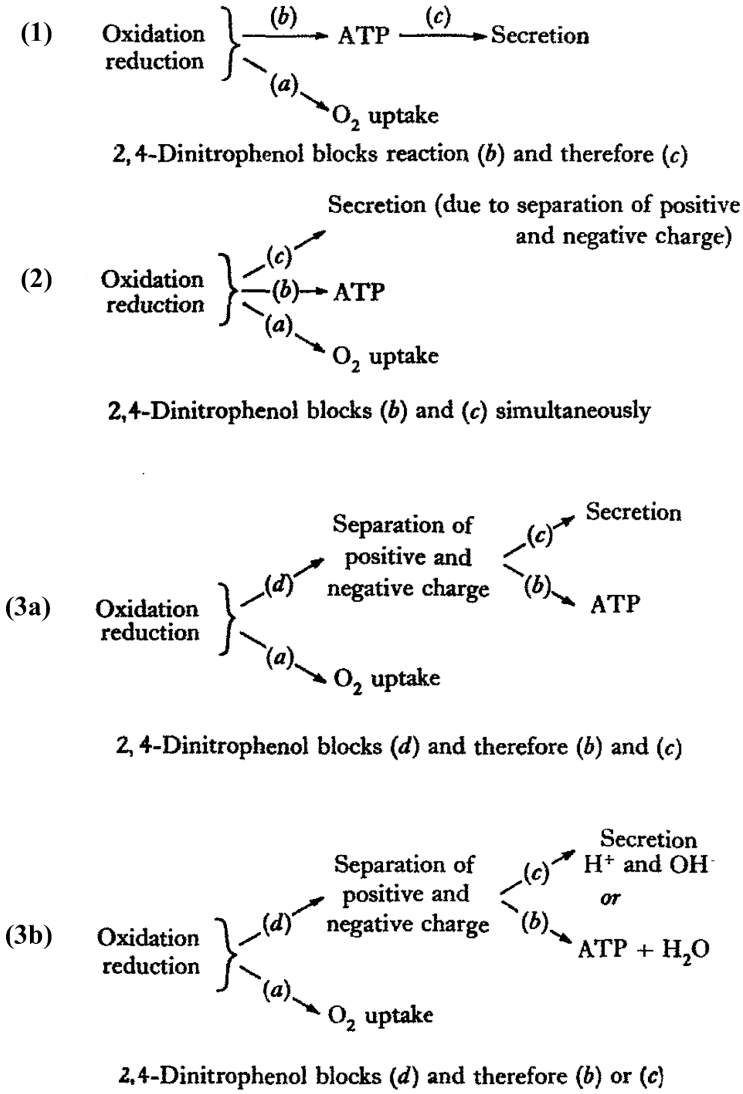

Fig. 3 The key figure from R. N. Robertson's "Ion Transport and Respiration" (1960). Robertson considers three of four possible causal structures to explain the blocking of both ATP synthesis and secretion by DNP. 1: DNP blocks ATP synthesis and therefore secretion. 2: DNP simultaneously blocks the independent processes of ATP synthesis and secretion. 3a: ATP synthesis and secretion depend on the mechanism of charge separation, and so blocking charge separation inhibits both. 3b: A variant of (3a), but with ATP and secretion being alternative rather than parallel outcomes of the same process. The fourth possible structure, in which an existing H^+ gradient is used to drive ATP synthesis, is not considered. Note that we renumbered Robertson's graphs for clarity of presentation.

Redox pumps were an active area of discussion in biochemistry at the time.[8] However, it would be going too far to say that Robertson anticipated Mitchell's theory. ATP synthesis in his scheme is *not* accomplished by harnessing the proton gradient produced by secretion. Rather, he proposed that the redox reactions of the respiratory chain are *directly* used for ATP generation: Reduced intermediates are involved in reactions leading to phosphorylation of ADP on the one hand and to charge separation on the other. In this respect, Robertson's proposal was much closer to Slater's chemical than to Mitchell's chemiosmotic hypothesis. Robertson's suspected mode of action for DNP reflects this. He did not suggest that DNP made the membrane permeable, thus depleting a gradient. In his scheme, this would not have explained DNP's effects, since ATP synthesis did not depend on a proton gradient. Instead, Robertson suggested that DNP caused the depletion of a reduced intermediate of the electron transport chain – thus preventing phosphorylation and charge separation, in effect "short-circuiting" the mechanism (p. 240). This is essentially reaction (3) in Slater's chemical hypothesis.

With his systematic and combinatorial approach, Robertson proposed three of four possible causal structures linking the known factors charge separation, ATP synthesis, secretion and DNP blocking. We can now see in what respect the chemiosmotic theory was "latent" in the Robertson review: the unexplored fourth causal structure is the latent element. Presumably, Robertson did not explore the fourth structure because he lacked a mechanism to make it plausible. In the next section, we will show how Mitchell was in a better position to explore the fourth structure due to his work on membrane transport. Mitchell introduced a key element to make the fourth causal structure plausible: a *reverse* ATPase to harness the proton gradient for ATP synthesis.

3.2.4 The reverse ATPase

We have seen that Robertson considered three possible causal structures linking ATP synthesis and secretion of protons: ATP driving secretion, ATP synthesis and secretion being independent processes, and both resulting from the same process. It was left for Mitchell to consider the fourth possibility: that secretion of protons is a cause of ATP synthesis, via a trans-membrane proton gradient.

