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Volume 5

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Proceedings of the fifth symposium on Regulation of Enzyme Activity and Synthesis in Normal and Neoplastic Tissues held at Indiana University School of Medicine Indianapolis, Indiana October 3 and 4 1966

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FOREWORD

Advances in Enzyme Regulation is now in its fifth volume. The appreciative reception of this series reflected the need for such a source of information, inspiration, and laboratory and teaching companion.

Volume 5 concentrates on subjects which have reached the stage of productive summarization and critical evaluation in the light of extensive new results. This book also lives up to its goal of advancing a few steps ahead of the general front of mammalian enzyme regulation studies.

It has been my editorial policy to impose as few restrictions as possible, emphasizing, however, the objectives of excellence of contribution, perfection in presentation, and penetration and scope in interpretation. This principle gives a wide range of freedom to the participants to express their concepts. Thus, the responsibility for detail—accuracy of reporting, preciseness of references, allocations of priority, expressions of judgment and evaluation—lies with the individual authors.

The Editor, who enjoyed the advice of leaders in the field, has been organizing the Symposia and selecting topics and speakers on the basis of immediate and long-range significance of the scientific contributions. It is hoped that the comments and suggestions of investigators and teachers in this field will continue to come to the Editor's office and contribute to shaping the course of forthcoming conferences and volumes.

Indiana University
1967

GEORGE WEBER, *Editor*

ACKNOWLEDGMENTS

THIS is the fifth in a series of Symposia dedicated entirely to problems and advances in regulation of enzyme activity and synthesis in mammalian systems.

I take great pleasure in expressing appreciation for the support and assistance I received in organizing and conducting this Conference. I wish to gratefully acknowledge that Indiana University School of Medicine, the American Cancer Society Institutional Fund, Burroughs Wellcome and Co., Eli Lilly and Co., Hoffman LaRoche, Merck Sharp and Dohme, and The Upjohn Co. provided the financial support for this Meeting.

In the planning of the program, selection of participants and arrangements for the Symposium the advice of the following was invaluable: J. Ashmore, G. F. Cahill, Jr., B. Chance, C. F. Cori, O. Greengard, N. Katunuma, W. E. Knox, Sir H. A. Krebs, N. Kretchmer, H. A. Lardy, H. P. Morris, V. R. Potter and S. Weinhouse.

I am very obliged to Drs. Cahill, Chance, Cori, Knox, Krebs, Kretchmer, Lardy, Olson, Potter, Weinhouse and Williams-Ashman for serving as chairmen of the sessions, and to all contributing authors for their cooperation in the preparation of this volume.

At Indiana University School of Medicine in the local organization of the Symposium I had the kind assistance of Provost Kenneth E. Penrod and Deans Glenn W. Irwin, Jr., A. D. Lautzenheiser and Doris H. Merritt. The efficient and competent help of R. Dault and J. P. Schall in accommodation arrangements and the expert assistance of J. Glore in the preparation of illustrations are much appreciated.

Thanks are due to Patsy J. Brown, Delores Cameron, Freida Jones, Dr. Patrick C. Logan and Nancy B. Stamm, members of my staff who assisted in the local arrangements and in the typing of the manuscripts.

My highest appreciation is due to my wife, Catherine E. Forrest Weber, whose contribution in the role of Technical Editor was invaluable in the assembling of this volume.

GEORGE WEBER
Symposium Chairman

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MICROFLUOROMETRIC STUDY OF RESPONSES OF THE CYTOSOL TO MITOCHONDRIAL SUBSTRATES

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AN EXACT knowledge of the time course of biochemical concentration changes in each compartment of the living cell is required for a proper understanding of metabolic control phenomena in normal and pathological states. Although many techniques have been developed, none is at present completely satisfactory. In this paper we shall describe an attempt to measure intracellular DPNH concentrations with a high degree of localization, a fast response, and a high chemical specificity. However, it is appropriate to review briefly the other methods currently available.

