ENERGY PRODUCTION AND PYRROLIDONECARBOXYLIC ACID FORMATION IN RAT LIVER NUCLEI

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The nuclear fraction obtained from rat liver has been shown to catalyze the conversion of L-glutamic acid to pyrrolidonecarboxylic acid[1]. This system differs from other enzymatic pathways promoting the formation of pyrrolidonecarboxylic acid from glutamic acid[2,3] in an apparent requirement for an energy-yielding source, as well as in subcellular distribution. The energy-yielding system which we have described previously contained glucose, NAD, cytochrome c, fumarate, ATP and Mg^{++}. These components were required for maximum production of pyrrolidonecarboxylic acid from glutamic acid.

The experimental data reported in this communication demonstrate that pyruvate or substrates of the tricarboxylic acid cycle plus ATP and Mg^{++} could be substituted for the multi-component system previously used. It was found also that this system required aerobiosis and was inhibited by the same compounds which inhibit ATP synthesis in calf thymus nuclei[4]. In contrast, however, to mitochondrial

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[1]Niwaguchi, T., Motohashi, N. and H.J. Strecker, Biochim. Biophys. Acta, 82, 635 (1964).

[2]Meister, A., and M.W. Bukenberger, Nature, 194, 557 (1962).

[3]Cliffe, E.E. and S.G. Waley, Biochem. J., 79, 118 (1961).

[4]McEwen, B.S. Allfrey, V.G., and A.E. Mirsky, J. Biol. Chem., 238, 758 (1963).

preparations capable of coupling energy production to substrate oxidation the system in nuclei, that couples pyrrolidonecarboxylic acid formation to substrate oxidation is solubilized readily.

Materials and Methods.-- Preparations: The nuclear fraction of rat liver was prepared and purified by the method of Hymer and Kuff[5]. The washed nuclear pellet was suspended in 3 ml of 0.1 M tris (hydroxymethyl) aminomethane solution, pH 7, per gram wet weight of original tissue, with the aid of the glass homogenizer described by Dounce et al.[6]. The suspension was centrifuged at 105,000 x g for 30 minutes. The clear, reddish supernate contained the active system. The concentration of protein was 1.5 - 2.0 mg/ml as determined by the method of Lowry et al.[7].

Enzyme assay: The incubation mixtures described in the headings of the tables were incubated for 2 hours at 37°. The reaction was terminated by adding 0.5 ml of 20% perchloric acid. After centrifugation to remove the denatured protein, the supernatant solution was neutralized with 6 N KOH and the potassium perchlorate precipitate removed by centrifugation. The supernatant solution was transferred to a 1 x 10 cm. column of Dowex 50 - X8 (H^+ form). Water was passed into the column and 10 ml of the initial eluate collected; this contained all of the pyrrolidonecarboxylic acid. A 2 ml aliquot of this eluate was heated with 0.5 ml of 5 N HCl at 100° for 15 minutes to hydrolyze the pyrrolidonecarboxylic acid to glutamic acid. After cooling, the resulting solution was analyzed by the ninhydrin method of Rosen[8]. The pyrrolidonecarboxylic acid concentration was calculated from a standard curve prepared with glutamic acid. The compound giving a positive

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[5]Hymer, W.C. and E.L. Kuff, J. Histochem. Cytochem., In Press.
[6]Dounce, A.L., Witter, R.F., Monty, K.J., Pate, S. and M.A. Cottone, J. Biophys. Biochem. Cytol., 1, 139 (1955).
[7]Lowry, O.M., Rosebrough, N.J., Farr, A.L. and R.J. Randall, J. Biol. Chem., 193, 265 (1951).
[8]H. Rosen, Arch. Biochem. Biophys., 67, 10 (1957).

ninhydrin reaction under the conditions described had been identified previously as pyrrolidonecarboxylic acid[1]. Prior to hydrolysis with HCl the eluate of the Dowex 50 - X8 column was always ninhydrin-negative. Amytal, which interfered with the ninhydrin reaction, was separated from pyrrolidonecarboxylic acid by elution with 1.5 M formic acid, after passing the Dowex 50 - X8 eluate into a 1 x 10 cm column of Dowex 1 - X8 formate.

Results.-- McEwen et al. have demonstrated that the oxidation of glucose supports the synthesis of ATP in thymus nuclei[4]. In our system that formed pyrrolidonecarboxylic acid in intact nuclei, the requirement for glucose and other components known to stimulate glycolysis and oxidation suggested that ATP was being generated and reacted directly or indirectly with the γ-carboxyl group of glutamic acid. It is known that γ-anhydrides and esters of glutamic acid such as γ-glutamyl phosphate and γ-glutamyl-S-glutathione readily cyclize to pyrrolidonecarboxylic acid[9]. Testing of other substrates which could generate ATP revealed that pyruvate, ATP and Mg^{++} together were as effective in promoting pyrrolidonecarboxylic acid formation as was the fortified glucose-containing system used previously[1].

Using the simplified incubation mixture to promote the formation of pyrrolidonecarboxylic acid, it was found that the entire system could be solubilized by extraction of the nuclear pellet with tris (hydroxymethyl) aminomethane solution as described in the methods section. With this soluble extract, pyruvate, ATP, Mg^{++} and aerobiosis are required for the maximum yield of pyrrolidonecarboxylic acid. Other components of the tricarboxylic acid cycle could substitute for pyruvate although they were not as effective when used in the same concentration (Table I).

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[9]Sachs, H. and H. Waelsch, J. Am. Chem. Soc., 77, 6600 (1955).