For this mechanism to work, Mitchell needed a membrane-bound proton-transporting ATPase running in reverse. One would expect Mitchell to point to the origin of this idea in the published literature, but his 1961 paper in *Nature* is scant help: When introducing the ATPase, Mitchell refers to his own earlier publication of the chemiosmotic mechanism in the *Proceedings of the Biochemical Society* (Mitchell 1961b). However, the *Proceedings* paper introduces the ATPase without giving any references for it! So where did the idea of a proton-transporting ATPase come from?

We cannot offer a definitive answer, but one possibility suggests itself very strongly: ion-transporting ATPases were already a major concern of the biochemical community in the 1940s and 1950s.[9] The two most studied ATPases at the time were the

[8] See Robinson (1997) for a discussion of the history of the redox pump hypothesis on pp. 113–116.

[9] See Joseph D. Robinson's invaluable history of membrane transport, especially chapter 3–8, for an in-depth discussion of these developments (Robinson 1997).

myosins of muscle-contraction and the ubiquitous Na^+/K^+-exchange ATPase. By the 1930s, it was known that the interior of cells is rich in K^+ while the extracellular fluid is rich in Na^+. Initial attempts to explain this distribution used passive models, in which no energy was expended for maintaining the ionic asymmetry. However, these models were soon abandoned in favor of energy-dependent systems. Remember also that the late 1930s and 1940s were the time of Hodgkin and Huxley's work on the action potential, which recognized a Na^+-current into the axonal cells as a main component. The question of how the Na^+ from action potentials was extruded was pressing.

By the mid-1950s, the ATP-dependent Na^+/K^+-exchange system was reasonably well characterized. However, the responsible membrane protein had not yet been isolated. This succeeded when Jens Christian Skou (1957) isolated a fraction of squid axon membrane whose characteristics permitted him to identify it as the sought-for Na^+/K^+-ATPase.[10] The number of citations of Skou's paper increased exponentially from around 1958 (Robinson 1997, p. 135): The Na^+/K^+-transporting ATPase was a hot topic in the membrane transport community, and we can safely assume Mitchell to have known about it. We can thus place the basic idea of a cation-transporting ATPase firmly in Nickelsen's category of "highly successful problem solutions used by others in one's own or related fields" (Nickelsen 2009, p. 74). Further evidence that Mitchell would have known about Skou's work is given by the fact that Mitchell and Skou attended the same conferences. Indeed, Mitchell's first suggestion of a reverse proton-transporting ATPase came in the proceedings to a conference in Stockholm in September 1960 (the proceedings are in Goodwin and Lindberg 1961, and Mitchell's contribution is Mitchell 1961a). This was two weeks after a conference in Prague attended by both Mitchell and Skou, who presented new results on the Na^+/K^+-ATPase (the proceedings are in Kleinzeller and Kotyk 1961, and see especially Skou 1961).

The evidence that Mitchell transferred the ATP-dependent cation-transporter from Skou's work is certainly not conclusive. However, the important point is that this component of Mitchell's mechanism does not challenge our discovery strategies. There was *at least* one plausible and ubiquitous source for the transfer. Moreover, the importance of such transporters in the field of membrane physiology may explain why neither Mitchell nor his referees saw any need to cite a source in the scientific literature.

Three steps were necessary to arrive at the reverse proton-transporting ATPase of the chemiosmotic theory. The first step is the the most trivial one: Did the mitochondrial membranes contain ion-transporting ATPases like the cell membranes? While this was far from settled, it was known that mitochondrial membrane fractions did contain ATPase activity (Lehninger et al 1958; Lehninger 1961). The second step was again of a combinatorial nature: Mitchell had to assume that if sodium and potassium transporting ATPases existed, so might proton transporting ones. The third step is the most daring: Mitchell had to consider the possibility that the ATPase might run in reverse. As we will show next, this idea emerges from Mitchell's considerations

[10] In recognition of the discovery, Skou received the Nobel Prize in Chemistry in 1997, together with Paul D. Boyer and John E. Walker.

of the mechanism of active membrane transport. Thus, the last step belongs firmly in Nickelsen's category of "one's own past achievements" (Nickelsen 2009, p. 74).

We have already seen that Mitchell had proposed a mechanism of membrane transport in which "translocases" are nothing but ordinary biochemical enzymes embedded anisotropically in a membrane. It remained for him to put the hypothesis on a firm thermodynamic footing by showing that transport could result from known enzyme dissociation constants along with appropriate intra- and extracellular concentrations of starting materials and products. He did this in his contribution to the proceedings of the Stockholm conference (Mitchell 1961a). On pp. 595–598, Mitchell first develops the standard equilibrium equation for enzymatic reactions such that it can take into account a pH-gradient across a membrane-embedded enzyme (where the gradient is produced by a "photoelectric effect or by a metabolic oxidoreduction involving a flow of electrons across the membrane", p. 597). Next, he calculates that a pH gradient of 3 would be sufficient for the enzyme glucose-6-phophatase to effect membrane transport, given the enzyme's equilibrium constant. It is a small step from glucose-6-phosphatase to other phosphatases, and Mitchell takes it immediately:

> I need hardly point out that a similar, but greater asymmetry of electrochemical hydrogen ion activity to that considered in the above example, could be responsible for converting the ATPases of the particulate systems of photosynthetic and oxidative phosphorylation into the ATP-synthesising catalysts. I hope to develop this interesting and important aspect of translocation catalysis on another occasion. (pp. 597–598)

Thus, the seemingly outlandish reversal of an ATPase into an ATP-synthase emerges naturally from Mitchell's theory of membrane transport: It is a consequence of the fact that the theory assumes transport to be accomplished by ordinary biochemical enzymes whose reactions were already known to be reversible (depending on the enzymes' equilibrium constants and the concentrations of starting materials and products).

