# METABOLIC DIFFERENCES BETWEEN MITOCHONDRIA ISOLATED FROM VARIOUS TISSUES.

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It is generally known that mitochondria of various differentiated animal tissues contain enzyme systems which under conventional experimental conditions perform apparently the same metabolic reactions (e.g., citric acid, fatty acid oxidizing, oxidative phosphorylation systems, cf. 1). Considerable difficulties arise when, on the basis of customary experimental models (e.g., measurement of overall rates, metabolic balance studies, etc.), tissue specific metabolic patterns are sought. That organ-specific metabolic responses exist is predictable from physiological phenomena, which are in sharp contrast to the apparent monotony of analytically oriented enzymology.

In the course of search for more specific experimental designs capable of detecting specific control phenomena, it was found that various specific enzyme inhibitors elicited different metabolic responses in multienzyme systems obtained from different animal tissues (2,3,4,5), even though the same enzyme was inhibited in all of these systems. We have expanded this experimental model in two ways. First, initial velocity of $O_2$ uptake of isolated mitochondria was determined in the presence of one substrate and various substrate pairs. In addition, the effect of monofluoro oxaloacetate ($F_1$OAA), a potent inhibitor of mitochondrial malate dehydrogenase (6,7), was tested in the presence of all selected substrates and substrate pairs. The rate limiting role of succinic dehydrogenase (with malonate, cf. 8,9), NADH oxidase (with rotenone, 10), in terminal electron transfer and the kinetic contribution of glutamate oxaloacetate transaminase (with a combination of mono- and difluoro oxaloacetates, cf. 6) were also determined. From the configuration of observed inhibition patterns it is evident that large "metaboli differences exist between mitochondria isolated from various rat tissues. Recognition of tissue specific control factors circumvents the conceptual difficulty whic arises if metabolic regulation is to be "explained" on the basis of monotonous metabolic "cycles", presumably operating in the same fashion in all tissues.

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EXPERIMENTAL METHODS. Isolation of mitochondria was carried out in 0.25 M sucrose, containing 0.05% EDTA (pH adjusted to 7.4) as described earlier (cf. refs. 2,3,4,5). Initial rates of $O_2$ uptake were determined polarographically by means of a Clark $O_2$ electrode attached to a chart recorder (Gilson Oxygraph). The reaction chamber of 5 ml volume was kept at 30° by thermostatic control provided by circulating water in the double wall of the vessel. Continuous stirring was achieved by a magnetic stirrer. Reaction time varied from 3 to 5 minutes. The reaction system contained: 0.15 M KCl, 0.01 M phosphate (pH 7.4), 0.002 M $MgCl_2$ and 0.002 M ADP. All substrates were used at a final concentration of $2 \times 10^{-3}$M, and $F_1$OAA at $2 \times 10^{-4}$M. The reaction was started by addition of ADP, since oxidizable substrates alone did not influence endogenous respiration of mitochondria. The absolute dependence of respiration on ADP in presence of substrates was taken as a measure of complete respiratory control.

The evaluation of enzyme inhibitory studies critically depends on the instantaneous penetration of monofluoro oxaloacetate ($F_1$OAA) into mitochondria. This has been previously determined (refs. 4,5) and recently reconfirmed by direct spectrophotometry of mitochondria by the Chance double beam instrument.* Since monofluoro oxaloacetate is a substrate of mitochondrial malate dehydrogenase ($V_{max}$ is 50 to 100 times smaller than with oxaloacetate, cf. 7), the recording of the reoxidation of intramitochondrial NADH by the highly sensitive double beam instrument, after addition of $F_1$OAA, is a precise index of penetration. This reaction takes place instantaneously. The partial reduction of $F_1$OAA to monofluoromalate (which is, however, quantitatively negligible under our experimental conditions) does not complicate the kinetic picture since monofluoromalate and $F_1$OAA have nearly the same $K_i$ for malate dehydrogenase. On the other hand, monofluoromalate itself penetrates mitochondria in an unpredictable fashion, thus cannot be used as an enzyme reagent. Decomposition of $F_1$OAA to fluoropyruvate, as studied previously (11), does not occur. Conversion of $F_1$OAA to the non-inhibitory isomer of fluorocitrate (cf. 2) is much too slow to alter the concentration of $F_1$OAA during short term polarographic measurements.

