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The discovery of oxidative phosphorylation: a conceptual off-shoot from the study of glycolysis

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ABSTRACT

The origins of oxidative phosphorylation, initially known as aerobic phosphorylation, grew out of three research areas of muscle metabolism, creatine phosphorylation, aerobic metabolism of lactic acid in muscle, and studies on the nature and role of adenosine triphosphate (ATP). Much of this work centred round the laboratory of Otto Meyerhof, and most of those contributing to the study of aerobic phosphorylation were influenced by that laboratory: particularly Lipmann and also Ochoa. The work of Engelhardt on ATP levels in blood also appears to have been influenced by the studies of Meyerhof's laboratory. However, with the work of Kalckar, influenced by Lipmann, biochemists began to realise the potential importance of the process. This was confirmed and extended by Belitzer and Ochoa and the theoretical contribution of Lipmann. Thus it is not easy to identify a single point that marked the initiation of studies in this field.

The early work was based on the use of tissue homogenates and cells but ultimately this approach limited research possibilities. The development of techniques based on mitochondria opened up new possibilities and brought this first phase of the study of oxidative phorphorylation to a close.

1. Introduction

Allchin has pointed out that the history of the understanding of cellular metabolism, which is a major achievement of the twentieth century, has received only limited attention in contrast to such areas of biology as evolution, genetics, molecular biology and so on.[1] One of the reasons for the paucity of interest in this field is the nature of the progress made, which has been mostly by small steps rather than by significant conceptual advances, although there have been exceptions such as the work of Hans Krebs and Peter Mitchell.[2] Thus in most areas conceptualisation has only proved possible after the accretion of many experimental facts. Hull has noted that historians have tended to prefer an approach where 'a very few scientists account for most change in science and these changes are made in short periods of time'.[3] The early study of aerobic phosphorylation (oxidative phosphorylation) was carried out by several individuals, each making limited contributions over three decades. Thus this field of cellular metabolism has received rather less attention than some other areas of the history of biology.

The present paper explores the emergence of a new field of study in cellular metabolism, phosphorylation associated with oxygen uptake. Although ultimately acquiring an identity of its own as oxidative phosphorylation, this area of investigation grew out of the research on glycolysis, particularly in muscle. Many of the facts of the story have been outlined by Joseph Fruton, Marcel Florkin and Dorothy Needham, who generally traced the origins of such studies to the work of Engelhardt while noting the work of Lundsgaard.[4] In contrast, the biochemist Lehninger perceptively commented that

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[1] Allchin (2002).
[2] See Holmes (1991, 1993); Prebble & Weber (2003, 2008).
[3] Hull (1988), p. 484.
[4] Fruton (1972), p. 388; (1999), p. 318; Florkin (1975), pp. 407–409; Needham (1971), p. 407.

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> With the advantage of hindsight, it is now possible to see evidence for the occurrence of oxidative phosphorylation in many early studies of phosphate metabolism. However, it was not until the work of Kalckar and of Belitzer . . . that the true significance of oxidative phosphorylation was grasped.[5]

Such a comment underlines the diffuse origins of oxidative phosphorylation.

Here I re-examine the development of the concept of aerobic or oxidative phosphorylation looking at the roots of these ideas, which lie in the oxidation of lactic acid in muscle. In particular I note three characteristics of this story. First, the origins of aerobic phosphorylation belong within the researches of the community investigating a different field, glycolysis. Only later did a community devoted to oxidative phosphorylation emerge. Second, initial studies proceeded by accretion of evidence. Only after the emergence of an oxidative phosphorylation community in the late 1940s did significant conceptualisation take place. Third, this initial phase of study of oxidative phosphorylation came to an end with the introduction of techniques based on the use of mitochondria, particularly cell fractionation. The new methodology appears to have been a major factor in the emergence of the oxidative phosphorylation community.

2. The dawn of metabolic studies on oxidation energy

The origins of aerobic phosphorylation are found within the extensive programme associated with the elucidation of the glycolytic pathway of metabolism. Three areas of research can be seen as the basis for this: aerobic lactic acid metabolism; phosphorylation studies, particularly of creatine; and the nature and role of adenosine triphosphate (ATP).

The first area stems from the earlier observations that Walter Fletcher (1873–1933) and Frederick Gowland Hopkins (1861–1947) made at Cambridge in 1907.[6] These studies were based on the earlier work of the German physiologist, Felix Hoppe-Seyler (1825–1895). They examined the production of lactic acid in isolated frog muscles and, among their observations, found that lactic acid disappeared in aerobic conditions. This work became the basis for studies of muscle metabolism in the early part of the twentieth century and was followed up by Otto Meyerhof (1884–1951) in Berlin, who examined the role of lactic acid in muscle with a view to understanding the mechanism of muscular contraction. As Peters described Meyerhof's studies on this point, 'All this work arose from the desire to place upon a firm basis the "lactic acid" theory of contraction according to which muscular contraction was due to the effect of the liberation of lactic acid upon the colloidal apparatus'.[7] In 1923 he shared the 1922 Nobel Prize for Physiology with Archibald Hill (1886–1977) working in London, with whom Meyerhof had begun collaboration shortly after the First World War. Meyerhof's prize was awarded 'for his discovery of the fixed relationship between the consumption of oxygen and the metabolism of lactic acid in the muscle'.

In the course of these studies Meyerhof concluded that the lactic acid that was produced in muscle anaerobically from carbohydrate, and that disappeared aerobically, was not all oxidised in a subsequent aerobic phase, but that most was resynthesized to glycogen. Only some was burnt (oxidised) and this provided energy for the synthetic activity. In 1927, he was still unclear whether the increased respiration was due to lactic acid oxidation or carbohydrate oxidation but he regarded the energy from respiratory oxidation as driving the synthesis of glycogen from lactic acid: 'lactic acid is reconverted to sugar, that is to glycogen, by means of energy furnished by the accompanying oxidation of carbohydrate'.[8] Thus, in the 1920s, Meyerhof was fully aware that respiratory oxidation provided energy for synthesis although there was little suggestion as to the processes whereby respiration would generate useable energy or how it might be used.