Mitchell's success thus depended on a false mechanistic hypothesis: Robertson's unexplored fourth causal structure – an ion gradient causing ATP synthesis – was accessible to him because of his orthodox biochemical theory of membrane transport. We now know that this is not how the ATP synthase works, and that it instead harnesses the proton gradient's energy by a series of conformational changes. The inapplicable mechanism nevertheless helped Mitchell to find the correct coarse-grained causal story: the ion gradient *is* the cause of ATP synthesis. This suggests a subtle interplay between a coarse-grained causal and a fine-grained mechanistic level of analysis: false mechanistic hypotheses can permit the exploration of true causal relationships, which then in turn form the basis for a revision of the mechanistic details.[11]

[11] Prebble (2000) notes that Mitchell "came up with mechanisms in large numbers" and "was allergic to systems that depended on processes which at the time could not be defined precisely at the molecular level" (p. 330). He defends Mitchell's penchant for inventing mechanisms with the suggestion that a few successes justify "so many apparently fruitless mechanisms". We would argue that no defense is needed: scientific discovery relies on the proposal of appropriate candidate mechanisms. Moreover, we are not sure to what extent Mitchell's insistence on molecular detail was unusual, since at least Robertson (as discussed above) also formulated hypotheses largely at the mechanistic level. Prebble mentions that many further

This concludes our discussion of the context of discovery (in a fairly narrow sense) of oxidative phosphorylation. Even though we have shown that Mitchell's hypothesis was well anchored in existing empirical and theoretical work, it was not born empirically justified or in its final form – but we omit the further details of the empirical investigation and conceptual revision of Mitchell's hypothesis, a process which terminates at the earliest over a decade later with the experiments by Racker and Stoeckenius (1974). This story has been well covered by the existing literature (cited above) and does not need retelling. We instead turn to our second case study of discovery in 20th century biochemistry: a story not of a grand novel idea but of painstaking detective work.

4 The Calvin-Benson Cycle

Our second case study is also from the field of bioenergetics: the Calvin-Benson cycle, that is, the mechanism for the reduction of carbon dioxide to the stage of carbohydrates in photosynthesis. It was discovered through the concerted action of a large, interdisciplinary research team working on the elucidation of the cycle from 1946 to 1954.

The investigation of the intermediate compounds of metabolic pathways became possible with the advent of radioactive tracer molecules in the early 1940s, in particular with the finding of carbon-14 that was proudly announced in Ruben and Kamen (1941). In order to explore the further implications of this discovery, a "Bio-Organic Chemistry Group" was set up at Berkeley, in 1946, headed by the chemist Melvin Calvin. Together with a former student of Ruben's, Andrew A. Benson, who was leading the photosynthesis division of the group, Calvin made skillful use of the radiotracer technique, and the group eventually succeeded in elucidating the so-called "dark" reactions of photosynthesis. The major part of the group's work on the cycle was completed by 1954, for which Calvin – unfortunately without the acknowledgment of Benson's contribution – was awarded the 1961 Nobel Prize in Chemistry.

In the following sections we will sketch the conceptual milestones without discussing the empirical foundations and the development of the radiotracer technique.[12] More historically detailed analyses of this case have been provided in Nickelsen (2012) and Nickelsen (in press, Ch. V; further references, in particular to numerous autobiographical accounts, are given therein). Useful background information is also available in the interviews of a large Oral History Project on the Berkeley group, available as Moses and Moses (2000). The account is structured along a sequence of model proposals as they were brought forward by the group in the years 1947/48, 1950, 1952 and, finally, 1954. Elucidating this cycle involved the same discovery strategies as the OxPhos case: the wholesale transfer of causal mechanisms or mechanism schemata to other contexts, the combination of existing elements of knowledge to create something new, and the reversal of known biochemical pathways.

mechanistic ideas exist in Mitchell's unpublished documents, and it will be worthwhile to investigate these in the light of a causal-mechanical understanding of discovery.

[12] This is not meant to suggest that the radiotracer techniques were unimportant. However, an appropriate treatment would double the paper's length.

4.1 The Rise of the Standard Hypothesis

The first principal assumption which in the 1940s guided the search for the carbon reducing mechanism was that photosynthesis formed the reverse of respiration. When the Calvin-Benson group started its work, the biochemical pathway of cell respiration had been largely uncovered, including:

1. the process of glycolysis – we have already seen the importance of glycolysis as a mechanism template in the first case study (cf. section 3.1);
2. the tricarboxylic acid cycle, first brought forward by Krebs and Johnson (1937), which is the stepwise oxidation of the carbon residue that resulted from glycolysis, whereby reducing equivalents are formed; and
3. first and very tentative ideas of how the final oxidation process was to be conceived of, presented in, e.g., Keilin and Hartree (1939).

