"SUBSTRATE-INDUCED EFFLUX OF ANIONS FROM BOVINE ADRENAL CORTEX MITOCHONDRIA AND ITS RELATIONSHIP TO STEROIDOGENESIS"

Evan R. Simpson and Rene Frenkel

Department of Biochemistry
University of Texas Southwestern Medical School at Dallas,
Dallas, Texas, 75235

Received May 9, 1969

SUMMARY: The efflux of DHAP and pyruvate from bovine adrenal cortex mitochondria, incubated with αGP and malate respectively, has been studied. With αGP as substrate, virtually all the DHAP formed leaves the mitochondria. With malate as substrate, 75% of the pyruvate formed leaves the mitochondria. These observations support the concept that the "α-glycerophosphate shuttle" and the "malate shuttle" can be operative in bovine adrenal cortex.

In recent years, an increasing amount of effort has been devoted to measuring the efflux of substances from mitochondria. This is due primarily to interest in shuttle mechanisms involved in the transfer of reducing equivalents across mitochondrial membranes (Boxer and Devlin, 1961), and to a desire to understand the reactions involved in gluconeogenesis (Lardy, Paetkau and Walter, 1965; Mehlman, Walter and Lardy, 1967).

Carefully prepared bovine adrenal cortex mitochondria display an impermeability towards pyridine nucleotides when incubated in the absence of Ca^{++} ions (McCarthy and Peron, 1968). Consequently it would be expected that shuttle mechanisms exist to effect the net transfer of reducing equivalents across the mitochondrial membrane, as has been proposed for mitochondria of other tissues (Estabrook and Sacktor, 1958; Boxer and Devlin, 1961; Williamson, Scholz and Thurman, 1968). This problem is of particular interest when mitochondria of adrenal cortex are concerned, because they contain, in addition to the normal respiratory chain, a second electron transport chain responsible for

Abbreviations: αGP - α-glycerophosphate; DHAP - dihydroxyacetone phosphate; GPDH - α-glycerophosphate dehydrogenase; LDH - lactate dehydrogenase; TRA - triethanolamine; DOC - deoxycorticosterone.

steroid mixed-function oxidase reactions (Harding and Nelson, 1966; Omura, Sanders, Estabrook, Cooper and Rosenthal, 1966; Suzuki and Kimura, 1967). This second electron transport chain requires NADPH rather than NADH (Sweat and Lipscomb, 1955), and it has been shown that in bovine adrenal cortex,the most likely source of NADPH for the mitochondrial mixed-function oxidases is malic enzyme located within the mitochondria (Simpson and Estabrook, 1969). Further, it has been postulated (Simpson and Estabrook, 1968) that, in bovine adrenal cortex, the cytosol and mitochondrial malic enzymes constitute a 'malate shuttle' to effect the net transfer of cytoplasmic generated NADPH reducing equivalents into the mitochondria (fig. 1).

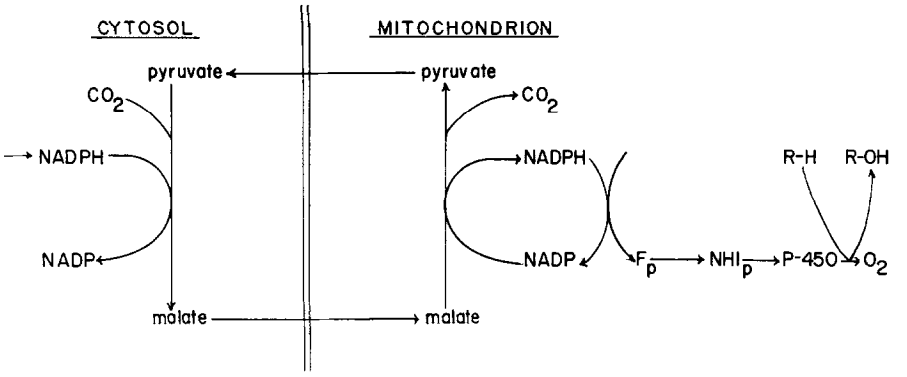

Figure 1. Proposed 'malate shuttle' of bovine adrenal cortex, showing how the cytosol and mitochondrial malic enzymes could function together to effect net transfer of NADPH reducing equivalents into the mitochondria.