A second area of research that contributed to early ideas on aerobic phosphorylation was related to the synthesis of phosphate compounds. Researches into the modern study of intermediary cellular metabolism were initially made possible by the work of Eduard Buchner (1860–1917), who in 1897 accidentally observed cell-free fermentation.[9] Arthur Harden (1865–1940) and William Young (1878–1942)[10] demonstrated that sugar breakdown in these juices had strong phosphate dependency. This observation led to a growing realisation of the importance of phosphate in fermentation and, in due course, a search for, and identification of, a number of phosphate compounds of significance in both fermentation and muscle metabolism. A few were labile and not detected until Philip Eggleton (1903–1954) and Grace Eggleton (1901–1970), working in Hill's department at University College London, used improved methods to detect a new substance they named phosphagen.[11] Cyrus Fiske (1890–1978) and Yellapregada Subbarow (1895–1948), working in the Harvard Medical School, independently found phosphagen and identified it as creatine phosphate.[12] The Eggletons found that phosphagen disappears during muscle activity, but that in aerobic conditions isolated muscle would synthesise phosphagen, although they did not try to interpret this result. They noted that 'in the presence of oxygen phosphagen rapidly reappears and an exactly equivalent amount of inorganic phosphate is lost'.[13]

Einar Lundsgard (1899–1968) developed this understanding. However, his key discovery was in the use of iodoacetate, which he had been using in a study of amino acid metabolism. Iodoacetate blocked the formation of lactic acid but not muscular contraction. More importantly he showed that in muscle treated with iodoacetate, phosphagen could be broken down to provide energy for muscular contraction. This led him to conclude (in contradistinction to Meyerhof's view):

> one must reckon that the phosphagen splitting directly yields the energy of contraction. The role of the lactic acid formation which is a more general anaerobic energy-yielding process demonstrable in almost all tissues, must then be sought . . . in this that it brings about a continuous resynthesis of phosphagen.[14]

Lundsgaard's work on iodoacetate and phosphagen caused a major revision in the understanding of muscle biochemistry (described as a revolution in muscle physiology and biochemistry by Hill[15]) and forced Meyerhof totally to reinterpret his understanding of the role

[5] Lehninger (1964), p. 87.
[6] Fletcher & Hopkins (1907).
[7] Peters (1954), p. 181.
[8] Meyerhof (1927), p. 533.
[9] See Kohler (1972) for an account of the significance of this work.
[10] Harden & Young (1906).
[11] Eggleton & Eggleton (1927b).
[12] Fiske & Subbarow (1927).
[13] Eggleton & Eggleton (1927a), p. 161.
[14] Lundsgaard (1930). For an English translation of part of this paper see Teich (1992), pp. 231–232.
[15] Hill (1932).

of lactic acid. Lundsgaard also extended the view of aerobic phosphorylation by showing, as the Eggletons had done, that phosphagen can be synthesised from creatine and phosphate. As he wrote in 1932, 'Even if lactic acid formation is blocked [by iodoacetate] an aerobic synthesis of the phosphagen can take place making the oxidation energy available to the contraction mechanism'.[16] Such a view implies that oxidation by molecular oxygen can supply the energy for the phosphorylation of creatine, that energy then being available for muscular activity. On the basis of this work Lundsgaard was later described as 'the first to herald oxidative phosphorylations'.[17] However, Lundsgaard did not pursue further the role of oxygen or the nature of phosphorylation, but his student, Kalckar, investigated such issues a few years later.

Another compound phosphorylated under anaerobic conditions (at least) was adenine nucleotide, where ATP was the product. One of the many developments to emerge from Meyerhof's group had been the discovery in 1929 of ATP by Karl Lohmann (1898–1978) and, simultaneously and independently, by Fiske and Subbarow.[18] The compound obtained from fresh muscle was seen as a cofactor[19] in the production of lactic acid from glycogen in muscle, and its addition was necessary for activity in dialysed muscle juice.

The relationship of ATP to phosphocreatine was soon seen in terms of a synthesis of the latter at the expense of ATP. The situation was summarised by Meyerhof and Lohman, who came to believe that:

> the endothermic and not spontaneous synthesis of phosphocreatine can take place through a coupling of this process with the exothermic and spontaneous breakdown of adenylpyrophosphate whilst the resynthesis of the adenyl pyrophosphate from adenylic acid and inorganic phosphate is brought about through the energy of lactic acid formation.[20]

Thus ATP was seen as being a possible source of energy in muscle and a cofactor in the conversion of carbohydrate to lactic acid. As we will see, these studies, which related the role of ATP to the process of carbohydrate breakdown, created the basis for Engelhardt to link ATP with aerobic phosphorylation.

The realisation of the importance of aerobic conditions was extended by the Polish biochemist Jacob Carol Parnas (1884–1949), who studied the release of ammonia from adenine nucleotides under anaerobic conditions. He found that in the presence of oxygen, levels of adenine nucleotide remained relatively high. He concluded

> that in the oxidative recovery processes, deamination of other substances brings about a resynthesis of adenine nucleotide from inosinic acid; and in this way a system is maintained which is over and over renewed and conserved, readily available for anaerobic instantaneous splitting of ammonia.[21]

Thus, by the beginning of the 1930s, there was an appreciation that aerobic metabolism provided the energy for synthesis of carbohydrate from lactic acid and that oxidation could provide the energy for the phosphorylation of creatine. All this was strongly illuminated by an emerging understanding of the role of ATP, which was seen as a cofactor, but an appreciation of its importance in muscle metabolism was dawning.