The idea that photosynthesis might use some of these reactions in reverse was strongly suggested by the blunt fact that the products of the one process were the starting materials of the other; the growing awareness that many enzymatic reactions were found to run in both directions gave additional support to this longstanding idea.

The second assumption that strongly influenced the modeling of photosynthetic carbon reduction was the idea that a cyclic pathway was involved. This was forcefully brought forward, for instance, by the plant physiologist Kenneth V. Thimann (1938), although at the time he was not yet aware of Krebs's work on the aforementioned tricarboxylic acid cycle – Thimann did cite, however, Krebs's earlier work on the urea cycle, Krebs and Henseleit (1932), as the first instance of a metabolic cycle to be completely illuminated (cf. Nickelsen and Graßhoff 2008; Holmes 1991). Since that time, cyclic pathways had become an integral part of biochemists' conceptual tool kit.

These two key assumptions of modeling the photosynthetic carbon dioxide reduction – the reversal of respiration and biochemical cycles – were strongly endorsed by an unexpected finding: Wood and Workman (1935; 1936; 1938) showed that the fixation (which implies reduction) of carbon dioxide was not confined to plants but was part of the metabolism of organisms ranging from bacteria to mammals, and this reduction process involved a partial reversal of the tricarboxylic acid cycle (for further accounts of this episode, see Singleton 1997; Krebs 1974). This pathway was henceforth taken as an obvious template for the carboxylation step in photosynthesis, while it was combined, as we will see, with the reversal of glycolysis as the postulated pathway for the synthesis of glucose.

Thus, the first proposal of the Berkeley group realizes at least two of the strategies we have already discussed in the OxPhos case: transfer and reversal. It combines a pathway from non-photosynthetic carbon dioxide fixation with the reversal of glycolysis. This model is reconstructed in figure 4 based on the chemical prose in Benson and Calvin (1947) and Calvin and Benson (1948). This graph also highlights the fact that the group around Benson and Calvin had (implicitly) modularized the problem in three partial processes: (1) carbon dioxide had to be incorporated into an acceptor molecule ("carboxylation"); (2) there had to be a pathway from the acceptor

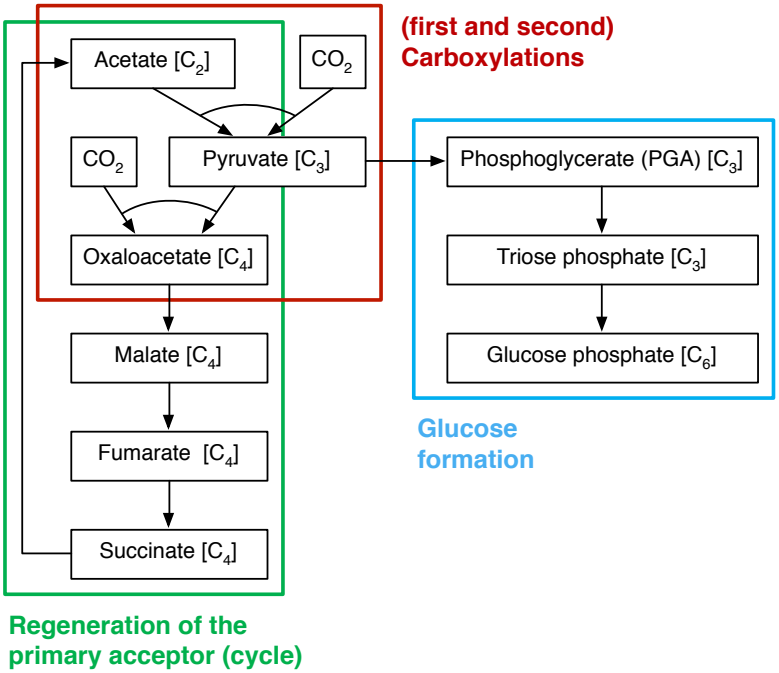

Fig. 4 The photosynthetic carbon cycle, as formulated by the Berkeley group in 1947/48. The formation of glucose was modelled as the reversal of glycolysis, which has remained in place ever since; the carboxylation of the acceptor molecule as well as its regeneration in a cyclic sequence were – following the template of heterotrophic carbon dioxide fixation – conceived of as the reversal of portions of the tricarboxylic acid cycle. The latter two modules were substantially revised in the course of the following years.

molecule to six-carbon sugars, eventually glucose ("glucose formation"); (3) the primary carbon dioxide acceptor molecule had to be regenerated so that further carbon dioxide molecules could be incorporated ("regeneration of the primary acceptor"). These three modules can be traced through later stages of their work, although they were continuously revised according to the latest evidence. This "decomposition" of a mechanism in several component operations that contribute to the overall functioning of the system is one of the strategies that Bechtel and Richardson (1993) identified as a typical move in the elucidation of mechanisms; the concept also looms large in Bechtel (2005). While it was by no means certain that the hypothesized modules corresponded to actual modules in the system, it was a valuable guiding assumption and facilitated the task of the group.

