OXIDATION OF SUCCINATE BY BLUEGILL LIVER MITOCHONDRIA

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ABSTRACT.—(1) The succinic oxidase system of bluegill (*Lepomis macrochirus*) liver mitochondria was investigated. (2) Neither oxygen nor phosphate uptake was metal-ion dependent, but magnesium and ferric ions increased oxygen uptake. (3) Zinc and cadmium ions inhibited oxygen uptake. (4) Dicoumarol, gramicidin, 2,4-dinitrophenol, and pentachlorophenol uncoupled phosphorylation from succinate oxidation but also inhibited oxygen uptake. (5) Antimycin A, sodium azide, and sodium cyanide inhibited oxygen uptake.

Oxidative metabolism of the bioenergetic systems of fishes has not been extensively investigated until recently. The reviews by McCay (1957), Gamohmann et al. (1958), and Tarr (1958) cited only one report of oxidative systems in fish up to that time. Since Tarr's review, several additional reports have appeared: Ekberg (1958) considered energy metabolism in goldfish tissues. Brown and Tappel (1959) reported on fatty acid oxidation, and Brown (1960) discussed glucose oxidation by carp liver mitochondria. Kanungo and Prosser (1959) investigated the role of oxidative phosphorylation in cold adaptation in goldfish, and Gorabmann and Tappel (1962) studied the tricarboxylic acid cycle in carp liver mitochondria.

The energy-producing systems of the bluegill (*Lepomis macrochirus*) liver mitochondria were selected for study as one means of exploring the effects of pollutants on aquatic organisms. Although fish are poikilotherms and live in environments having restricted oxygen, they must produce energy via oxidative pathways. A preliminary report (Hiltibran 1964) indicated from oxygen uptake data that bluegill liver homogenates and mitochondria could oxidize various substrates of the tricarboxylic acid cycle. On the basis of these data, an investigation of oxidative phosphorylation with succinate as substrate was undertaken.

MATERIALS AND METHODS

Native, wild bluegills obtained from various bodies of water in central Illinois were maintained in aerated laboratory aquaria at 25°C. Preparation of the mitochondria and procedures for estimating oxygen and phosphate uptake have previously been reported (Hiltibran and Johnson 1965). Oxygen data were converted to μlO_2 per hour per mg of tissue nitrogen, and changes in the phosphate content of the reaction medium were converted to micromoles of phosphate per hour per mg of tissue nitrogen. All values were corrected for endogenous enzyme activity. The data presented are the average values from two or more experiments.

RESULTS AND DISCUSSION

Effect of homogenizing medium and time of centrifugation: The mitochondria for preparatory experiments were obtained from homo-

TABLE 1.—The effect of homogenizing medium on O_2 and PO_4 uptake.

Medium	$\mu l O_2$/hr/mgN	μmoles PO_4/hr/mgN
0.25 M sucrose	123[a]	20
0.15 M sucrose	65[b]	6
	157[a]	32
	114[b]	25
	122[c]	26
0.08 M sucrose	100[d]	(−) 24
	175[a]	19
0.04 M sucrose	152[a]	22
Water	88	(+) 18
	66	(+) 20

[a] Tissue divided between 0.25 and 0.15 M sucrose.
[b] Second experiment, between 0.25 and 0.15 M sucrose.
[c] Tissue divided between 0.15 and 0.08 M sucrose.
[d] Average of 10 experiments (0.25 M sucrose).
[e] Average of 18 experiments (0.08 M sucrose).
(+) Denotes increase of inorganic phosphate in reaction medium.

genates prepared in 0.25 M sucrose by centrifuging in a Servall refrigerated centrifuge for 20 minutes at 21,000 x g after preliminary centrifugation for 10 minutes at 800 x g (Schneider and Hogeboom 1950). Very loosely-packed mitochondrial pellets were obtained, whose oxygen uptake was variable. Since the results indicated that the conditions might not have been suitable for the preparation of bluegill liver mitochondria, an investigation was undertaken to determine the effects of centrifugation time and homogenization in various media on mitochondria.

The full oxidative potential of the homogenates was obtained by centrifugating the homogenates for 10 minutes at 21,000 x g after a preliminary centrifugation for 5 minutes at 800 x g to remove cellular debris.

The oxygen and phosphate uptake by mitochondria prepared in various sucrose media are summarized in Table 1. The results indicated that either 0.08 or 0.15 M sucrose was a better homogenizing medium than was 0.25 M sucrose. The mitochondria prepared in 0.15 M sucrose appeared to have greater phosphorylation efficiency and the mitochondria prepared in 0.08 M sucrose utilized more oxygen.