The purpose of this report is to demonstrate two examples of substrate-induced efflux of anions from bovine adrenal cortex mitochondria: - (a) the efflux of DHAP when αGP is the substrate, and (b) the efflux of pyruvate when malate is the substrate. These observations support the concept that the 'α-glycerophosphate shuttle' and the 'malate shuttle' are operative in bovine adrenal cortex mitochondria.

METHODS: Bovine adrenal cortex mitochondria were prepared as previously described (Cammer and Estabrook, 1967) except that the mitochondria were washed with 0.25M sucrose containing 1% bovine serum albumen, pH 6.8. The mito-

chondria were incubated at 24°C in a buffer containing 0.25M sucrose, 20mM KCl, 15 mM TRA-HCl buffer pH 7.4, 10mM potassium phosphate buffer pH 7.4, and 5mM $MgCl_2$. Efflux of substances from the mitochondria was measured directly by coupling the enzymic reduction of the substance as it leaves the mitochondria to the oxidation of NADH (Suranyi, Hedman, Luft and Ernster, 1963) which was measured fluorometrically.

Thus for the measurement of DHAP efflux, GPDH and NADH were used; for the measurement of pyruvate efflux, LDH and NADH were used. Efflux was also measured directly by filtration of aliquots of the mitochondrial incubation mixture on Millipore filters (Millipore Corp., Bedford, Massa.), using a 0.8 micron filter on top of a 0.65 micron filter. The filtration time was about 5 seconds. Reactions were stopped with 7% perchloric acid and assays were performed on neutralized aliquots by the enzymatic methods of Estabrook and Maitra (1962).

RESULTS AND DISCUSSION: Fig. 2 shows DHAP formation and efflux from bovine adrenal cortex mitochondria supplied with αGP. The solid triangles show total DHAP in aliquots of a reaction mixture incubated in the absence of GPDH

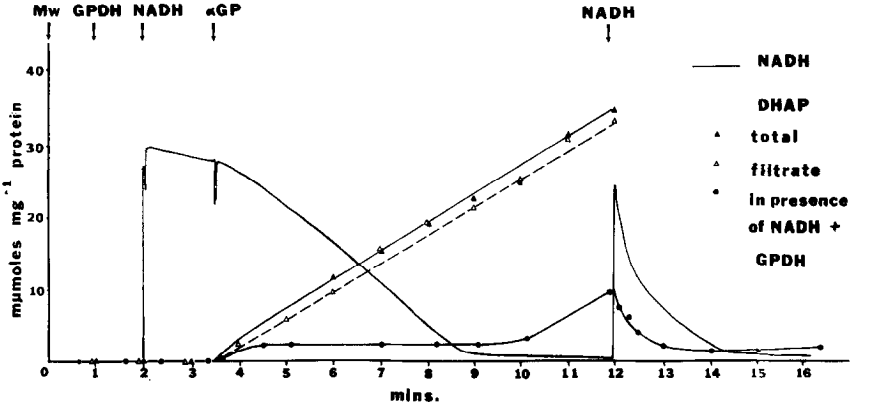

Figure 2. Formation and efflux of DHAP from bovine adrenal cortex mitochondria incubated with αGP. Final concentrations: Mitochondria-2.1 mg/ml; αGP - 800 μM; NADH - 57 μM; GDPH - 6.7 μg/ml. The NADH trace was taken directly from the fluorimeter recording.