The further exposition of these partial processes clearly fits our idea of a combinatorial approach, as Benson and Calvin (1947) saw no reason to to conceal: "Using some of the reactions already established in animal tissue and bacteria", they found it possible to account for all their experimental findings (pp. 448-49). The resulting model, the authors underlined, had the definitive advantage that it was exclusively based on reaction steps which were part of standard biochemical knowledge.

The principal idea of this model was rather simple: Acetate, which is a two-carbon compound [C_2],[13] was thought to act as the first carbon dioxide acceptor, the carboxylation of which would lead to the formation of pyruvate [C_3]. The latter would be subject to a second carboxylation, which resulted in the formation of oxaloacetate [C_4]. Via the stages of malate [C_4], fumarate [C_4] and succinate [C_4], the acceptor molecule would be regenerated by the splitting of succinate into two molecules of acetate [C_2]. Starting from pyruvate, the way was also open to the formation of glucose via the intermediate steps of 3-phosphoglyceric acid (PGA) and triose phosphates [C_3]. As to the latter reaction step, Calvin and Benson (1948) underlined that it could be taken as "fairly certain" that "the hexose synthesis proceeds by a reversal of the usual glycolytic split of fructose diphosphate" (p. 478). The energy required for all these reactions was thought to be provided by reducing equivalents, which were assumed to come from the photochemical parts of photosynthesis.

4.2 Modification of the Standard Hypothesis

Two years later, the Berkeley team had to contract their model: the scant empirical evidence for the intermediates succinate, fumarate and malate turned out to be artifactual. And while the occurrence of substantial amounts of PGA was strongly confirmed, the same could not conclusively be maintained for triose phosphates, which were highly probable intermediates on the assumed pathway from PGA to glucose. Nevertheless, the group clung to the hypothesis that the hexoses, that is, the end products of photosynthesis, were formed from the PGA in a reversal of the process of glycolysis; and, interestingly, although evidence only accumulated slowly, this assumption was no longer seriously challenged. The new model was described in Benson and Calvin (1950) as follows:

> It is essentially a dicarboxylic acid cycle in which a two-carbon acceptor molecule is converted to oxaloacetate by two successive carboxylations. Upon splitting the four-carbon acid, two new acceptor molecules are formed. The intermediates of photosynthesis are diverted from this cycle for synthesis of fats, amino acids and carbohydrates. (p. 33)

The 1950 cycle was modified as carefully and minimally as possible. It now started with a hypothetical [C_2]-acceptor. The group still suspected that acetate had this function but as they had not been able to produce any evidence for this assumption, they left this position open. The acceptor was carboxylated (and phosphorylated) to form PGA [C_3]. Then PGA would either be supplied to form glucose-6-phosphate in what constitutes the reversal of glycolysis (although, as was mentioned earlier, the presence of triose phosphates, which were the central compounds of this path, was not undisputed), or it would be rearranged to form phosphopyruvate [C_3]. The latter was thought to be subject to the second carboxylation reaction, yielding oxaloacetate [C_4], which would regenerate the acceptor by splitting into two [C_2] fragments. Aspartate and alanine, two amino acids which were also among the compounds identified to occur in photosynthesizing cells, could easily be formed from oxaloacetate

[13] The abbreviation [C_2] denotes that this compound contains two carbon atoms, usually as a backbone chain. Within a compound the carbons are given numbers according to their position in the chain.

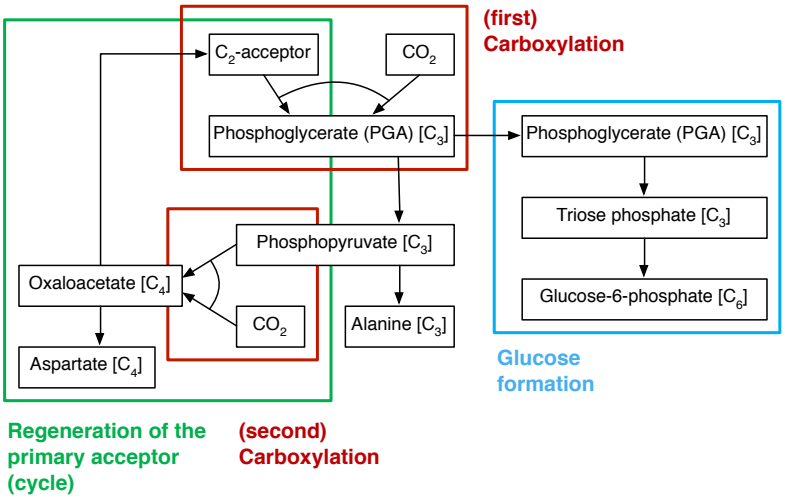

Fig. 5 The photosynthetic carbon cycle, as formulated by the Berkeley group in 1950. The standard hypothesis of 1947/48 was only minimally revised: the formation of glucose is still the reversal of glycolysis; the group still assumed two instances of carboxylation, although they left the first carbon acceptor open; the regeneration of the latter still was thought to be brought about by the split of a 4-carbon compound (oxaloacetate), although the intermediates malate, fumarate and succinate were dropped.

or pyruvate. In reaction to the revised empirical evidence, malate, succinate and fumarate were no longer listed as intermediates on the path (and would not return). Yet, it was well-known that all these [C4]-compounds were easily formed starting from oxaloacetate – and vice versa.