Since the full oxidative potential could be obtained by shorter periods of centrifugation and since the mitochondria prepared in 0.15 M sucrose utilized more phosphate, the mitochondria for the experiments reported here were prepared in pH 7.5 phosphate-buffered 0.15 M sucrose and were obtained from homogenates by centrifugation for 10 minutes at 21,000 x g following preliminary centrifugation for 5 minutes at 800 x g. The mitochondria were resuspended in the homogenizing medium, recentrifuged, and resuspended in the original volume of buffered sucrose.

Most investigators have used mitochondria in 0.25 M sucrose prepared according to Schneider and Hogeboom (1950). Brown and Tappel (1959) used carp liver mitochon-

dria prepared in fortified 0.20 M sucrose for the investigation of fatty acid oxidation. Gumbmann and Tappel (1962) used mitochondria obtained by 10 minutes of centrifugation at 11,000 x g. These data suggest that in fishes 0.25 M sucrose or prolonged centrifugation may not be desirable for the preparation of mitochondria or other cellular components.

Effect of cofactors: Oxygen uptake was drastically reduced in the absence of adenosinetriphosphate (ATP) and as much as 80 per cent of the oxygen uptake shown in the presence of ATP was restored by adenosinediphosphate (ADP). Coenzyme A (Co A) or ADP added to ATP did not appreciably alter either oxygen or phosphate uptake. Co A and ADP without ATP did not restore the oxygen uptake level above that shown by ADP alone. For maximum uptake of oxygen in the presence of succinate, ATP was required by the bluegill liver mitochondrial systems.

Bluegill liver mitochondria exhibited coupled oxidation and phosphorylation in the absence of a phosphate trap, but the conversion of inorganic phosphate into organic phosphate was increased by the presence of the glucose-hexokinase phosphate trap. Associated with the increased utilization of phosphate was a slight increase in oxygen utilization. Glucose alone was without effect on either oxygen or phosphate uptake. The oxygen uptake by unwashed mitochondria was increased by bovine serum albumin, but washed mitochondria did not exhibit this requirement. Cytochrome C added to the reaction medium resulted in a 30 per cent increase in oxygen utilization, presumably by increased electron transport. The above data indicate that ATP, cytochrome C, and the glucose-hexokinase trap are required for maximal oxygen and phosphate uptake.

Since electrons from succinate are not transferred to the cytochrome chain by diphosphopyridine nucleotide (DPN), the presence of DPN in the reaction medium would not be expected to have caused any effect. However, in all experiments in which DPN was added to the reaction medium, oxygen uptake was substantially reduced. Triphosphopyridine nucleotide (TPN), on the other hand, did not have any effect on the oxidation of succinate. Thirty micromoles of succinate gave maximum oxygen uptake.

The effect of pH on the oxygen and phosphate uptake was limited to the effective range (pH 7 to 9) of Trishydroxyaminomethane (Tris) buffer. A broad range of activity was found from pH 7.2 to 7.8, and the oxygen and phosphate uptake activity decreased above and below those pH values. All subsequent analyses were determined at pH 7.5.

Effect of metal ions: The effects of several metal ions on the oxygen and phosphate uptake are summarized in Table 2. Oxygen and phosphate uptake occurred in the absence of any added metal ion, indicating that neither oxygen nor phosphate uptake was dependent upon the presence of ions. In the following experiments, the effects of the presence of metal ions were estimated.

Ten micromoles of the divalent magnesium ion and five micromoles

TABLE 2.—Effect of metal ions on oxygen and phosphate uptake.

Metal	μmoles added	$\mu l O_2$/hr/mgN	μmoles PO_4/hr/mgN
Control	none	76	(−) 12
Mg	10	125	(−) 20
Ferric	5	131	(−) 11
Manganese	5	29	(+) 22
Calcium	5	22	(+) 34
Zinc	1	.....	(+) 30
Cadmium	1	.....	(+) 41

of the trivalent ferric ion increased oxygen uptake. Magnesium ions, but not ferric ions, increased the conversion of inorganic to organic phosphate. The ratio of phosphate to oxygen utilized in the presence of the magnesium ions was approximately the same as the ratio in the control vessels. These observations suggest that since magnesium ions are necessary for maximal conversion of inorganic phosphate to tissue organic phosphate, the effect on oxygen uptake is indirect and due primarily to the increase in phosphorylation promoted by magnesium ions. Low levels of ferric ions increased the oxygen uptake but did not alter the phosphate uptake even though there was additional substrate oxidized and presumably additional energy available for phosphate conversion. The energy was apparently not utilized. Whereas 10 micromoles or more of ferric ions inhibited oxygen uptake, magnesium ion levels up to 30 micromoles did not further alter either the oxygen or phosphate uptake.