RESULTS. Metabolic rates in the Table are expressed as μmoles of $O_2$/1 g protein/30 minutes at 30°. These values are extrapolated from initial rates, measured in the course of 2-4 minutes following initiation of the reaction (by ADP). This unit to define metabolic rate was chosen in order to compare values with other published data obtained by the Warburg manometric procedure (defined previously as "pseudo-steady state" condition, in contrast to the polarographic method, called "initial rate" condition, cf. 4). The numerical values obtained by the two methods are in good agreement (cf. 4,5,12). Comparison of metabolic rates of mitochondria pre-

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pared from rat liver, kidney, brain and heart reveals the following characteristic patterns: a) Endogenous respiration is completely inhibited by $F_1$OAA in all instances. b) While oxaloacetate in liver mitochondria is not oxidized, its metabolism by kidney, brain and heart mitochondria is completely blocked by $F_1$OAA. Oxaloacetate augments the endogenous rate of kidney mitochondria 3-fold, that of brain mitochondria 2-fold, and heart mitochondria close to 4-fold. c) Pyruvate alone is not oxidized by kidney and brain mitochondria, but approximately doubles the endogenous rate of both liver and heart mitochondria. $F_1$OAA in liver mitochondria diminishes the augmentation of $O_2$ uptake caused by pyruvate to the level of endogenous rate, and completely inhibits respiration in heart sarcosomes. d) Combination of oxaloacetate and pyruvate markedly increases respiration even in mitochondria where one substrate alone had no effect. This respiration is completely insensitive to $F_1$OAA in liver, kidney and brain, but not in heart mitochondria. e) The rate of oxidation of malate varies significantly depending on the mitochondria studied. This rate is approximately proportional to malate dehydrogenase content of various types of mitochondria (cf. 7). $F_1$OAA completely blocks malate oxidation in all mitochondria. f) Combination of malate with pyruvate results in large respiration in all mitochondria. This respiration is partially sensitive to $F_1$OAA in heart sarcosomes, but is completely insensitive to this inhibitor in kidney and brain and only to a small extent in liver mitochondria. g) Metabolism of glutamate is inhibited to varying degrees by $F_1$OAA, depending on the type of mitochondria studied (cf. 4,5,12). In presence of glutamate, added oxaloacetate increased $O_2$ uptake, even in mitochondria which do not oxidize oxaloacetate alone (e.g., liver). $F_1$OAA completely blocks this respiration. h) Isocitrate is almost completely inert in kidney, brain and heart mitochondria, although there is high isocitrate dehydrogenase activity of all mitochondrial extracts. Isocitrate oxidation by liver mitochondria is completely inhibited by $F_1$OAA. i) Fumarate oxidation is only partially inhibited by $F_1$OAA in liver mitochondria, but is completely blocked in all other systems. j) Combination of isocitrate and fumarate increases $O_2$ uptake of all types of mitochondria. This respiration is insensitive to $F_1$OAA in liver mitochondria, but is completely inhibited in brain mitochondria. Kidney and heart mitochondria are only partially inhibited by $F_1$OAA under the same conditions. k) Among all substrates tested, succinate is oxidized at the highest rate in liver and kidney mitochondria. This respiration is insensitive to $F_1$OAA. Brain mitochondria do not exhibit maximal $O_2$ uptake with succinate (compare with malate + pyruvate) and are significantly inhibited by $F_1$OAA. Heart mitochondria, which are known to contain large amounts of succinate dehydrogenase, have surprisingly low $O_2$ uptake in presence of succinate. This reaction is completely inhibited by $F_1$OAA. l) The magnitude of the oxidation of $\alpha$-ketoglutarate by different types of mitochondria and the degree of inhibition by $F_1$OAA vary significantly. Oxidation of

α-ketoglutarate by brain mitochondria is insensitive to $F_1$OAA.

