OXIDATION OF ALPHA-KETOGLUTARATE BY BLUEGILL LIVER MITOCHONDRIA

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ABSTRACT. — The alpha-ketoglutarate oxidase of bluegill (*Lepomis macrochirus*) was investigated. Neither oxygen nor phosphate uptake was metal ion dependent, but magnesium ions increased oxygen and phosphate uptake. Zinc, cadmium, manganese, and calcium ions inhibited oxygen uptake and promoted the hydrolysis of tissue organic phosphates. Dicoumarol, gramicidin, 2, 4-dinitrophenol and pentachlorophenol uncoupled phosphorylation from alpha-ketoglutarate oxidation. Antimycin A, sodium azide, sodium arsenite, and sodium cyanide inhibited oxygen uptake.

The energy production by enzyme systems from fishes has not been extensively investigated (Gumbmann et al 1957; Tarr 1958), but Ekberg (1958) and Kanungo and Prosser (1959) discussed energy production by goldfish tissues; Brown and Tappel (1959), Brown (1960) and Gumbmann and Tappel (1962) discussed oxidative metabolism in carp liver mitochondria; Hiltibran and Johnson (1965), and Hiltibran (1965, 1966) reported on energy production by bluegill liver mitochondria.

The energy production by enzyme systems of the bluegill (*Lepomis macrochirus*) is being investigated as one approach to determine the effects of pollutants on energy production of the bluegill. An investigation of the oxidative phosphorylation with alpha-ketoglutarate as substrate was initiated to determine whether the oxidation of alpha-ketoglutarate might not be altered by pollutants. A preliminary report (Hiltibran 1964) has been presented, and the effect of sodium amytal and rotenone on the alpha-ketoglutarate oxidase has been reported (Hiltibran and Johnson 1965).

MATERIALS AND METHODS

Native wild bluegills obtained from central Illinois waters were utilized. The bluegills were held approximately two weeks at 25° C in aerated laboratory aquaria. Preparation of the mitochondria and the procedures used for estimating oxygen and phosphate uptake have been reported previously (Hiltibran & Johnson 1965). Oxygen data are expressed as QO_2 values (μl 0_2/hr/mgN) and phosphate data as micromoles (μmoles) of PO_4/hr/mgN and all values have been corrected for endogenous activity. The data represent the values from five or more experiments.

RESULTS AND DISCUSSION

Effect of Homogenizing Medium.— Less oxygen and phosphate uptake occurred with mitochondria prepared in phosphate buffered (pH 7.5) 0.15 or 0.08 M sucrose when alpha-ketoglutarate was the substrate than when succinate was the substrate. Although there was net phosphate uptake, this uptake was almost masked by the large increase in the inorganic phosphate content of the reaction media. Various co-factors added to the reaction media did not alter either

oxygen or phosphate uptake. These data indicate that the mitochondria prepared in phosphate-buffered sucrose apparently were not capable of maximum utilization of alpha-ketoglutarate.

Oxygen uptake was increased approximately 30 per cent and there was a 3-fold increase in phospate uptake by mitochondria from liver tissue homogenized in buffered sucrose containing 0.001 M ethylenediaminetetraacetic acid (EDTA).

In other experiments the liver tissue was divided—one part of the tissue was homogenized and the mitochondria were resuspended in buffered sucrose. The other part was homogenized and the mitochondria were resuspended in buffered sucrose containing EDTA. The mitochondria prepared and resuspended in EDTA buffered sucrose utilized more oxygen and phosphate than the mitochondria prepared in non-EDTA buffered sucrose. Oxygen and phosphate uptake by the mitochondria prepared in buffered sucrose were increased by the presence of EDTA in the reaction vessels. These data further support the need for EDTA during the mitochondrial preparation and incubation period for the maximum use of oxygen and phosphate. Neither niacin, nicotinamide, bovine serum albumin (BSA) nor 0.002 M EDTA in either the homogenizing media or in the reaction vessels had an effect.

These preparation conditions are similar to those observed by Brown and Tappel (1962) who used a fortified medium to prepare the mitochondria in their study of the fatty acid oxidation by carp liver mitochondria. Gumbmann and Tappell (1962) used carp liver mitochondria prepared in EDTA-sucrose for the investigation of the TCA cycle in carp liver mitochondria.

Hiltibran (1965) proposed that buffered 0.15 M sucrose was a suitable medium for the preparation of mitochondria from bluegill liver tissue in studies of the succinic oxidase and adenosinetriphosphatase (ATPase). In the latter study, EDTA was found to increase cadmium ATPase activity, but decreased the other ATPase activities, particularly the Mg ATPase activity.