and NADH. The open triangles show DHAP in parallel aliquots which were passed through a Millipore filter. It can be seen that almost all the DHAP was extra-mitochondrial. In a parallel experiment, GPDH and NADH were added to the mitochondrial suspension and the oxidation of the NADH followed fluorometrically. The rate of this oxidation was in good agreement with the rate of DHAP efflux. After the initial aliquot of NADH was oxidized, a second aliquot of NADH was added. This was initially oxidized rapidly and then the oxidation rate slowed down to the previous rate. This initial rapid burst of NADH oxidation indicates that DHAP accumulated outside the mitochondria during this time period when no NADH was present, and that the extra-mitochondrial coupled enzyme system was not serving to facilitate the diffusion of DHAP out of the mitochondria. These observations were confirmed by measurement of DHAP in aliquots from the incubation mixture containing GPDH and NADH. In this case, the DHAP maintained a low level, in agreement with the difference between total and filtrate in the experiment conducted in the absence of the reducing system. When the NADH was oxidized, the DHAP accumulated until the second addition of NADH. It then dropped to the steady-state level over the same time interval as the rapid phase of NADH oxidation.

The rate of DHAP formation from both the direct measurement and the rate of NADH oxidation was 4 mμmoles $min.^{-1}$ $mg.\ protein^{-1}$, and the steady-state level within the mitochondria was about 2 mμmoles $mg.\ protein^{-1}$. The α-glycerophosphate shuttle may be important as an energy source in adrenal cortex, as NADH oxidation in adrenal cortex mitochondria may be inhibited during steroidogenesis (Simpson and Estabrook, 1969; Stoppani, De Brignone and Brignone, 1968).

Cammer, Cooper and Estabrook (1967) and Simpson and Estabrook (1969) showed that malate-supported 11β-hydroxylation of DOC was accompanied by pyruvate formation due to the action of mitochondrial malic enzyme in supplying NADPH for 11β-hydroxylation. Fig. 3 shows pyruvate formation and efflux from bovine adrenal cortex mitochondria supplied with malate and DOC. The solid

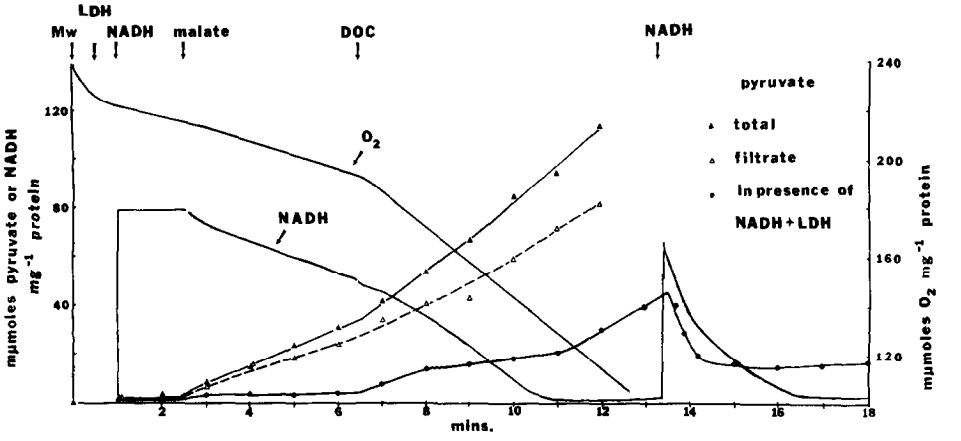

Figure 3. Formation and efflux of pyruvate from bovine adrenal cortex mitochondria incubated with malate and DOC. Final concentrations: mitochondria - 1.1 mg. protein/ml; malate - 3.1 mM; DOC - 140 μM; NADH 78.5 μM; LDH - 5μg/ml. The NADH trace is the fluorimeter recording from which the contribution of endogenous pyridine nucleotide has been subtracted.