3. Engelhardt and aerobic ATP synthesis

Work that focused on ATP synthesis itself came from a Russian biochemist Vladimir Engelhardt (1894–1984), whose contribution requires special consideration since many historians have identified his work as the 'discovery' of oxidative phosphorylation. The ever growing understanding of the role of phosphate in metabolism, and especially the fact that ATP had significance in carbohydrate breakdown in muscle, prompted Engelhardt to investigate phosphate metabolism while working at Kazan in the Russian Federation.[22] At the outset he noted it was generally accepted that phosphate plays a role in oxidative metabolism probably just as important as in fermentation.

Engelhardt's chosen experimental material was red blood cells, where initially he studied phosphate levels during glucose breakdown under aerobic and anaerobic conditions.[23] Concurrently, Barron and Harrop had investigated glycolysis in red cells from various sources with the use of methylene blue, which mediated oxidation. They noted that mammalian and avian cells carry out glycolysis, but only the avian cells show respiratory activity.[24]

In 1930, Engelhardt developed these studies further using mammalian red cells where glycolysis had been depressed by fluoride. He observed a conversion of inorganic phosphate into organic phosphate (pyrophosphate or ATP) in aerobic conditions, and where the dye methylene blue was used.[25] In the absence of methylene blue oxidation, levels of inorganic phosphate were low. These experiments gave little information about the role of oxygen (since respiration induced by methylene blue is artificial), although they directed attention to the link between oxidation and phosphorylation in the glycolytic pathway. It is not clear however, that Engelhardt appreciated he was in fact measuring glycolytic phosphorylation but he believed he was studying aerobic metabolism.

More importantly, he also carried out these experiments with nucleated avian red cells, which do respire naturally. Here he also observed similar effects of aerobic conditions without methylene blue. Concentrations of ATP were maintained during respiration, but in anaerobic conditions ATP was decomposed. Cyanide, a potent inhibitor of respiration, also inhibited the maintenance of ATP levels. Engelhardt concluded that ATP was broken down in anaerobic conditions but was being actively synthesised during respiration. Additionally he noted that the products of ATP breakdown promoted respiration. The results showed that ATP synthesis itself can be driven by respiration.[26]

He interpreted his experiments as showing that ATP had the same role in respiration as Meyerhof and Lohmann had described for muscle glycolysis where ATP was synthesised and was part of

[16] Lundsgaard (1932). For an English translation see Kalckar (1969), p. 349.
[17] Kruhøffer & Crone (1972), p. 5.
[18] For a full account of the discovery of ATP see Maruyama (1991).
[19] Early in the study of fermentation and glycolysis, substances essential for the processes were separated by dialysis. Although, except for phosphate, their identity remained obscure, they were known not to be proteins or to have enzymic activity and were regarded as cofactors.
[20] Meyerhof & Lohmann (1932). For an English translation of part of this paper see Teich (1992), pp. 232–233.
[21] Parnas (1929). For an English translation of part of this paper, see Teich (1992), p. 228.
[22] A detailed consideration of the experiments described in these papers is given in Slater (1981).
[23] Engelhardt & Braunstein (1928).
[24] Harrop, Jr. & Barron (1928); Barron & Harrop, Jr. (1928).
[25] Engelhardt (1930); Engelhardt & Ljubimova (1930).
[26] The terms *respiration* and *respiratory chain* as used by biochemists refer to cell respiration involving a terminal enzyme complex, the respiratory chain that uses oxygen to oxidise the products of intermediary metabolism. If methylene blue or other redox dyes are used as here, the dye oxidises a cellular component and is oxidised by oxygen; the respiratory chain may not be involved. It is distinct from *respiration*, as traditionally used by physiologists to refer to processes associated with breathing. The history of respiration demonstrating the changing use of the word is explored by Keilin (1970).

the associated coenzyme system.[27] He concluded that ATP was part of a coenzyme complex associated with respiration:

> Es erscheint berechtigt, der Adenylpyrophosphorsäure eine Co-Fermentfunktion bei der Atmung zuzuschreiben, und das Adenylpyrophosphat als einen Bestandteil des Co-Fermentkomplexes der Atmung zu betrachten.[28]

Although Engelhardt's work was an advance on previous studies, he did have a claim to have discovered oxidative phosphorylation, as Slater has noted. The association of ATP synthesis with respiration and the observation that breakdown products of ATP stimulated respiration are the basis for Slater's view that Engelhardt discovered oxidative phosphorylation.[29] However, there are other aspects that need to be noted in order to place Engelhardt's work in context. Although Slater argues that Engelhardt's work had distinguished between glycolytic and respiratory oxidation, it is not clear that Engelhardt himself did so when he wrote his paper. He appears to equate natural respiration and methylene-blue-induced respiration. Moreover, Runnström and Michaelis place Engelhardt's methylene blue work alongside natural respiratory synthesis of phosphagen in muscle without distinction. Lipmann pointed out that such dyes facilitate oxidation in glycolysis.[30] It should also be noted that at this stage ATP was one of two phosphate compounds (the other being creatine phosphate) identified as synthesised in association with respiration. This work was followed up in the Soviet Union by V. A. Severin, who obtained similar results with respiration studying the oxidation of keto-acids.[31]

Engelhardt's contemporaries do not appear to have recognised his work as a major discovery, although phosphorylation with methylene blue was pursued during the 1930s, demonstrating the importance of oxidation in glycolytic phosphorylation.[32] The author of a review on the chemistry and metabolism of nucleic acids recorded the observations that were placed alongside the work of Lohmann on ATP as a cofactor.[33] The identification of a cofactor for respiration seemed but a minor advance in a world where major discoveries on glycolysis, on the urea cycle and on enzymes were being made, and where, as Engelhardt later remarked, respiration was emerging from being 'nothing more than the removal of the anaerobically formed products ... a kind of scavenging function'.[34] Kalckar (1908–1991) also noted that the early work on aerobic phosphorylation did not attract much attention, owing to

> the lack of appreciation of the importance of phosphorylation in cellular physiology in most leading biochemical and physiological circles at that time. It was not uncommon to consider phosphocreatine and ATP as nice 'cellular buffers' and phosphorylation as an artefact found in broken cells.[35]

4. Kalckar and Belitzer: establishing oxidative phosphorylation

Phosphorylation linked to oxygen metabolism became much more clearly established by the work of Kalckar in Copenhagen. Around 1934, Lundsgaard transferred his interests from muscle physiology to questions of glucose transport in the intestine and kidney. Theories at that time led him to look at glucose phosphorylation and dephosphorylation, which seems to have encouraged Kalckar to take up studies on phosphorylation and dephosphorylation, particularly in kidney, which possessed a strong phosphatase. Kalckar was influenced in the choice of kidney, rather than pigeon breast muscle, by the ready availability of kidney tissue within the department, thus avoiding the need to kill pigeons, an unattractive activity for Kalckar. The interest in phosphorylation was strongly encouraged by Kalckar's mentor, Lipmann.