Interestingly, a carboxylation module as envisioned in the 1947/48 model – involving malate as a central intermediate – actually *does* exist in other organisms than green algae. The first indication of the existence of this pathway was found in the late 1950s in sugar cane plants, but the findings were published only years later as Kortschak et al (1965). This paper immediately prompted a group in Brisbane, Australia, to pursue the question; and further research revealed the aforementioned pathway that today is called "C_4 photosynthesis", since it includes a number of C_4 organic acids (cf. Hatch and Slack 1966; Hatch et al 1967; retrospective accounts are given in Hatch 1992, 2002). Around the same time Evans et al (1966) reported the occurrence of a "reductive carboxylic acid cycle" in some bacteria, which in fact was a tricarboxylic acid cycle run in reverse in order to produce organic compounds from carbon dioxide in water.

4.3 The Fall of the Standard Hypothesis

The Berkeley perspective on the path of carbon completely changed, however, when Benson found evidence in the chromatograms for the existence of two entirely unexpected compounds among the products of short-time photosynthesis: sedoheptulose monophosphate [C_7] (Benson et al 1951) and ribulose diphosphate [C_5] (Benson

1951).[14] The finding of a sedoheptulose, in particular, was hard to comprehend. So far, these elusive seven-carbon sugars had been reported to be present only in succulents (therefore the name, which refers to the genus *Sedum*), while now they seemed to perform a vital function in photosynthesis! The sedoheptulose was found to be formed prior to hexose phosphates, while it was too different in structure to be one of the latter's direct precursors. Thus, Benson assumed that the sedoheptulose had a function somewhere in the regenerative cycle. Furthermore, parts of its structure were so similar to the five-carbon sugar, the ribulose diphosphate, that it was an obvious step to assume that the two were directly linked to each other, in a precursor-product relationship. In principle, this could have been possible in both direction, that is, there was no obvious preference whether the one or the other was the precursor. However, Benson then was able to establish that ribulose diphosphate readily disintegrated into PGA [C_3] and phosphoglycolic acid [C_2]. Benson found this extremely suggestive and concluded his report by announcing that "a discussion of its [the ribulose diphosphate's] importance as a C_2 donor in the cycle for regeneration of the CO_2 acceptors will be published" (Benson et al. 1951, p. 2972).

The irritating fact was that these newly found substances were neither involved in respiration nor in non-photosynthetic carbon dioxide fixation. This implied, first, that there was no well-known alternative cause for their formation except photosynthesis. A second, very significant conclusion also followed: namely, that at least parts of the photosynthetic pathway substantially diverged from the biochemical processes that so far had been used as modeling schemata. Thus, a more substantial revision of the model was required to accommodate these unusual compounds.

The first revision was put forward in Benson et al (1952). The authors stressed the striking similarities between the five-carbon sugar and the seven-carbon sugar, and also speculated how the sugars might be related to the unknown [C_2] primary acceptor of carbon dioxide. In contrast to the earlier models of 1947/48 and 1950, this time they were forced to include a number of reaction steps which so far had *not* been reported in the literature. But because the molecular structures of the compounds were known, likely reaction steps could be inferred on the basis of typical reactions of similar compounds. Thus, the space of possible causal structures was populated based on biochemical background knowledge, which allowed the unknown compounds to be integrated into the pathway. We see a strong attempt to transfer causal knowledge from other contexts at work here as well as a creative combinatorial approach.

One of the resulting hypotheses was that in the course of the regenerative cycle, sedoheptulose monophosphate would be split into the unknown [C_2] acceptor and ribulose diphosphate [C_5], while the latter would be degraded as well and give rise to another [C_2] primary acceptor molecule and triose phosphate [C_3]. However, we are skipping the further details of this model since its lifetime was extremely limited: immediately after it had been published, subsequent studies in Berkeley revealed that empirical evidence made it unlikely that the seven-carbon sugar was the precursor of the five-carbon sugars – in fact, the data were highly confusing and apparently incoherent; yet, they effectively rendered the latest, carefully crafted model obsolete.

[14] This compound is known today as "ribulose bisphosphate"; in order to be consistent with the historical sources, the older name has been adopted for the paper.

4.4 Solving the Puzzle

The decisive step towards the eventual solution of the puzzle came from experiments of the year 1953, the full results of which were published only two years later in Wilson and Calvin (1955). Having developed a sophisticated steady-state apparatus to monitor the course of photosynthetic carbon reduction under varying conditions, Wilson was able to demonstrate that PGA and the unknown five-carbon sugar were related in a reciprocal precursor-product relationship: whenever one of them accumulated in the system, the concentration of the other went down in exact proportion. This was highly suggestive of a causal relationship in the spirit of Mill's method of "concomitant variation" (recently discussed in Scholl 2013). It also neatly aligned with earlier experiments along similar lines which were published in Calvin and Massini (1952) (although they had brought less clear-cut results). The important conclusion was that "that RuDP [i.e. ribulose diphosphate] is the actual CO_2 acceptor in photosynthesis, or alternatively is related to it by a vanishingly small reservoir, and that PGA is the first observable product of the carboxylation" (Wilson & Calvin 1955, p. 5952). This was the solution that finally brought to an end the hunt for the two-carbon acceptor: there was no two-carbon acceptor. Only a five-carbon acceptor existed, namely the ribulose diphosphate, which, the authors surmised, was split into two halves immediately after carboxylation, yielding two three-carbon molecules of PGA.