Effect of inhibitors: The effects of various enzyme inhibitors on the oxidation of succinate are summarized in Table 3. Antimycin A and sodium cyanide completely inhibited

TABLE 3.—Effect of inhibitors on oxygen and phosphate uptake.

Inhibitor	Amt/ml of reaction mod. (μmole)	Ave. change in $\mu l O_2$/hr/mgN	Ave. change in μmoles PO_4/hr/mgN
Azide	1.7	(−) 35	(+) 10
Antimycin A	0.6	(−) 138	(+) 19
Cyanide	1.7	(−) 158	(+) 14
Fluoride	1.7	(±) 37	(−) 3
Malonate	1.0	(−) 108	(−) 1
Arsenate	1.7	(±) 27	(+) 29
Arsenite	1.7	(−) 26	(+) 9
Pentachlorophenol	0.017	(−) 56	(+) 55
DNP	0.1	(−) 150	(+) 46
Gramicidin	0.5*	(−) 102	(+) 14
Dicoumarol	0.017	(−) 35	(+) 15

* Micrograms.

oxygen uptake. The level of antimycin A used (0.033 μg per ml of reaction medium) was less than that reported by Kanungo and Prosser (1959) for the inhibition of oxygen uptake by goldfish liver mitochondria. Sodium azide was less inhibitory to oxygen uptake.

The effect of several oxidative phosphorylation uncouplers was investigated. Sodium arsenate or sodium arsenite did not alter oxygen uptake. Sodium arsenite did not greatly affect the phosphate uptake, but sodium arsenate uncoupled the phosphate uptake from oxidation of the substrate.

The effect of 2,4-dinitrophenol (DNP) on succinic oxidase was investigated over a concentration range from 0.003 to 6.7 micromoles per ml of reaction medium. Oxygen uptake was inhibited at all levels of DNP tried. At the lowest level of DNP, inhibition of oxygen and phosphate uptake was slight. At 0.5 micromoles of DNP, oxygen utilization was reduced approximately 50 per cent. Maximum phosphate uncoupling was observed between 0.3 to 0.5 micromoles of DNP.

Since oxygen and phosphate uptake occurred in the absence of magnesium ions, the effect of DNP at levels from 0.3 to 3 micromoles per ml of reaction medium was estimated in the absence of magnesium ions, and the effect of DNP on both oxygen uptake and phosphate was reduced. These data suggest that the primary effect of DNP is on the enzyme sequence converting morganic phosphate to organic phosphate.

Gramicidin at 0.5 micrograms per ml of reaction medium reduced oxygen uptake approximately 25 per cent, but maximum phosphate uncoupling effect was observed at gramicidin levels of one or more micrograms.

Dicoumarol reduced oxygen uptake and interfered with phosphate incorporation at levels as low as 0.001 micromoles per ml of reaction medium. Oxygen uptake was reduced approximately 50 per cent at a dicoumarol level of 0.05 micromoles and was not further affected by higher levels. The maximum effect on phosphate occurred at a dicoumarol level of 0.1 micromoles and above.

Very low levels of pentachlorophenol (0.001 micromoles) reduced oxygen uptake approximately 50 per cent, and the phosphate uptake was reduced approximately 20 per cent. Maximum phosphate effect was observed in the presence of 0.01 micromoles of pentachlorophenol. At this level, however, oxygen uptake was reduced approximately 75 per cent.

Since oxygen uptake was inhibited by most of the oxidative phosphorylation uncouplers at levels which did not result in maximum effect on phosphate, caution will have to be exercised in interpreting the actions of other substances which may interfere with the phosphate or oxygen uptake in the bluegill liver mitochondria systems.

Of all the phosphate uncouplers investigated, all except sodium arsenite and sodium arsenate inhibited oxygen uptake to various degrees. Similarly, the effect on phosphate utilization varied with the concentration of inhibitor, and this effect seemed to be independent of the effect on oxygen uptake.

