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INTRACELLULAR RESPIRATION

Phosphorylating and Non-Phosphorylating Oxidation Reactions

Edited by

E. C. SLATER, *Amsterdam*

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PREFACE

THIS volume forms the proceedings of one of the symposia held on the occasion of the Fifth International Congress of Biochemistry in Moscow on 11–15 August, 1961. Speakers invited to participate in the symposia were asked to send a manuscript suitable for publication not later than 31 January 1961. Although this deadline was met by most of the authors, some because of illness sent in their paper considerably later, and one paper was not received until the first day of the symposium. All except this paper were printed and made available to members of the Congress at registration in Moscow. In view of the long time-interval between writing the paper for publication and presenting it at the symposium, and because the time available for presentation was often very short, the paper presented often differed considerably from the preprint. The general principle adopted in editing the proceedings was that both the preprint and the paper presented formed part of the proceedings of the symposium. The preprint was treated as an uncorrected proof, and no alterations of substance were allowed. The date of receipt of the original manuscript is printed.

The author was given the opportunity of submitting an addendum to this paper within one month of the close of the Congress. The purpose of this addendum was to draw attention to material introduced into the talk which did not appear in the preprint, or to where the author had changed his views between submitting the manuscript and presenting his paper. The only restriction was that material not given in the talk should not be included in the addendum. The date of receipt of the addendum has also been recorded. Two authors preferred to replace the preprint by a new version, corresponding exactly to the paper presented, which was given to the secretariat of the symposium before presentation of the paper. The date of receipt of these papers was altered accordingly. One invited speaker was unfortunately unable to be present at the symposium, so that his paper, although it appeared in preprint, is not included in the published proceedings.

The preparation for publication of the discussions, which were for the most part lively and interesting, gave considerable difficulties. A complete

transcript of the discussion was not available, but all participants were invited and indeed urged by the secretariat of the symposium to write down their comments as soon as possible after making them. Most of the contributions to the discusions were recorded in this way and were typed and arranged by the secretariat in Moscow. In practice, however, it was impossible for those who had taken an active part in the discussions to find the time during the busy programme of the Congress to write down all their remarks. Moreover, it is clear that the type of discussion printed at the end of Session I, for example, required more reflection than was possible during the symposium itself. Some participants were, therefore, given the opportunity of submitting, after they had returned to their own laboratory, an account of what they thought they had said.

This procedure inevitably resulted in the written discussion departing somewhat from that actually presented. In some cases, some expansion of the comment was allowed, when this resulted in a clarification of an important point. No topics which were not actually discussed in the symposium have been introduced into the printed report. Some discussions which the editor considered were fruitless, which went into uninteresting side-paths, or which were based on a misunderstanding of the question which was clarified by later discussion or correspondence, have been omitted.

The organization of this symposium and the preparation for publication of its proceedings presented some unique difficulties. I should like to close this preface by expressing my thanks to our Moscow colleagues, to the publishers in England, to the printers in Poland, and to participants from all over the world, whose co-operation and good humour in unfamiliar surroundings made the symposium possible.

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CHAIRMAN'S INTRODUCTION

by E. C. SLATER

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IT IS most appropriate that, on the occasion of the Fifth International Congress of Biochemistry in Moscow, a symposium should be held dealing with oxidative phosphorylation. The pioneer studies in the thirties by Engelhardt, Runnstrom and Kalckar culminated in the publication in 1939 from Professor Engelhardt's laboratory of the classic paper by Belitzer and Tsibakowa, which established the concept of what we now call respiratory-chain phosphorylation. Quite soon afterwards, and independently, this was confirmed by Ochoa working in the laboratory of Professor Peters in Oxford. We are very glad that Sir Rudolph Peters will be presiding at one of our sessions.

On behalf of those interested in intracellular respiration, I should like to express our thanks to the organizers, and in particular to Professor Severin, for giving us this opportunity, the first on such a scale at an International Congress, of coming together to discuss our problems. We are, in fact, doubly fortunate, because not only do we have a symposium to ourselves, but yesterday we had the privilege of hearing one of the mos distinguished workers in the field, Professor Green, giving the opening lecture of the Congress.