The rate limiting contribution of NADH oxidase is clearly shown by the observation that rotenone (4 μg/5 ml) completely blocks $O_2$ uptake in presence of all substrates except succinate. Succinate dehydrogenase appears to be rate limiting only in liver mitochondria when glutamate or succinate is the added substrate (complete inhibition of $O_2$ uptake occurs in presence of $4 \times 10^{-3}$M malonate), while 50% inhibition is obtained at the same malonate concentration when glutamate-pyruvate, oxaloacetate-pyruvate or malate-pyruvate substrate pairs are oxidized. Oxidation of oxaloacetate by kidney mitochondria is uninfluenced by malonate unless glutamate-aspartate transaminase is inhibited simultaneously (by difluoro oxaloacetate, cf. 6). This pattern indicates that transaminases have a hitherto unrecognized metabolic significance, probably related to substrate transfer systems as discussed elsewhere (cf. 5). Succinate oxidation in kidney mitochondria is not inhibited when glutamate-aspartate transaminase is completely blocked by $2 \times 10^{-3}$M $F_2$OAA.

DISCUSSION. Metabolic differences were identified experimentally by a comparison of initial rates of $O_2$ uptake by various types of mitochondria in the presence of well-known substrates (or intermediates) of generally recognized metabolic "cycles." Enzymes acting on all substrates tested have been isolated and characterized by many biochemists. Based on this knowledge and on general properties of the electron transport system, it might be expected that if a universal metabolic cycle operates in all types of mitochondria, it should make no difference which substrate initiates the cycle, since all intermediate substances can be derived from the added substrate provided an open system is created by unhindered electron flow (i.e., by ADP and orthophosphate). The observed deviation from the simplest (i.e., cyclic) multienzymatic pattern clearly shows that conditions within phosphorylating mitochondria do not permit the realization of kinetic coupling of enzymatic reactions in the most elementary thermodynamically predictable fashion. The major contributing factor resulting in apparently "unreasonable" multienzymatic kinetics is oxidative phosphorylation, which is known to cause "reversals" of electron transfers and coupling of thermodynamically not feasible oxidation reduction systems (e.g., succinate $\longrightarrow$ NADH reaction, cf. 13 a,b,c). A combination of the function of energy transducing mechanisms with primary kinetic regulation of enzyme systems (e.g., in terms of variations in rate limiting steps) was shown to result in a linear multienzymatic kinetic organization of the glutamate system in kidney and in a distributive multienzymatic organization in liver (cf. 4,5). From the descriptive data shown in the Table, it is not possible to formulate at this time an enzyme chemical mechanism for each experimental observation. It is predictable, however, that just as in the case of the glutamate system (4,5,12), detailed studies will reveal unexpected multienzymatic patterns exhibiting to some extent varying degrees