Initially, Kalckar discovered that there was 'a coupled reaction between oxygen consumption and phosphorylation' of glucose.[36] Inhibition of respiration with cyanide also inhibited phosphorylation. In the light of work by Albert Szent-Györgyi (1893–1986),[37] these experiments were extended using a range of substances including dicarboxylic acids, which stimulated both respiration and phosphorylation. He also examined various phosphate acceptors including adenylic acid, carbohydrates, glycerol and pyruvic acid. The conclusions from these studies were that some substances, particularly the dicarboxylic acids that promoted phosphorylation, might do so by providing phosphate acceptors.[38] In summary, 'A coupling between the phosphorylations and the respiration in kidney tissue undoubtedly exists since the phosphorylation and the respiration are inhibited equally by cyanide and stimulated by different dicarboxylic acids'.[39] Although the link between phosphorylation and respiration was thus established, the initial phosphate acceptor remained unclear.

Kalckar's papers were noted by Carl Cori (1896–1984) and Gerty Cori (1896–1957) at the Washington University School of Medicine, St. Louis. The Coris were seeking a system for glucose phosphorylation in glycogen synthesis and re-examined the aerobic phosphorylation of glucose described by Kalckar. After advice on the need for shaking the tissue in order to observe the phosphorylation,[40] they confirmed the earlier work and concluded that 'it is evident that phosphorylation of glucose in kidney extracts is not dependent on the dehydrogenation of glyceraldehyde phosphate [phosphorylation driven by the glycolytic pathway] but on the oxidation by molecular oxygen of any one of a number of dicarboxylic acids'.[41]

Although Kalckar's work attracted attention, it was in many respects the work of the Russian, Vladimir Aleksandrovich Belitzer (1906–1988), that put the phenomenon of aerobic phosphorylation beyond reasonable doubt. Probably the key to the impact made by

[27] Lohmann (1931).
[28] Engelhardt (1932), p. 368.
[29] Slater (1981, 1984).
[30] Runnström & Michaelis (1935); Lipmann (1941), p. 139.
[31] Severin (1937).
[32] See, for example, Lennenstrand & Runnström (1935).
[33] See Cerecedo (1933), p. 121.
[34] Engelhardt (1975). See p. 62.
[35] Kalckar (1966), p. 4. The latter part of this comment was probably based on Sacks (1940, 1943–1944). The point is noted by Florkin (1975), who regards it as an attack on the phosphate cycle originally arising from the work of Lundsgaard but elaborated by Lipmann in his 1941 review.
[36] Kalckar (1937), p. 51.
[37] The Hungarian biochemist Albert Szent-Györgyi had proposed that the dicarboxylic acids play a role in respiration as hydrogen carriers (Annau et al., 1935). The experiments but not their interpretation were important in Krebs' formulation of the citric acid cycle.
[38] Kalckar (1939a).
[39] Kalckar (1939b), p. 209.
[40] See Kalckar (1991), p. 10.
[41] Colowick et al. (1940), p. 371.

his work with Tsybakova was the measurement of the P/O ratio,[42] which was substantially higher than that of Kalckar so that aerobic phosphorylation could not be confused with glycolytic phosphorylation. Importantly, he tentatively concluded that adenylic acid was the primary substance phosphorylated even though most of the experiments concerned creatine phosphate formation.

Belitzer interpreted the earlier work, including Kalckar's, as showing a strictly respiratory synthesis of phosphate esters such as phosphagen and ATP, and set out to investigate the matter further using muscle tissue from pigeon breast and rabbit heart. Together with Elena Tsybakova, he made measurements of phosphorylation against oxygen uptake and concluded that the P/O ratio lay between 2 and 3.5. They estimated that this phosphorylation was some ten times greater than might be obtained from glycolysis. They also noted that glycolysis was 'unconditionally' (obligatorily) coupled to phosphorylation, whereas phosphorylation linked to respiration was 'conditionally' coupled to respiration: that is, respiration could occur without phosphorylation. Indeed, elsewhere Belitzer demonstrated a dependence of respiration on creatine phosphorylation in muscle.[43] Although higher levels of phosphorylation were obtained with creatine, they concluded that there was nothing to 'prevent one from assuming that the adenylic system is a primary acceptor of phosphate in the synthesis of phosphagen'. 'In general, the results of the investigation supported the assumption that adenylic acid is capable of playing the role of acceptor of phosphate in phosphorylations linked with respiration'.[44] Adenylic acid phosphorylation was thus seen as the primary event in phosphagen synthesis.