The scope of the symposium is indicated in the title *Intracellular respiration: phosphorylating and non-phosphorylating reactions.* Perhaps it would be as well to spend a minute to discuss what we mean by non-phosphorylating reactions. The oxidation of β-hydroxybutyrate to acetoacetate by rat-liver mitochondria is accompanied by three phosphorylation reactions, and oxidation is slow in the absence of phosphate or phosphate acceptor. Most of the oxidation is phosphorylating. Two parts of this reaction—the reduction of DPN^+ to DPNH catalyzed by β-hydroxybutyrate dehydrogenase, and the oxidation of DPNH by oxygen—can be studied separately from one another by using enzyme preparations derived from mitochondria. But these oxidation reactions are not coupled with phosphorylation—they are non-phosphorylating, or non-coupled. Here "non-" has no implications concerning mechanism. It is purely descriptive and neutral, meaning "not".

It seems likely, in fac, that the reaction between DPN and β-hydroxybutyrate is also non-phosphorylating or non-coupled in the intact mitochondria. There is, therefore, little reason to expect that the reactions studied with purified dehydrogenases differ from these occurring *in vivo*. On the other hand, the oxidation of DPNH by intact mitochondria is a coupled reaction which has become *un*coupled during the preparation of the fragments. In English, the word *un*coupled goes further than *non*-coupled. It means that something had to be done to uncouple a previously coupled oxidation.

Although the main emphasis of the symposium is on coupled oxidations, a number of contributions will deal with the detailed mechanisms of *non*-coupled or *un*coupled reactions. I am sure that studies of the non-phosphorylating reactions of dehydrogenases or of flavoproteins have much to teach those studying coupled reactions. The mechanism of *un*coupled oxidative reactions catalyzed by, for example, purified DPNH dehydrogenase or cytochrome oxidase form an essential part—but only a part—of the coupled reaction. I hope that this symposium might help to persuade those studying these mechanisms that their task is not yet finished.

The papers are divided rather roughly into four groups. The grouping starts logically—with dehydrogenases, then with pyridine nucleotides—and ends with cytochrome oxidase. But we had to give the flavoproteins tomorrow's session to themselves, which means that the quinones will to-day precede the flavoproteins. No conclusions concerning the sequence of components in the respiratory chain should be drawn from this purely organizational decision. On the third day of the symposium, rather more emphasis will be placed on phosphorylation than on electron transport (except for Dr. Hess' paper which is really displaced from to-day's session), while up to the last paper the last day will be devoted to the mechanism of uncoupling and to the possible significance of non-phosphorylating pathways, which are always present to a greater or less extent in preparations of isolated mitochondria. As already mentioned, the last paper of all will be on cytochrome oxidase.

Fundamental for all our studies is a knowledge of the way the oxidation-reduction potential of components of the respiratory chain behaves to changes in the phosphorylation potential. No one has succeeded better in solving the enormous technical difficulties which lie in the way of a direct attack on this problem than Professor Chance. He will now tell us about the results which he and Dr. Hagihara have obtained by direct spectroscopic measurements of interaction of components of the respiratory chain with ATP, ADP, phosphate, and uncoupling agents.

DIRECT SPECTROSCOPIC MEASUREMENTS OF INTERACTION OF COMPONENTS OF THE RESPIRATORY CHAIN WITH ATP, ADP, PHOSPHATE, AND UNCOUPLING AGENTS

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(Received 13 January 1961)

INTRODUCTION

KEILIN's pioneer observations of increased reduction of the cytochromes of the flight muscle of the wax moth provided the first evidence of the relationship between functional activity and the oxidation–reduction state of cytochrome. In fact, Keilin stated in 1925,[1] "The state of cytochrome as seen spectroscopically denotes only the difference between the rates of its oxidation and reduction". From this observation there subsequently developed the current concepts of the steady state and of the relationship between the oxidation–reduction level of the cytochromes in the mitochondria and the metabolic and physiologic state of the cell. Keilin's visual spectroscopic observations showed that "in natural conditions cytochrome is in the oxidized form and that during exertion, however great, cytochrome becomes only partially reduced". Further studies have shown that the response of cytochrome *c* to increased respiratory activity may not be a higher degree of reduction as observed visually in the wax moth, but may be an increased oxidation as observed in rat liver mitochondria[2] or in pigeon heart mitochondria as will be pointed out in this paper.

The understanding of Keilin's ideas on the steady state evolved slowly due to confusion between thermodynamic oxidation–reduction potential and dynamic steady-state levels; the Cold Spring Harbor Symposium of 1939[3] indicated some progress toward the recognition of this phenomenon. Baumberger[4] stated: "An appreciable percentage of reduced cytochrome

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occurs in the presence of measurable oxygen only when strong exogenous metabolism exists."