The classical methods of cell fractionation[1, 2] are, in general, satisfactory for the major cellular organelles: the mitochondria, microsomes, nuclei, etc. In principle, the extraction of these organelles is achieved without disturbance of their internal concentrations. While this is never strictly correct, it is more true for the organelles than for the remainder of the cell, termed by Lardy the "cytosol".[3] The latter is completely disrupted, not only in its structural nature but also in its chemical constitution. Unfortunately, it is in this portion of the cell that many important control points of metabolism are found.

The total metabolite assay of single living cells is likewise unsatisfactory, since the concentrations of metabolites which are constituents of more than one organelle are usually averaged in the total cell analysis so that the transport of a control metabolite from one compartment to another may occur unobserved. This problem becomes acute in the study of control phenomena in ascites tumor cells, where compartmentation of ATP has been postulated.[4, 5]

The possibility that cell samples may be withdrawn from localized portions of the cell and submitted to a complete biochemical analysis is not yet within our grasp; however, developments in the laboratories of Lowry[6] and Hyden[7] are suggestive that such methods may ultimately be developed.

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The procedure studied extensively by Bücher[8, 9] and further employed by Krebs[10] utilizes DPN-linked dehydrogenase couples to indicate the DPN/DPNH ratios in the portions of the cell in which they are located. While, theoretically, this procedure pinpoints the DPN/DPNH ratio to the exact location of the particular enzyme, the problems in connection with its practical employment have been discussed elsewhere.[9, 11] The difficulties hinge largely upon the problem of identifying a pool of reduced pyridine nucleotide which is in thermodynamic equilibrium with a single dehydrogenase having a single, known intracellular location.

Intracellular organelles can be used as sensitive indicators of localized concentrations of metabolites for which they are specific. A case in point is provided by the mitochondrial response to ADP and phosphate in muscle[12] or ascites tumor cells.[13] Cytochrome *b* has been shown to be a highly sensitive indicator of changes in the concentration of ADP in the medium in which the mitochondria are located; responses to increases in intracellular ADP concentrations of about 5 mμmoles per g of tissue in times as short as 0.1 sec have been achieved.[12] Such measurements are presently limited to homogeneous volumes of tissue such as skeletal muscle, or to homogeneous suspensions of cells such as ascites[13] or yeast.[14]

Similar measurements of cytochromes in single cells have been made possible by improvements to the basic Caspersson–Thorell microspectrophotometric methods[15, 16] carried out in collaboration with Åkerman on instrumental problems and with Thorell and Perry on biological aspects.[17, 18] Although 6000 molecules of mitochondrial cytochrome *b* are detectable by this method, the response time is greatly inferior to that obtainable from the larger volumes of homogeneous cells. Moreover, the sensitivity of this technique diminishes so greatly in the ultraviolet region that it is inadequate, at the present time, for studies of DPNH absorption in localized portions of single cells.

The enhanced fluorescence of DPNH bound to dehydrogenases[19] and to mitochondria[20] affords a basis for microfluorometric measurements of this component in mitochondrial and extramitochondrial portions of the living cell. By appropriate electronic and instrumental developments,[21] it has been possible to construct a differential microfluorometer that is sensitive enough to detect 30,000 molecules of mitochondrial DPNH.[22] This instrument was first employed to study the localized mitochondrial aggregate, or nebenkern, of the grasshopper spermatid[23] and then that of the cricket.[24] Not only could the mitochondrial DPNH pool be detected, but also the kinetics of changes of fluorescence resulting from alterations in the redox state of the cell could readily be followed.

The method was further applied to ascites tumor cells grown on glass,[25]

past which a number of reagents could be flowed to cause varying oxidation-reduction states of reduced pyridine nucleotide in the mitochondrial, cytoplasmic, and nuclear compartments. Characteristic fluorescence increases were obtained in the cytoplasmic and nuclear spaces on perfusing with glucose[26] and in the mitochondrial space on perfusing with Amytal.[27]

The great limitation to this method (and, in fact, to all methods so far described) is imposed by cell impermeability. The possibility of localized fluorometric assay of cellular compartments suggested that it would be worthwhile to develop a technique in which microfluorometric observation was combined with microinjection of appropriate substrates and cofactors into localized portions of the cell. This development has been undertaken by my colleagues, the Kohens, in collaboration with V. Legallais, and they have recently achieved results of extraordinary importance to problems of metabolic control. I shall report one result particularly relevant to today's discussion topic; others are described in a general report to be submitted elsewhere (Chance, Kohen, Legallais, and Kohen, in preparation).