By the end of the 1930s, respiration linked to phosphorylation had been firmly established by the work of Kalckar and Belitzer and shown to be separate from glycolytic phosphorylation. They also observed the phenomenon of coupling and uncoupling. Indeed some, such as David Green, have credited Kalckar and Belitzer with the discovery of oxidative phosphorylation, while others regarded them as initiating the study of the process.[45]

Meanwhile work on glycolysis continued to influence the understanding of aerobic phosphorylation. Needham and Pillai, working at Cambridge, showed a link between phosphorylation of adenylic acid by inorganic phosphate with oxidation in the glycolytic breakdown of sugar phosphate.[46] However, perhaps the most significant event in the study of glycolysis to influence the understanding of phosphorylation linked to respiration was the work on triose phosphate oxidation. Thus, in his 1941 review[47] Kalckar placed much weight on recent work from Warburg's laboratory showing the way in which inorganic phosphate was used in glucose breakdown (glycolysis) and linked to the synthesis of ATP.[48] He noted that

> The clarification of the coupling between the triose phosphate oxidation and the uptake of inorganic phosphate represents one of the greatest advances in modern biology ... For the first time since this recent discovery of the Warburg school, a complete description of a biological coupling is possible. By following this new line a clarification of other energetic couplings is to be expected.[49]

He went on to consider respiration linked to phosphorylation and also other phosphorylations. Indeed, the oxidation of triose phosphate became a model for understanding phosphorylation linked to respiration. Such a comparison reinforced the idea that ATP should be the first-formed phosphorylated compound. By the beginning of the 1940s respiration linked to phosphorylation could be seen (without total confidence) as the addition of phosphate to adenosine diphosphate (ADP) to form ATP.

5. Lipmann and Ochoa: placing aerobic phosphorylation on the biochemical map

In the early 1940s two contributions confirmed the fact that biochemists now understood aerobic phosphorylation as an important process in the cellular metabolism of aerobic cells. The first centred on the metabolic role of ATP while the second developed the experimental understanding of the process, which now became known as oxidative phosphorylation.

The studies on phosphorylation, both those linked to oxygen and those linked to glycolysis, were rationalised by Fritz Lipmann (1899–1986), who at the beginning of the 1940s emerged as a key figure in the development of cell bioenergetics. He was probably the first to think comprehensively about the importance of phosphate in energy metabolism and to develop the understanding of energy in cell metabolism and the role of ATP. From the work of the 1930s, he provided the basis for recognising ATP as the energy currency of the cell. Such a step was an essential element to understanding both the nature and importance of oxidative (aerobic) phosphorylation.

In the late 1930s Lipmann had studied the metabolism of pyruvate by a bacterium. He found that when pyruvate was oxidised, ADP was phosphorylated to ATP. Subsequently he showed the intermediate in this process to be acetyl phosphate.[50] These observations, which provided an example of phosphorylation similar to that involving glyceraldehyde phosphate oxidation studied by Warburg's group, reinforced the view (developed during the 1930s) that phosphorylation is associated with oxidation. Lipmann's thinking about the role of phosphate, particularly in terms of energy transformations in the cell, was revealed in his classic 1941 review on phosphate bond energy.[51] He considered those bonds, which the cell uses to convey energy from breakdown systems (like glycolysis) to energy consuming systems, as energy rich-phosphate bonds, which he denoted as ~ph. In contrast, other phosphate bonds that he regarded as not energy rich were denoted –ph. The use of Lipmann's squiggle (~) became customary in biochemistry for at least two decades as a means of conveying some thermodynamic aspects of metabolism.[52] It was extensively used in discussions of oxidative phosphorylation to convey the conservation of energy from oxidation in the

[42] The P/O ratio is the number of phosphorylations per atom of oxygen consumed. This became the standard measure of phosphorylation in the 1940s and I have used it here. Belitzer used a P/O_2 ratio which gave values numerically twice the P/O ratio.

[43] Belitzer (1939).

[44] Belitzer & Tsybakova (1939). For an English translation, see Kalckar (1969), pp. 211–227.

[45] See Ziegler et al. (1956), Slater (1966).

[46] Needham & Pillai (1937a,b). Note that Meyerhof (1937) simultaneously came to similar conclusions with slightly different experiments.

[47] Kalckar (1941). In his autobiographical essays, Kalckar (1991) referred to this paper as his 'ATP review', while Singleton (2008) regards it as having influence in the field. A similar review (Kalckar, 1942), more suited to biologists was published in *Biological Reviews*.

[48] Warburg & Christan (1939); Negelein & Brömel (1939). It should be noted that Warburg and Christian's mechanism for the phosphorylation, while being influential, was subsequently shown by Racker to need significant revision; see Racker (1965), pp. 17–29.

[49] Kalckar (1941), p. 131.

[50] Lipmann (1939, 1940)

[51] Lipmann (1941).

[52] Lipmann designated ATP as ad–ph~ph~ph, distinguishing between the energy available from hydrolysis of the first phosphate from that of the other two.

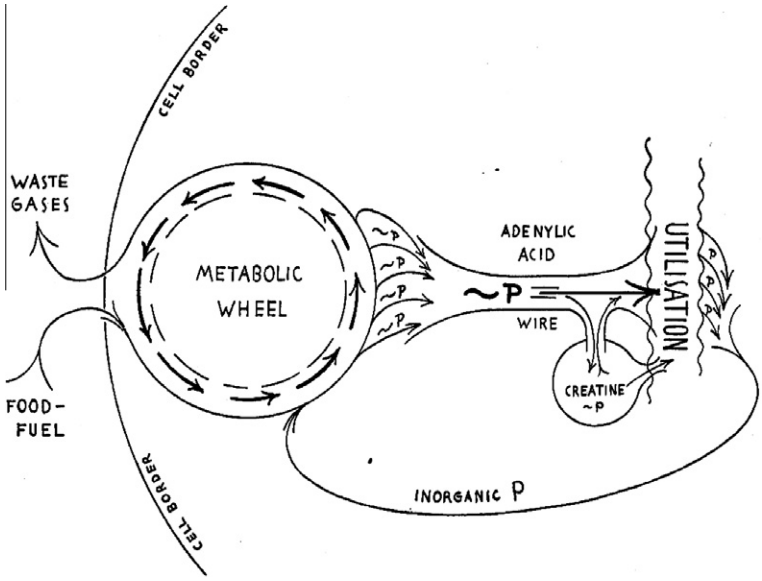

Fig. 1. Lipmann's diagram, which showed the role of ATP as an energy currency (from Lipmann, 1941, p. 122). ATP is here shown as ~**P**, representing Lipmann's high-energy bond. It conveys energy from the oxidation process to the energy-requiring aspects of cell function, which releases phosphate as inorganic phosphate.

respiratory chain. The notion of energy through ~ph was graphically presented in a diagram (Fig. 1), which shows metabolism (essentially breakdown of foodstuffs) generating (~**P**), ATP, which provides the energy for synthesis and muscular contraction (represented by the reference to creatine phosphate). Thus ATP now had a central role in metabolism, and oxidative phosphorylation was its *raison d'être*. Prior to Ochoa's estimate of the P/O ratio, Lipmann estimated that the value of the ratio would be between 2 and 4,[53] thus recognising the process as the principal source of ATP in the cell.