triangles show total pyruvate concentration in aliquots of a reaction mixture which was incubated in the absence of LDH and NADH. As expected, addition of DOC resulted in a stimulation of pyruvate formation concomitant with increased oxygen uptake. The open triangles show pyruvate concentrations in parallel aliquots which were passed through Millipore filters. Thus about 75% of the pyruvate was extra-mitochondrial. In an experiment conducted in the presence of LDH and NADH, there was oxidation of NADH corresponding quite well to the pyruvate in the filtrate. Addition of a second aliquot of NADH once again resulted in an initial rapid burst of oxidation of the reduced nucleotide followed by a rate of oxidation of the nucleotide at the previous rate, again showing that pyruvate was accumulating outside the mitochondria during the interval when no NADH was present, and that the extra-mitochondrial coupled enzyme system was not facilitating the diffusion of pyruvate out of the mitochondria. Measurement of pyruvate in the presence of LDH and NADH gave levels that corresponded to the difference between total and filtrate in the reaction mixture incubated in the absence of LDH and NADH. When all the NADH was oxidized, pyruvate

accumulated until the second addition of NADH. It then dropped to the steady-state level over the same time interval as the initial rapid burst of NADH oxidation.

It has previously been shown (Peron and Caldwell, 1968; Simpson and Estabrook, 1968) that pyruvate + CO_2 + NADPH are a good source of malate for 11β-hydroxylation, in the presence of bovine adrenal cortex cytosol malic enzyme. The observations reported here on pyruvate efflux from bovine adrenal cortex mitochondria are consistent with the scheme shown in Fig. 1. This 'malate shuttle' involves the cytosol and mitochondrial malic enzymes working together to effect the net transfer of NADPH across the mitochondrial membrane, and thus would bring the mitochondrial steroid hydroxylase systems of bovine adrenal cortex under the control of extra-mitochondrial generation of NADPH.

Supported by U. S. P. H. S. Grant No. GM 16488.

REFERENCES

Boxer, G. E. and Devlin, T. M., Science, 134, 1495 (1961).
Cammer, W., Cooper, D. Y. and Estabrook, R. W. in "Functions of the Adrenal Cortex" ed. McKerns, Appleton-Century-Crofts, 943 (1968).
Cammer, W. and Estabrook, R. W., Arch. Biochem. Biophys., 122, 721, (1967).
Estabrook, R. W. and Maitra, P. K., Anal. Biochem, 3, 369 (1962).
Estabrook, R. W. and Sacktor, B., J. Biol. Chem., 233, 1014 (1958).
Harding, B. W. and Nelson, D. H., J. Biol. Chem., 241, 2212 (1966).
Lardy, H. A., Paetkau, V. and Walter, P., Proc. Natl. Acad. Sciences, 53, 1410 (1965).
Mehlman, M. A., Walter, P. and Lardy, H. A., J. Biol. Chem., 242, 4594 (1967).
McCarthy, J. L. and Peron, F. G., in "Functions of the Adrenal Cortex", ed. McKerns, Appleton-Century-Crofts, 261 (1968).
Omura, T., Sanders, E., Estabrook, R. W., Cooper, D. Y. and Rosenthal, O., Arch. Biochem. Biophys., 117, 660 (1966).
Peron, F. C. and Caldwell, B. V., Biochim. Biophys. Acta, 164, 396 (1968).
Simpson, E. R. and Estabrook, R. W., Arch. Biochem. Biophys., 126, 977 (1968).
Simpson, E. R. and Estabrook, R. W.,Arch. Biochem. Biophys., 129, 384 (1969).
Stoppani, A. O. M., De Brignone, C. M. C. and Brignone, J. A., Arch. Biochem. Biophys. 127, 463 (1968).
Suranyi, E. M., Hedman, R., Luft, R. and Ernster, L., Acta Chem. Scand., 17, 877 (1963).
Suzuki, K. and Kimura, T., J. Biol. Chem., 242, 485 (1967).
Sweat, M. L. and Lipscomb, M. D., J. Am. Chem. Soc., 77, 5185 (1955).
Williamson, J. R., Scholtz, R. and Thurman, R. G., Bari Symposium (1968) in press.