After this experimental breakthrough, the group spent some weeks on combining the established elements into one comprehensive and consistent pathway. The "ultimate" model was presented in Bassham et al (1954). The manuscript was received by the journal in October 1953 and printed in April 1954; a rough schema of the regenerative cycle, as formulated in this paper, is depicted in figure 6. Ribulose diphosphate, the five-carbon sugar, was presented as the acceptor of carbon dioxide, while the resulting six-carbon compound was assumed to be highly unstable and immediately split into two molecules of PGA, or, alternatively, one molecule of PGA and one of phosphoglyceraldehyde. The further processing of the PGA required an energy input from the photochemical reactions (in the form of reducing equivalents) and resulted in the formation of triose phosphate. This then became the starting point both of the formation of hexose phosphates and of the cyclic regeneration of ribulose diphosphate.

Given the knowledge of the time, the carboxylation of ribulose diphosphate was easily construed on paper (carboxylation of the ribulose diphosphate at the second carbon atom, hydrogenation on the third carbon atom, and a subsequent splitting right between these two carbon atoms); however, no direct evidence for its occurrence was available. The authors tentatively judged that the alternatives were less likely (p. 1766). However, only a month later (in May 1954) the group reported in Quayle et al (1954) the successful carboxylation of ribulose diphosphate to PGA in a cell-free system: obviously the group felt the urgent need to prove, at the very least, the actual in-vitro existence of this reaction step, however plausible it looked on paper.

The seven-carbon sugar, finally, also found its place. We mentioned earlier that, based on the similarity of the compounds' structures, the group immediately suggested a precursor relationship to ribulose diphosphate, which, however, the empiri-

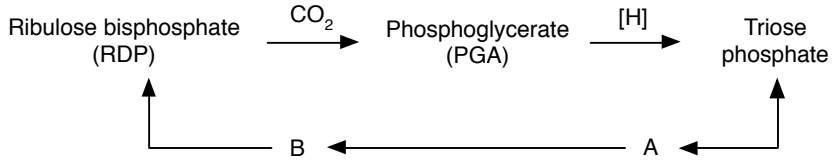

Fig. 6 A rough schema of the photosynthetic cycle. Ribulose diphosphate is the carbon dioxide acceptor, upon which two molecules of PGA are formed. The latter on the one hand gives rise to the formation of triose phosphates and, eventually, hexoses, on the other hand initiates the regeneration of ribulose diphosphate. After Bassham et al (1954), p. 1766.

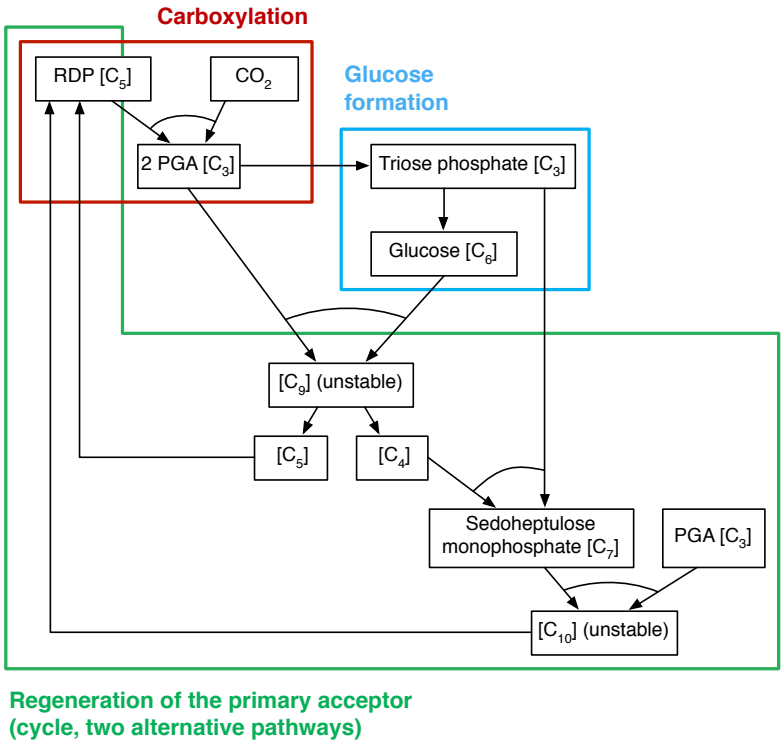

Fig. 7 The cyclic model of 1954. The path of glucose formation has remained unchanged since 1947/48, while carboxylation was limited to one instance, the acceptor of which was ribulose diphosphate. The regeneration of ribulose diphosphate then was a complex combination of two alternatives passing through an unstable [C_9] molecule. Reconstructed from Bassham et al (1954).

cal data did not support. Pondering the quality of the data, the structures of the compounds and the potential types of reactions, the group came up with a quite unexpected solution: sedoheptulose monophosphate, after all, *was* a precursor to ribulose diphosphate; but they were linked by *two different pathways*, which explained the confusing set of data. A two-carbon fragment from the top of the sedoheptulose would combine with triose phosphate to yield ribulose diphosphate; then, the remaining five-carbon fragment of the former sedoheptulose would be rearranged to the same effect. This aligned extremely well with the quality of the data. However, at the time the transition from the five-carbon fragment to the ribulose diphosphate was extrapolated from knowledge of the *reverse* reaction – by now, we know this strategy well.