Later Lipmann, who strongly influenced the bioenergetics of the post-Second World War era, shared the Nobel Prize for Physiology in 1953 with Hans Krebs for his work on acetylation, especially the discovery of acetyl coenzyme A, which not only completed the jigsaw of Krebs' citric acid cycle but also gave a basis for the understanding of large areas of metabolism. He also proposed a simple theory of oxidative phosphorylation that reflected the views that emerged in his 1941 review.[54]

Severo Ochoa (1905–1993) complemented the work of Lipmann by providing apparently very accurate measurements of the number of phosphorylations per oxygen atom taken up (the P/O ratio) in systems catalysing phosphorylation linked to oxygen metabolism. Significantly he was the first to use the term 'oxidative phosphorylation' in the title of a paper devoted to a study of pyruvate oxidation in brain.[55] Influenced by Lipmann's study of pyruvate metabolism in bacteria, Ochoa measured the phosphorylation of the sugars and had reasonable grounds to suppose that this phosphorylation involved the synthesis of ATP as the first step. His P/O ratio was 2.

Since Ochoa's preparations contained an enzyme that broke down ATP, he reasoned that the P/O ratio was in fact low owing to some ATP destruction. He carried out experiments with heart muscle preparations where ATP breakdown was measured alongside phosphorylation and was therefore able to calculate what he regarded as a true P/O ratio. The result was P/O = 3.0.[56] This figure became the basis for further elucidation of oxidative phosphorylation for the following two decades. The understanding of the mechanism of oxidative phosphorylation up into the 1960s was based on glycolytic phosphorylation and this would predict an integer for the P/O ratio. Thus Lehninger wrote in 1951 'Today it appears reasonably certain that the average P:O ratio of the Krebs cycle oxidations is 3.0'.[57] Even in 1966, it was still sufficiently widely believed for Slater to sound a note of caution.[58] However, like other valuable advances in the early period, it ultimately proved incorrect and misleading. Nevertheless it resonated well with the discovery of Chance and others that they could identify three sites of phosphorylation in the respiratory chain.[59]

Thus by the mid 1940s, oxidative phosphorylation had become a recognised part of cell metabolism with a key role in providing energy through the synthesis of ATP. However, the basis for this view remained less than substantial.

6. Origins in the glycolytic community

The foregoing discussion illustrates the point that gaining an understanding of aerobic phosphorylation proceeded by limited steps; there was no overarching discovery of aerobic (oxidative) phosphorylation. It was only with the work of Kalckar and Belitzer that phosphorylation associated with respiration began to loosen its links with the study of glycolysis. Both conceptually and methodologically the phenomenon of aerobic phosphorylation belonged to the glycolytic paradigm associated with the breakdown of carbohydrate in mammalian systems, especially muscle. The key techniques for phosphate measurement were those developed by the Eggletons and, particularly, Fiske and Subbarow, and used by Lohmann in Meyerhof's laboratory. In essence the study of fermentation in yeast, mostly using juices, belonged to the same research

[53] This was an extension of calculations carried out by Meyerhof.
[54] Lipmann (1946).
[55] Ochoa (1940).
[56] Ochoa (1943).
[57] Lehninger (1951), p. 344.
[58] Slater (1966), see p. 334.
[59] See Chance & Williams (1956).

programme, but this system did not carry out aerobic phosphorylations.

The link to glycolysis can also be seen in the social relationships and influences of the investigators involved in the earlier developing ideas on aerobic phosphorylation. Thus it was essentially the study of muscle that brought about an awareness of aerobic phosphorylation, particularly the studies carried out in Meyerhof's laboratory by Meyerhof and his collaborators. As Teich has noted, 'Of all the active tissues of the organism, muscle is the only one in which we can readily compare the chemical changes going on with the work done or the energy set free as heat'.[60] This was exploited by Otto Meyerhof, who described his work as 'How chemical energy in the body is transferred into mechanical work' or later, more specifically: 'All these investigations are connected with the problem of energy transformation in muscle'.[61] In fulfilling this mission, much of his work centred on the formation of lactic acid, which initially he saw as central to muscular contraction, and its metabolism in aerobic conditions. It was the oxygen-related aspect of this work that laid the basis for aerobic phosphorylation, which Meyerhof recognised as providing the energy for resynthesis of glycogen.

The establishment of the phenomenon of aerobic phosphorylation was carried out by a disparate group of workers, none of whom engaged in a programme of work to elucidate the matter further with the partial exception of Kalckar. Almost all of this group were influenced directly or indirectly by Meyerhof's laboratory. Meyerhof himself, initially interested in psychiatry and philosophy, was early attracted into cell physiology by Otto Warburg (1883–1970).[62] From 1913 to 1924 he worked at the Physiological Laboratory at Kiel, although he found life there uncongenial. In 1924, Warburg made space for Meyerhof at the Kaiser Wilhelm Institute for Biology in Berlin-Dahlem. During the years after the First World War, he built up collaboration with Archibald Hill in London, in whose department the Eggletons demonstrated the synthesis of phosphagen in aerobic conditions. Later Meyerhof was given the directorship in Physiology at the Kaiser-Wilhelm Institute for Medical Research at Heidelberg, where he remained until 1938 when he escaped Germany, ultimately arriving in Philadelphia. Karl Lohmann, who carried out critical work on ATP, so essential to developing the phenomenon of oxidative phosphorylation, was an important part of Meyerhof's laboratory both in Berlin and in Heidelberg.[63] In both places, Meyerhof entertained many visitors; notable was Einar Lundsgaard from Copenhagen, who showed that lactic acid could not be intimately connected with muscular contraction and brought about the 'revolution in muscle physiology'.