Five micromoles of sodium fluoride did not appreciably affect oxygen

uptake, but phosphate uptake was reduced. Higher fluoride levels inhibited oxygen uptake, and phosphate uptake was correspondingly reduced. Since the primary effect of high fluoride appears to be on oxygen uptake, a reduction in the oxidation of succinate would cause a decrease in the phosphate uptake. These data are in contrast to those of Kamango and Prosser (1959) who reported that 1.0×10^{-3} M sodium fluoride was necessary for maximum phosphate uptake by goldfish liver mitochondria. Therefore, sodium fluoride was not routinely added to the reaction medium.

Malonate, a competitive inhibitor of succinic acid oxidation, was found to reduce oxygen uptake at levels of 20 micromoles. Further studies revealed, however, that oxygen uptake was reduced by 30 per cent at 0.1 micromoles of malonate and that levels of malonate of 1.0 to 1.5 micromoles completely abolished oxygen uptake. Similar results have been reported by Gumbmann and Tappel (1962).

Although oxidation of succinate by the bluegill liver systems apparently follows the usual scheme for the oxidation of succinate as shown by all other systems, there are some differences. Suitable mitochondrial preparations were obtained from less dense homogenizing media and by shorter periods of centrifugation. This also is indicated by the work of Brown and Tappel (1959) and Gumbmann and Tappel (1962), but other workers apparently did not investigate the effect of homogenizing media or the time of centrifugation on the tissue preparations.

In the work described above, all couplers used except sodium arsenite the oxidative phosphorylation and sodium arsenate inhibited oxygen uptake at levels in which uncoupling action was greatly reduced. Frequently, low levels of inhibitors were used and the effect on oxygen or phosphate was slight; the change from control vessels approached the experimental error of the experiments so that further work with lower levels did not appear feasible under such conditions.

The effect of malonate is interesting. If malonate blocks succinic oxidase activity by irreversibly combining with the active succinic acid enzyme sites, this would indicate that relatively few succinic acid sites may be available and that the turnover number of the succinic acid oxidase is relatively great.

In this regard, oxygen uptake by bluegill liver mitochondria in the presence of sodium amytal was increased about 90 per cent and in the presence of rotenone about 60 per cent (Hiltibran and Johnson 1965). Presumably, this effect would be due to the inhibition of DPN-linked oxidative steps preventing the formation of oxalacetic acid, a powerful inhibitor of succinic oxidase. These data also indicate, however, that bluegill liver mitochondria can rapidly oxidize succinic acid.

Acknowledgment

The author gratefully acknowledges the technical assistance of Messrs. John Cralley, John Boulos, Lawrence Goodwin and Michael Johnson.

Literature Cited

Brown, W. D. 1960. Glucose metabolism in carp. J. Cell. and Comp. Physiol. 55:81-86.

Brown, W. D., and A. L. Tappel. 1959. Fatty acid oxidation by carp liver mitochondria. Arch. Biochem 85: 149-158

Ekhert, D. R. 1958. Respiration in tissues of goldfish adapted to high and low temperatures. Biol. Bull. 114: 308-316.

Gumbmann, M., W. D. Brown, and A. L. Tappel. 1959. Intermediary metabolism of fishes and other aquatic animals. U. S. Fish and Wildl. Serv. Spec. Sci. Rept. Fish. Ser. No. 288.

Gumbmann, M., and A. L. Tappel. 1962. The tricarboxylic acid cycle in fish. Arch. Biochem. 98:262 270.

Hultman, E. C. 1964 Oxidative phosphorylation by bluegill liver mitochondria. Absts. 6th International Congress of Biochemistry. p. 750.

Hultman, R. C., and M. G. Johnson. 1965. The effect of rotenone on oxygen uptake by mitochondria of the bluegill, *Lepomis macrochirus*. Trans. Ill. Acad. Sci. Vol. 58:140-143.

Kanungo, M. S., and C. L. Prosser. 1959. Physiological and biochemical adaptation of goldfish to cold and warm temperatures. II. Oxygen consumption of liver homogenate: Oxygen consumption and oxidative phosphorylation of liver mitochondria. J. Cell. and Comp. Physiol., 54:265-274.

McCay, C. M. 1937. The biochemistry of fish. Ann. Rev. Biochem., 6:445-468.

Schneider, W. C., and G. H. Hogeboom. 1950. Intracellular distribution of enzymes. V. Further studies on the distribution of cytochrome c in rat liver homogenates. J. Biol. Chem., 183:123-128.

Tarr, H. L. A. 1958. Biochemistry of fishes. Ann. Rev. Biochem., 27:223-244.

Manuscript received February 9, 1965.