We skip the further details of the complex pathway, which is graphically reconstructed in figure 7. With this paper, the explanation of the "dark" reactions of photosynthesis had been accomplished. All the key problems in elucidating the way from starting materials to products were solved: integrating the puzzling five-carbon and seven-carbon sugars; finding the carbon dioxide acceptor; and establishing a full sequence of reaction steps that was in agreement with all the relevant empirical evidence. The resulting model, with minor modifications and expansions, is still part of the commonly accepted knowledge in biochemistry.

5 Conclusions

One of the promises of the causal-mechanical point of view is that it will allow us to recognize the appropriate "units of recombination" of hypothesis generation in the biomedical sciences: the specific components of older theories which are reused to construct new ones. These components include, among others, types of entities, types of interactions, and entire mechanism schemata. Instead of speaking in general terms of new hypotheses emerging from previous ones "by analogy", the causal-mechanical approach tells us something about the source and granularity of the analogies. Our studies suggest four systematic conclusions about this process which we consider to be particularly important.

First, it speaks to the power of the causal-mechanical approach that it is able to illuminate the genesis of two hard cases of scientific discovery: both oxidative phosphorylation and the Calvin-Benson cycle were far from trivial extensions of existing knowledge. Despite the simplicity of the discovery strategies here discussed, they are evidently powerful. It is therefore plausible that a large number of less complex scientific discoveries can be explained on the basis of these or similar strategies.

Second, there has long been a debate about whether "general" philosophical insight into biological hypothesis generation is possible, or whether ultimately all discovery strategies are specific to particular bodies of empirical knowledge (or even individual, narrowly circumscribed empirical problems). These opposites are discussed for instance by Marcel Weber (2005, chapter 3), who concludes that, despite claims to the contrary, most instances of discovery essentially depend on domain-specific knowledge. Our studies, however, suggest a middle course between the extremes of substrate-neutral algorithms and domain-specific knowledge: While the strategies un-

der study are to some extent substrate-neutral, they are applied to previous causal and mechanistic knowledge which is domain-specific. Thus, in causal-mechanical hypothesis generation we see an interaction of general strategies and domain-specific knowledge. The challenge lies in elucidating the nature of this interaction more thoroughly.

Third, a particularly interesting instance of the interaction between general strategies and domain-specific knowledge is Robertson's exploration of the relationship between ATP synthesis and secretion. Recall that Robertson explored three of four combinatorially possible causal structures (section 3.2.3), while Mitchell – having additional mechanistic resources at his disposal – explored the fourth. The notion that there is a limited search space of causal explanations for observed correlations is quite general. Yet the productive use of this strategy depended on highly specialized empirical knowledge about the effects of DNP on ATP synthesis, secretion and oxygen uptake. The general strategy of defining a causal search space became productive only because it was applied to a highly circumscribed empirical problem of mid-20th-century biochemistry. Similar dynamics are apparent in the Calvin-Benson study: consider how the group dealt with the finding of unexpected five- and seven-carbon sugars. In order to integrate these into a sensible reaction schema, the group turned to biochemical knowledge on structurally similar compounds and investigated a list of plausible reaction steps. Their rather general combinatorial approach was enabled by a narrowly defined empirical question.

Fourth and finally, the Mitchell case reveals a subtle interplay between causal and mechanistic hypotheses. Both Robertson and Mitchell produced causal hypotheses that had a much longer shelf life than the mechanisms underlying them. Nevertheless, both authors seem to have thought in terms of concrete mechanisms for generating their more successful causal hypotheses: It is a striking fact that Robertson, despite his combinatorial spirit, considered only three of four possible causal structures, neglecting the one for which he lacked a mechanism. Mitchell was better positioned to pursue this fourth structure, which required the notion of a reversible, H^+-transporting ATPase – a notion which was accessible to him because he held a *false* hypothesis about the mechanism of membrane transport. Nevertheless, Mitchell's hypothesis survived because it was correct about matters of causal connectivity: the H^+-gradient is in fact a cause of ATP synthesis. Thus, hypothesis generation generally seems to occur on the basis of mechanisms, but continuous development of a theory in the medium-term is intelligible partly on the more abstract level of causal interactions. We here agree with Levy and Bechtel (2013), who recently emphasized that completeness and concreteness are not always necessary or useful in the exploration of mechanisms: sometimes a more abstract perspective in terms of patterns of causal connectivity is needed. Their view is strongly supported by our third and fourth conclusions: thinking in terms of causal connectivity rather than mechanistic detail is required both for the definition of a causal search space (third conclusion) and for recognizing the medium-term continuity of theory development (fourth conclusion).

It may be objected that our conception of scientific discovery does not truly offer an explanation of Mitchell's or Calvin and Benson's achievements, since we trace their development only a few steps back. From where came the knowledge – here treated as given – of lipid biological membranes, of group-transfering enzymes, of

biochemical cycles, or of the reaction possibilities of ribulose diphosphate? We agree that these further steps should be pursued in great detail. The result would be nothing less than the internal history of biochemistry.

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