Arguably the most significant and influential person to participate in Meyerhof's laboratory was Lipmann, who moved there in 1927, became interested in phosphate chemistry and worked with Karl Lohmann. He later remarked about his time with Meyerhof, 'most of what I was to do later was preformed then, I would almost say by assimilation from the environment'.[64] Here he met, among others, Lundsgaard and Ochoa (who felt the atmosphere in Meyerhof's laboratory was enormously stimulating[65]). Later, he worked in the Carlsberg laboratory in Copenhagen,[66] close to Lundsgaard and Kalckar, from 1932 to 1939, after which he moved to the United States.[67] Another biochemist who met Lipmann during his Berlin days was Vladimir Engelhardt, who had graduated from the Faculty of Medicine at Moscow University and worked in the Biochemical Institute in Moscow under Alexei Bach (1857–1946), one of the founding fathers of Russian biochemistry.[68] It was while working under Bach that Engelhardt spent time at Peter Rona's laboratory in Berlin, from where he was able to meet Fritz Lipmann, Karl Lohmann, Otto Meyerhof and Otto Warburg, among others, and seems to have been strongly influenced by Lohmann.[69] Engelhardt was initially directed by Bach into immunology; he later started investigations into the role of phosphate in metabolism. In 1929 he became Professor of Biochemistry at the University of Kazan, almost 500 miles east of Moscow, in the Russian Federation.[70] Although he did not work with Engelhardt, Vladimir Belitzer, who graduated from Moscow University in 1930, worked initially in the A. N. Bach Institute of Biological Chemistry and then in the Institute of Experimental Medicine in Moscow.[71] While some link certainly existed between the two, it was more tenuous than Slater has implied.[72] In fact, Belitzer was part of the Russian school of biochemists who were pursuing issues of phosphate metabolism.

Kalckar was a student of Lundsgaard in Copenhagen and pursued some of the latter's interests in phosphorylation. During his early years the two major influences on Kalckar were 'his two great teachers', Lundsgaard and Lipmann.[73] The latter arrived in Copenhagen in 1932 and, after Lundsgaard had become taken up with running his department, Lipmann became Kalckar's chief mentor and advised him to pursue 'respiration obligatory for phosphorylation; I would forget all your other theories'.[74]

Thus the origins of aerobic phosphorylation lie with a group of biochemists whose interest was in glycolysis mainly in muscle, but also in other tissues (red cells, kidney cortex and brain). Most of them shared the influences of the Meyerhof laboratory, and some, like Kalckar and Ochoa, were influenced by Lipmann, himself deeply affected by his time in that laboratory.

A new situation arose in the late 1940s where a growing group of workers began to apply their skills to the problems of oxidative phosphorylation and established this as a defined research field. One may ask what initiated the new situation. It is true that a whole raft of new methodologies was being developed in biochemistry—particularly enzymology—and these were being applied to the multitudinous problems of metabolism. However, in the field of oxidative phosphorylation, one development in particular, the realisation of the importance of the mitochondrion, can be seen as providing the driving force to create the new social structure and conceptual framework.

[60] Teich (1992), p. 219.
[61] Taken from Meyerhof (1924), see p. 61, and Meyerhof (1927).
[62] For a short biography of Meyerhof, see Peters (1954).
[63] For views of the relative merits of Lohmann and Meyerhof in the life of that laboratory, see the discussion of Engelhardt (1975).
[64] Lipmann (1971), see p. 7.
[65] See Ochoa's contribution to the discussion of Engelhardt (1975).
[66] Lipmann migrated to Denmark, since he found even in 1930 that being Jewish was already a great handicap if one was looking for a university position.
[67] For a brief autobiography, see Lipmann (1971).
[68] For a short biography of Bach, see Kretovich (1983).
[69] See Engelhardt (1975); see pp. 62–63 and particularly the discussion.
[70] Engelhardt (1982).
[71] For a brief biographical note on Belitzer, see Varetskaya (1989).
[72] I can find no evidence for the assertion by Slater (1976), p. 329, that Belitzer was working in Engelhardt's laboratory when he published his key paper. He was in the University of Moscow while Engelhardt was in Kazan. While some link no doubt existed between the two, it was more tenuous than Slater implies.
[73] For autobiographies, see Kalckar (1990, 1991); for a biographical note see Singleton (2008).
[74] Kalckar (1966), p. 2.

7. The new stimulus: the cellular location of oxidative phosphorylation

Even in 1943, there remained very fundamental questions about the phenomenon of oxidative phosphorylation. Was the phosphorylation linked directly to the respiratory chain recently described in some detail by David Keilin (1887–1963)? What was the nature of the phosphorylating system and what was the mechanism for the process? These questions were not readily answered with a methodology based on the study of glycolysis. The key event that made a new approach to research in this field possible was the development of techniques based on the use of the subcellular organelle, the mitochondrion. The early work on oxidative phosphorylation used isolated tissues or cell homogenates, but nothing was known of the intracellular location of the processes studied. Keilin, who had provided by 1940 a basic account of the respiratory chain, particularly that part involving cytochromes, had used preparations of fragmented heart muscle tissue (known as Keilin–Hartree muscle preparations) that were incapable of phosphorylation but very valuable in studying respiration.[75] In fact many early results, excepting those with blood cells, were obtained with disrupted cell preparations such as minces, which thereby avoided issues of cell structure.