The degree of oxidation and reduction of cytochrome under conditions of steady-state metabolism is difficult to measure quantitatively with visual spectroscopic methods since they require such high concentrations of cell suspension that the dissolved oxygen is rapidly exhausted at room temperatures. At lower temperatures, the dehydrogenase activity may be sufficiently lessened that very little reduction of cytochromes in the steady state occurs.

Sensitive spectrophotometric methods, originally developed to study catalase intermediates,[5] recorded a partial reduction of all components of the respiratory chain in succinate and oxygen metabolism.[6] In nonphosphorylating preparations, the percentage reduction of particular components was shown to be a function not only of the presence of substrate but of the type of substrate as well; in such preparations, reduced diphosphopyridine nucleotide (DPNH) causes further reduction of cytochromes than does succinate.* The effect of oxygen concentration on the steady-state levels of the components was also studied and the oxygen affinity of the oxidase determined.[7]

In studies of phosphorylating mitochondria it was soon recognized that factors other than the presence of substrate and oxygen could have a dominant effect on the steady-state oxidation–reduction levels of respiratory carriers; consequently it was necessary to differentiate five different metabolic states of mitochondria on the basis of the presence of oxygen, substrate, and adenosine diphosphate (ADP) plus inorganic phosphate (P_i).[8] The most important of these states are those of starvation (state 2), active metabolism (state 3), rest (state 4), and anaerobiosis (state 5).

It is the purpose of this paper to review the ways in which ADP and P_i can affect the steady state of the respiratory carriers in pigeon heart mitochondria, which are particularly responsive to these substances. In addition, a new type of response of the steady states of mitochondria will be described: an anaerobic response of cytochrome to adenosine triphosphate (ATP) or an ATP reversal of electron transfer.[9, 10]

The results of such studies are important to our knowledge of the mechanisms of enzyme reactions, especially those that occur where the enzymes are bound tightly to particles from which they are not diffusible as required by the law of mass action. In addition, the study of the response of mitochondria to ADP, P_i, and ATP provides a new method for intracel-

* B. Chance, unpublished data.

lular measurement of these substances appropriately localized in strategic places in the muscle, nerve, or brain cell.[11, 12] Application of such indicators to studies of internal concentrations of intracellular levels of ADP has already been presented.[12]

EXPERIMENTAL

Preparation of the Mitochondria

A pigeon heart is washed in a cold medium (either 0·21 M mannitol, 0·07 M sucrose, 0·1 mM EDTA or 0·3—0·35 M mannitol alone containing 0·1 mM EDTA) and sliced. 4 g of the sliced muscle are suspended in 40 ml of the same medium to which have been added 20 mg of crystalline *B. subtilis* proteinase and Tris-phosphate buffer (pH 7·6 at 0·5 M) to 0·01 M Tris. The suspension is kept at 0°C for 20 min and stirred occasionally. It is then homogenized gently in a very loose homogenizer and kept again at 0°C for another 20 min. After a second addition of 40 ml of the medium* (no proteinase, no buffer), the suspension is further homogenized slowly in a regular homogenizer.

The suspension is centrifuged at 500 × *g* for 5 min to remove residue, taking great care that the heavy washed mitochondria (M_w) are not lost in the residue; the M_w are recovered at 12,000 × *g* for 10 min. The loosely packed white pellet is removed by shaking the tube with a small volume of the medium (brown M_w are very tightly packed). The M_w are suspended in 10 ml of the medium and centrifuged at 8000 × *g* for 5 min.† They are then resuspended in a small volume of the medium and are ready for use.

With this method of preparation, respiratory-control ratios greater than 5 (usually 8–10) are obtained with glutamate as substrate and greater than 3 (usually 4–5) with succinate as substrate (see below).

Reaction Medium

Two types of media are used: (a) mannitol-sucrose-Tris containing 0·23 M mannitol, 0·07 M sucrose, 0·02 M Tris (final pH 7·4)[13]; (b) 0·25 M mannitol, 0·01 M KCl, 0·01 M Tris-Cl buffer, 0·005 M K-phosphate buffer, 0·02 mM EDTA, final pH 7·2. Temperature 22°C ((O_2) = 0·27 mM). The medium was shaken well at 22°C before use.

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* A better yield is obtained with large volumes of the homogenizing medium.

† With further washing, poorer respiratory-control ratios are obtained. A drawback to reproducibility of the results may be the difficulty in recovering the heavy M_w from the residue.