After EDTA had been found to increase oxygen and phosphate uptake in the presence of alpha-ketoglutarate, all the mitochondria used in oxygen uptake studies were prepared in EDTA-sucrose. Later, when comparing oxygen and phosphate values from succinic oxidase experiments using mitochondria prepared in EDTA-sucrose, with the oxygen and phosphate values obtained with mitochondria prepared in sucrose only, it was observed that the oxygen uptake was greater by mitochondria prepared in sucrose, but the phosphate uptake was not altered. In split-preparation experiments, oxygen uptake was found to be severely inhibited in mitochondria prepared and resuspended in EDTA-sucrose. These effects of EDTA on the succinic oxidase system were not unexpected as EDTA in the reaction media had been found to reduce oxygen uptake about 30 percent. These data indicate a differential effect of EDTA on the succinic and alpha-ketoglutarate oxidase systems. While 0.15 M sucrose appears to be a suitable preparation medium for the preparation of mitochondria, the presence of EDTA-sucrose promotes oxygen and phosphate uptake with alpha-ketoglutarate as substrate, but EDTA in either the mitochondrial preparation media, or in the reaction vessels inhibits oxygen uptake when succinate is used as substrate. The previous reports by Kanungo and Prosser (1959) and Gumbmann and Tappel (1962) do not indicate whether EDTA has a differential effect on the succinic oxidase systems and on the other systems described in goldfish and carp liver mitochondria.

The data from EDTA-sucrose succinate experiments have been combined and the average oxygen and phosphate values have been inserted into TABLE 1.

Slater and Cleland (1953) reported that on incubation, rat liver mitchondria lost their ability to oxidize alpha-ketoglutarate, and that this loss could be prevented by EDTA. They found that EDTA bound calcium ions, which were associated with the loss of oxidative ability. Lester and Hatefi (1958) found that EDTA prevented the release of particle-bound pyridine nucleotide from beef-heart mitochondria. Chefurka (1966 a, b) reported that the protective effect of the EDTA was attributed to the binding of calcium ions. The calcium ions promoted the release of fatty acids from the mitochondrial lipids, and the fatty acids released were the disruptive agents (Chefurka and Dumas 1966). Since EDTA is necessary for the oxidation of alpha-ketoglutarate by bluegill liver mitochondria, it would appear that EDTA protects the oxidation of alpha-ketoglutarate by preventing the release of fatty acids. The effect of EDTA on the succinic oxidase system is not understood.

In other experiments it was found

TABLE 1.—The Effects of Homogenizing Media on Oxygen and Phosphate Uptake.

Homogenizing Data	$\mu l O_2$/hr/mgN	μmoles/PO_4/hr/mgN
Alpha-ketoglutarate Oxidase		
Homogenized and resuspended in buffered sucrose...	64	(—) 9
Homogenized in EDTA media, resuspended in Sucrose	74	(—) 27
Homogenized in buffered Sucrose +EDTA mitochondria resuspended in Sucrose.....	55[1]	(—) 8
Liver tissue homogenized in EDTA + Sucrose mitochondria resuspended in EDTA Sucrose.....	107[1]	(—) 27
Average values of 18 experiments + co-factors.....	167	(—) 44
Succinic Oxidase System		
Homogenizing and resuspended in 0.15 M Sucrose...	189[2]	(—) 28
Homogenized in EDTA–Sucrose — resuspended in EDTA–Sucrose or Sucrose only.....	100[2]	(—) 19

[1] Liver tissue divided and treated as indicated.

[2] The data from split preps and other experiments have been combined into one average value.

TABLE 2.—Summary of the Effects of Co-factors on Alpha-ketoglutarate Oxidation.

Co-factors	Avg. Change in $\mu l O_2$/hr/mgN	Avg. Change in μmoles/PO_4/hr/mgN
—ATP.....	(—) 35.24	(—) 16.38
—ADP.....	(—) 54.36	(+) 27.73
—BSA.....	(±) 42.35	(±) 29.29
—CoA.....	(—) 22.21	(±) 4.57
—COA—ADP.....	(±) 44.95	(—) 16.34
—Cyto C.....	(—) 47.28	(±) 12.36
—Glucose.....	(—) 31.91	(+) 21.17
—Kinase.....	(±) 42.44	(+) 25.50
+NAD.....	(±) 55.94	(±) 9.54
+DPT.....	(±) 19.88	(±) 7.57
NAD + DPT.....	(±) 27.07	(—) 11.19

that the centrifugal force could be reduced from 21,600xg to 12,800xg without any decrease in oxygen or phosphate uptake. However, the period of high speed centrifugation, i.e., 10 minutes, could not be reduced. The latter centrifugal force is approximately that used by Gumbmann and Tappel (1962). These data indicate that suitable mitochondrial preparations can be obtained from fish liver homogenates under more mild conditions than those proposed by Schneider and Hogeboom (1950).

Effects of Co-factors.—Attempts to in-

crease oxygen and phosphate uptake by the addition of various co-factors to the reaction media were not successful until the mitochondria were prepared in EDTA-sucrose. Only Coenzyme A (CoA) and adenosinediphosphate (ADP) increased oxygen and phosphate uptake. The requirement of each for maximum oxygen and phosphate uptake was confirmed in experiments in which one component of the complete system was omitted and its effect on the oxygen and phosphate utilization was noted. These data are summarized in Table 2. However, for all subsequent experiments CoA and ADP have been added. Nicotinamide adenine dinucleotide (NAD) and diphosphothyamine (DPT), separately or together, did not greatly alter the oxygen and phosphate uptake. Likewise, BSA was not very effective and its use was discontinued.