However, there were contrasting approaches to the relationship of cell structure to metabolism in the early decades of the twentieth century. As Morgan has noted,[76] Hopkins, regarded by many as the father of British biochemistry, favoured a research programme that essentially ignored issues of structure, concentrating on the events involving small molecules in the aqueous phase, 'simple substances undergoing comprehensible reactions.' The view was re-enforced in his later lecture on organicism.[77] In contrast, Warburg was convinced of the importance of cell structure, and this question was explored by Meyerhof in his 1924 lectures, for example: 'there also exists a close connection between the speed of respiration and cell structure'.[78] Indeed, Warburg had used a simple cellular fractionation technique to demonstrate that small granules from liver cells were capable of consuming oxygen and evolving CO_2.[79] Such studies appear to have little influence on research at that time and concerted investigations on the relation of structure to aspects of oxidative phosphorylation came from a different source.

The modern study of subcellular particles stems from the work of Robert Bensley and Normand Louis Hoerr in 1934 at the University of Chicago, and was followed up by Albert Claude and others at the Rockefeller Institute in the 1940s. It was here that the basis was laid for the isolation of intact mitochondria. Although this group had demonstrated the presence of succinoxidase and cytochrome oxidase (respiratory enzyme activities) in their large granules (mitochondria), the mitochondria entered the world of biochemistry with the work of Albert Lehninger, who demonstrated their biochemical activities.[80]

Albert Lehninger (1917–1986) was born in Connecticut and, after obtaining his BA at Middletown University, moved to the University of Wisconsin, where he worked on ketone bodies and lipid metabolism, an interest that continued when he moved in 1945 to the University of Chicago.[81] Later he transferred to Johns Hopkins School of Medicine. At Chicago, together with Eugene Kennedy, he exploited the results of Palade's research on isolating mitochondria to demonstrate that fatty acid oxidation, the citric acid cycle and oxidative phosphorylation all occurred in the mitochondria.[82] Rather than using cell homogenates, oxidative phosphorylation could now be studied in preparations of mitochondria prepared by the cell fractionation methods developed in Palade's laboratory using the centrifuge. This gave much cleaner, simpler material to work with. The new technique known as cell fractionation[83] using cooled centrifuges was put to use by Lehninger in demonstrating that in mitochondria phosphorylation was linked directly to the respiratory chain.[84] This link had been anticipated by Belitzer in his classic work in 1939, but since respiration could readily be observed without phosphorylation occurring, experimental justification was necessary.

Cell biologists[85] provided a second major insight on mitochondrial biochemistry through the provision of electron micrographs showing the two membranes of the mitochondrion.[86] In due course, oxidative phosphorylation was shown to be a membrane-bound process associated with the inner of these two membranes.

The introduction of mitochondria into biochemical research not only gave a new stimulus to research on oxidative phosphorylation and related issues, it created a new association of biochemists (known to their colleagues as mitochondriacs!) who addressed these questions, developed their own methods and shared their understanding. An example of the importance of mitochondria to the new research programme can be seen in the work of Britton Chance (1913–), who successfully developed sensitive spectrophotometric equipment and techniques. These were used to study the oxidative phosphorylation system, particularly the respiratory chain and its relationship to phosphorylation.[87] Reflecting on the importance of the mitochondrion he noted 'that the Keilin and Hartree preparation was significantly denatured and that an understanding of the respiratory chain could only come from studying mitochondria directly'.[88] Similarly, the pioneering studies of the enzyme responsible for phosphorylation, the ATP synthase, carried out by Efraim Racker (1913–1991), were only made possible by the use of mitochondria and submitochondrial particles.

8. Conclusions

The early history of aerobic or oxidative phosphorylation is a scattered series of events within the study of glycolysis, only becoming a coherent field of study with the use of mitochondria. Although the influence of the glycolytic paradigm had weakened by the end of the 1930s, it remained in evidence even in the 1950s, when glycolytic phosphorylation (the oxidation of glyceraldehyde phosphate) was the model for understanding the mechanism of oxidative phosphorylation. This model was known as the

[75] See, for example, Keilin (1930); Keilin & Hartree (1939, 1940). It is noteworthy that although Keilin was undoubtedly the foremost authority on respiration between 1925 and 1950, his interests did not extend to phosphorylation. Indeed, his heart muscle preparations were incapable of significant oxidative phosphorylation.
[76] Morgan (1990).
[77] Hopkins (1913), see p. 137; Hopkins (1927).
[78] Meyerhof (1924), p. 10.
[79] Warburg (1913).
[80] For a fuller account of these developments, see Lehninger (1964) pp.7 ff.; Prebble (1981), pp. 93–106; Bechtel (2006), pp. 162–188; Bechtel & Abrahamsen (2007).
[81] For a short biography of Lehninger, see Allchin (2008).
[82] Kennedy & Lehninger (1949).
[83] Using different degrees of centrifugal force, the constituents of homogenised cells could be separated—one fraction being primarily mitochondria.
[84] Friedkin & Lehninger (1949); Lehninger (1949).
[85] The contribution of cell biologists to the study of metabolism has been explored in detail by Bechtel & Abrahamsen (2007).
[86] Palade (1952) and Sjöstrand (1953) published the first electron micrographs of mitochondria.
[87] See, for example, Chance & Williams (1956).
[88] Chance (1991), p. 6.

chemical theory. It was propounded by Edward C. Slater (1917–),[89] an Australian working initially in Cambridge, England under the influence of David Keilin but later in the Netherlands. The theory became a major influence in the development of many research programmes in the 1950s.

However, the introduction of techniques using mitochondria in the study of oxidative phosphorylation marked the end of the opening phase of research in that field. Phosphorylation linked to respiration had been clearly established, but the nature of that link remained elusive and became the basis for a second phase using mitochondria. Thus research shifted from the study of cells (blood cells) and homogenised tissues to the study of a subcellular particle, the mitochondrion. This shift has similarities with an almost concurrent shift in the development of molecular biological research from fruit flies and so on to micro-organisms and viruses.[90]

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[89] Slater (1953, 1974).

[90] For a recent comment on the historical phases of molecular biology, see Rheinberger (2009).

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