TABLE I

Requirements for Pyrrolidonecarboxylic Acid (PCA) Formation by Soluble Extracts of Rat Liver Nuclei

Components	PCA formed μmoles
Complete system, aerobic	0.92
Complete system, anaerobic	0.13
- Pyruvate	0.12
- ATP	0.55
- Mg^{++}	0.83
- Glutamate	0
Complete system with heated extract	0
Complete system with the following substrates in place of pyruvate	
Citrate	0.50
α-Ketoglutarate	0.68
Succinate	0.72
Fumarate	0.70
Malate	0.59

The incubation mixture contained 1.0 ml of nuclear extract, (protein concentration 2 mg/ml) 40 μmoles of potassium pyruvate, 2.1 μmoles of ATP, 24 μmoles of $MgCl_2$, 50 μmoles of potassium L-glutamate and 150 μmoles of potassium phosphate, pH 7.5. The acids tested in place of pyruvate were at the same concentration (40 μmoles in 3 ml) and were all added as the potassium salts. The heated extract was prepared by subjecting the extract to 100° for 15 minutes.

In order to determine if the complete system responsible for the conversion of glutamic acid to pyrrolidonecarboxylic acid was related to the system reported to catalyze ATP synthesis in nuclei, a number of substances were tested for inhibition of pyrrolidonecarboxylic acid formation. The first six compounds listed in Table II have been reported to inhibit ATP synthesis in thymus nuclei[4] and are known to have the same effect on mitochondrial preparations. Fluoroacetate inhibits the citric acid cycle in mitochondria and appears to have the same effect in nuclei[10]. Malonate also inhibits the citric acid cycle in mitochondria but was found to have no effect on nuclear catalyzed oxidations[10]. Ca^{++} and methylene blue uncouple oxidative phosphorylation in mitochondrial but not in nuclear preparations[4].

[10]McEwen, B.S., Allfrey, V.G., and A.E. Mirsky, J. Biol. Chem., 238, 2579 (1963).

The data presented in Table II demonstrate that substances affecting ATP synthesis or oxidation in thymus nuclei have a paralleling influence on pyrrolidonecarboxylic acid formation in rat liver nuclear extracts. In addition, arsenite was a very effective inhibitor of pyrrolidonecarboxylic acid formation.

TABLE II

Effect of Inhibitors of ATP Synthesis on Pyrrolidonecarboxylic Acid Formation in Nuclear Extracts

Inhibitor	Conc. M	PCA formed(μmoles)	Per cent inhibition
None	-	0.97	-
Potassium arsenite	3.3×10^{-4}	0	100
Potassium cyanide	3.3×10^{-4}	0	100
Dinitrophenol	3.3×10^{-4}	0	100
Sodium azide	1×10^{-3}	0	100
Antimycin A	1 μg /ml	0.20	79
Dicumarol	3×10^{-5}	0.72	26
Amytal	1×10^{-3}	0.47	52
Fluoroacetate	1×10^{-2}	0.25	73
Malonate	1×10^{-2}	0.77	21
Ca^{++}	4×10^{-3}	0.88	14
Methylene blue	2.5×10^{4}	1.0	0

The incubation conditions were the same as for Table 1. In the experiment with Ca^{++}, 150 μmoles of triethanolamine hydrochloride, pH 7.5 was substituted for potassium phosphate.

Discussion.-- McEwen et al. have shown that thymus nuclei catalyze the aerobic synthesis of ATP and have concluded that this synthesis depends on both glycolysis and the citric acid cycle. The synthesis of ATP by thymus nuclei was inhibited by compounds which inhibit the operation of the citric acid cycle (fluoroacetate), or inhibit electron transport in mitochondrial preparations (amytal, antimycin A, CN^-, N_3^-) or uncouple oxidative phosphorylation in mitochondria (dinitrophenol). Ca^{++} and methylene blue, which uncouple oxidative phosphorylation in mitochondrial preparations, had little effect on the nuclear system. The effect of these compounds on the conversion of glutamic acid to pyrrolidonecarboxylic acid by rat liver nuclei was practically the same as on ATP synthesis, and would suggest that ATP synthesis was required for pyrrolidonecarboxylic acid formation. However, ATP synthesis in

nuclei has been found to be quite labile and disappears when nuclei are damaged[11,12]. Preliminary experiments with our soluble extracts has not provided evidence for synthesis of ATP; inorganic phosphate is rapidly formed from ATP, ADP or AMP either in the absence or presence of the complete system. Nevertheless, the evidence indicates that the conversion of glutamic acid to pyrrolidonecarboxylic acid is coupled to the oxidation of substrates of the citric acid cycle and that the enzymes catalyzing these reactions have been solubilized. The effects of the inhibitors of aerobic ATP synthesis on this conversion suggest that a high energy intermediate other than ATP and required for pyrrolidonecarboxylic acid formation may be produced during oxidation. The formation of the intermediate would appear to be inhibited by arsenite, cyanide, azide, amytal, antimycin A, and dinitrophenol but not by Ca^{++} or methylene blue.

The inhibition by malonate, which did not inhibit ATP synthesis in thymus nuclei, may be explained by the proposal of McEwen et al. that malonate and other dicarboxylic acids do not enter intact nuclei[4,10].

The availability of a soluble preparation that can couple an energy utilizing reaction to substrate oxidation and which is inhibited by many of the same compounds which inhibit the synthesis of high energy intermediates in mitochondrial preparations may furnish a valuable tool for study of the mechanism of synthesis of high-energy intermediates. Although Triton X-100 is used during the preparation of the nuclei, it is not necessary for the preparation of the soluble extract. Active extracts also can be obtained from rat liver nuclei which are not treated with this detergent although such preparations are more contaminated with extra-nuclear material.

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[11]Allfrey, V.G., Mirsky, A.E. and S. Osawa, J. Gen. Physiol., 40, 451 (1957).
[12]Osawa, S., Allfrey, V.G. and Mirsky, A.E. J. Gen. Physiol., 49, 491 (1957).