Since the flow of electrons from alpha-ketoglutarate to the cytochrome chain is via NAD, apparently bluegill liver mitochondria contain sufficient functional NAD and the flow of electrons was not impaired regardless of the mitochondrial preparation media. It is possible also that structural integrity of the mitochondria is maintained when the mitochondria are prepared and assayed in the presence of EDTA.

Effects of Metal Ions.—The effects of five metal ions on the oxygen and phosphate uptake by bluegill liver mitochondria are summarized in Table 3. Neither oxygen nor phosphate uptake was dependent on any divalent metal ion, although magnesium ions increased both oxygen and phosphate uptake. Manganese, calcium, zinc and cadmium ions inhibit oxygen uptake; the latter two ions completely inhibited oxygen uptake. These same two ions also inhibited the succinic oxidase system (Hiltibran 1965) and promoted the hydrolysis of ATP by bluegill liver mitochondria (Hiltibran 1966).

TABLE 3.—Effect of Metal Ions on Alpha-ketoglutarate Oxidation.

Metal	μmoles added	$\mu l O_2$/hr/mgN	μmoles/PO_4/hr/mgN
Control	none	97.23	(—) 61.40
Mg	10	136.96	(—) 67.56
Mn	5	52.37	(±) 23.52
Ca	5	19.98	(±) 29.11
Zn	1	.....	(±) 22.27
Cd	1	.....	(±) 26.25

Effects of Inhibitors.—The effects of various enzyme inhibitors on the oxidation of alpha-ketoglutarate are summarized in Table 4. Sodium azide, Antimycin A and sodium cyanide inhibited oxygen uptake. Previous reports indicate that levels of Antimycin A of 0.33 μg/ml of reaction medium completely inhibited oxygen uptake. This was a lower level than the required values of goldfish liver mitochondria reported by Kanungo and Prosser (1959). In this investigation, .17 μg/ml of reaction medium completely inhibited oxygen uptake.

Sodium fluoride inhibited oxygen uptake approximately 30 percent and therefore was not routinely used. However, Kanungo and Prosser (1959) reported that fluoride was required for optimum phosphate uptake by goldfish liver mitochondria in the presence of alpha-ketoglutarate as substrate.

All the oxidative phosphorylation uncouplers used to date in the investigation except sodium arsenate inhibited oxygen uptake. One unexpected observation was that sodium arsenite (0.017 μmoles/ml of reaction media) severely inhibited oxygen uptake. However, arsenite did not

TABLE 4.—Effect of Inhibitors on Oxygen and Phosphate Uptake.

Inhibitor	Amt/ml of reaction ml (μmole)	Avg. Change in O_{O_2}	Avg. Change i. μmoles/PO_4/hr/mgN
Azide	1.7	(—) 68	(+) 8
Antimycin A	0.017	(—) 39	(+) 6
Cyanide	1.7	(—) 72	(+) 38
Fluoride	1.7	(—) 28	(+) 17
Arsenate	1.7	(±) 42	(+) 35
Arsenite	1.7	(—) 108	(+) 40
Pentachlorophenol	0.017	(—) 114	(+) 41
Dinitrophenol	0.017	(—) 54	(+) 22
Gramicidin	0.5[1]	(—) 26	(+) 17
Dicoumarol	0.017	(—) 81	(+) 30

[1] Micrograms.

greatly alter oxygen uptake by the succinic oxidase system of bluegill liver mitochondria, and its uncoupling of phosphorylation from oxidation of succinate was less severe than the uncoupling shown by arsenate. Sodium arsenate uncoupled the phosphorylation from alpha-ketoglutarate oxidation.

Pentachlorophenol and dicoumarol altered oxygen and phosphate uptake more severely than did dinitrophenol (DNP) or gramicidin (Table 4). Dicoumarol altered oxygen uptake more in the alpha-ketoglutarate oxidase system than in the succinic oxidase system, whereas DNP, gramicidin, and pentachlorophenol altered oxygen uptake more in the latter system. These data indicate that pentachlorophenol may exert its effect at some point common to both the succinic and alpha-ketoglutarate systems, but DNP, dicoumarol, and gramicidin may not. These oxidative phosphorylation uncouplers are capable of inhibiting oxygen uptake and all except gramicidin are capable of altering phosphate metabolism by bluegill liver mitochondria. Dinitrophenol and gramicidin increased the hydrolysis of ATP. These data suggest that if the toxic action of these oxidative phosphorylation uncouplers is mediated via the energy producing systems of the bluegill, then the toxicity levels of these uncouplers would vary. Pentachlorophenol is apparently more toxic to fish than is DNP (McKee and Wolf 1963).

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