Extramitochondrial Responses to Mitochondrial Substrates

Figure 1 represents the results of a series of experiments in which approximately 10^{-11} liters of 1 M mitochondrial substrate were injected

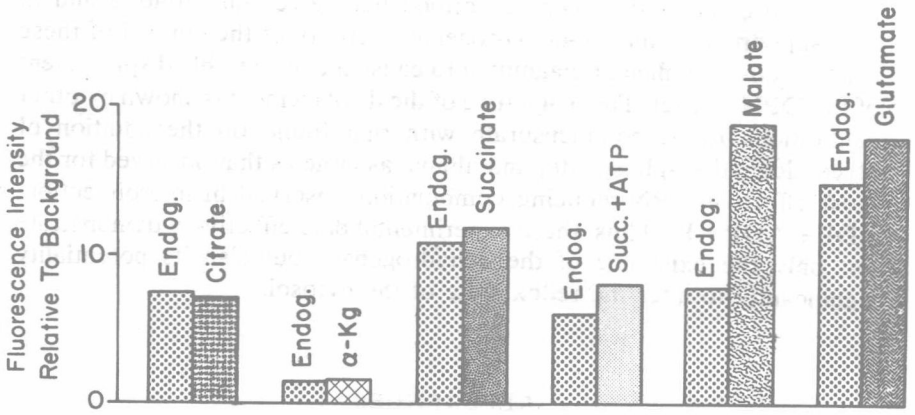

FIG. 1.
Extramitochondrial responses to mitochondrial substrates. A single ascites tumor cell (X-irradiated EL-2 "giant" $80 \times 20\ \mu$) microinjected into the cytosol with citric acid cycle substrates as shown. The fluorescence change in the extramitochondrial space is monitored by a fluorescence microscope technique and the per cent changes caused by the microinjection with respect to the cell prior to microinjection are indicated in the chart.

into the cytoplasm of a glass-grown EL-2 "giant" cell[28] about $80 \times 20\mu$, with the aid of microelectrophoresis (10^{-7} amps for 6 sec).[29] The various substrates were injected into portions of the cell adjacent to those under observation. The mitochondria are aggregated around the nuclear region in a manner characteristic of these multi-nucleate cells, and observation of the extramitochondrial responses is therefore readily obtained in areas of the cell where the mitochondrial concentration is minimal. The approximately 10μ aperture of the microfluorometer is focused upon this area, and fluorescence is measured prior to the injection of a substrate and at a time after injection corresponding to maximal response. Variations in the fluorescence intensity for the column marked "Endogenous" are due to variations in the thickness of the portions of the cell under observation as well as to the endogenous substrate content. It is apparent that the typical mitochondrial substrates, citrate, α-ketoglutarate, succinate + ATP, and glutamate, cause a barely detectable effect. Malate, however, has a pronounced effect. Control experiments show that malate, α-ketoglutarate, succinate + ATP, and glutamate all cause a large fluorescence increase in the mitochondrial space, indicating that these substrates were injected properly and were not already present in saturating amounts in the mitochondrial space.

DISCUSSION

The particular experiment described here gives quantitative and *in vivo* support for a malate-dehydrogenase activity of the cytosol of these ascites cells of sufficient magnitude to cause a considerable displacement of the DPNH level. The magnitude of the displacement is shown by other calibrations to be commensurate with that found on the addition of glyceraldehyde-3-phosphate, and almost as large as that observed for the most effective DPN-reducing combination observed in microinjection, FDP + ADP + P_i. Thus, these experimental data effectively demonstrate not only the existence of the dehydrogenase but also its potentiality significantly to alter the redox state of the cytosol.

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