| SOURCE OF MITOCHONDRIA | CONDITIONS | SUBSTRATES* | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | OAA | Pyr | OAA + Pyr | Mal | Mal + Pyr | Glu | Glu + OAA | Iso | Fum | Iso + Fum | Suc | α-Ktgl |
| LIVER | No Inhibitors | 120 | 120 | 220 | 340 | 240 | 650 | 660 | 920 | 290 | 195 | 650 | 10,000 | 560 |
| | $F_1$OAA** | 0 | 0 | 120 | 340 | 0 | 530 | 440 | 0 | 290 | 97 | 650 | 10,000 | 410 |
| | % Inhibition | 100% | *** | 46% | 0% | 100% | 19% | 33% | 100% | 0% | 50% | 0% | 0% | 27% |
| KIDNEY | No Inhibitors | 260 | 840 | 260 | 1220 | 1220 | 2350 | 650 | 820 | 300 | 370 | 770 | 15,000 | 1000 |
| | $F_1$OAA** | 0 | 0 | 0 | 1220 | 0 | 2350 | 325 | 0 | 0 | 0 | 335 | 15,000 | 755 |
| | % Inhibition | 100% | 100% | *** | 0% | 100% | 0% | 50% | 100% | *** | 100% | 56% | 0% | 24% |
| BRAIN | No Inhibitors | 72 | 151 | 76 | 340 | 240 | 680 | 204 | -- | 91 | 119 | 149 | 415 | 250 |
| | $F_1$OAA** | 0 | 0 | 68 | 340 | 0 | 680 | 69 | -- | 0 | 0 | 0 | 150 | 250 |
| | % Inhibition | 100% | 100% | *** | 0% | 100% | 0% | 66% | -- | -- | 100% | 100% | 64% | 0% |
| HEART | No Inhibitors | 400 | 1500 | 890 | 2000 | 1200 | 2200 | 1500 | -- | 440 | 900 | 1500 | 600 | 1500 |
| | $F_1$OAA** | 0 | 0 | 0 | 900 | 0 | 1500 | 0 | -- | 0 | 0 | 1000 | 0 | 900 |
| | % Inhibition | 100% | 100% | 100% | 55% | 100% | 46% | 100% | -- | *** | 100% | 33% | 100% | 40% |

ABBREVIATIONS:
0 = endogenous
OAA = oxaloacetate
Pyr = pyruvate
Mal = malate
Glu = glutamate
Iso = isocitrate
Fum = fumarate
Suc = succinate
α-Ktgl = α-ketoglutarate

* = $2 \times 10^{-3}$M
** = $2 \times 10^{-4}$M
*** = not oxidized.

of tissue specificity. Metabolic "flexibility" of certain mitochondria is particularly noticeable by substrate dependent variations in the rate limiting role of malate dehydrogenase. It is predictable that rapid and complex metabolic regulation can be brought about by these mechanisms, without actual changes in enzyme content. On the other hand, changes in the concentration of the same enzyme in various tissues can result in different systematic responses. These problems require further theoretical (14) and experimental approaches.

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REFERENCES.

1. Lehninger, A. L.: The Mitochondrion. W. A. Benjamin, Inc., New York, 1964.
2. Fanshier, D. W., Gottwald, L. K., and Kun, E.: J. Biol. Chem. 237, 3588 (1962).
3. Fanshier, D. W., Gottwald, L. K. and Kun, E.: J. Biol. Chem. 239, 425 (1964).
4. Kun, E., Ayling, J. E. and Baltimore, B. G.: J. Biol. Chem. 239, 2896 (1964).
5. Kun, E., Loh, H. H., and Ayling, J. E.: J. Biol. CHem. (in press).
6. Kun, E., Gottwald, L. K., Fanshier, D. W. and Ayling, J. E.: J. Biol. Chem. 238, 1456 (1963).
7. Kun, E. and Volfin, P.: Biochem. Biophys. Res. Comm. 22, 187 (1966).
8. Quastel, J. J. and Wooldridge, W. R.: Biochem. J. 22, 689 (1928).
9. Thorn, M. B.: Biochem. J. 54, 540 (1953).
10. Burgos, J. and Redfearn, E. R.: Biochim. Biophys. Acta 110, 475 (1965).
11. Kun, E. and Williams-Ashman, H, G.: Biochim. Biophys. Acta 59, 719 (1962).
12. Kun, E., Loh, H. H. and Volfin, P.: Biochem. Biophys. Res. Comm.
13. Chance, B. and Hollunger, G.: a) J. Biol. Chem. 236, 1534 (1961); b) ibid. 236, 1555 (1961); c) ibid. 236, 1562 (1961).
14. Goodwin, B. C.: Temporal Organization of Cells. Acad. Press, London, 1963.