THE EFFECT OF ROTENONE ON OXYGEN UPTAKE BY LIVER MITOCHONDRIA OF THE BLUEGILL, *LEPOMIS MACROCHIRUS*

ROBERT C. HILTIBRAN and MICHAEL C. JOHNSON
Illinois Natural History Survey, Urbana

ABSTRACT.—The effect of sodium amytal and rotenone on the alpha-ketoglutarate, oxidase, succinate oxidase, and adenosinetriphosphatase of bluegill liver mitochondria was investigated. Amytal and rotenone inhibited oxygen uptake with alpha-ketoglutarate as substrate but not with succinate. At amytal levels, which did not inhibit oxygen uptake, there was no effect on phosphate uptake; but at rotenone levels, which did not alter oxygen uptake, there was a decrease in phosphate uptake. Neither amytal nor rotenone increased the hydrolysis of adenosinetriphosphate; however, high concentrations of amytal and rotenone inhibited the hydrolysis.

Rotenone is widely used as a fish toxicant. This insecticide has been shown to reduce oxygen uptake by fishes (Danneel, 1933) and grasshoppers (Tischler, 1945). Later rotenone was thought to cause destruction of gill tissue (Danneel, 1933; Scheuring & Heuschmann, 1935) and to block the circulation in gill tissue (Hamilton, 1941). Oberg (1959) reported that in severe rotenone poisoning, the circulation in the gills was normal and the destruction of gill tissue was apparently due to secondary changes. Lindahl and Oberg (1961), studying the effect of rotenone on cellular respiration, reported that rotenone inhibited the uptake of oxygen in the presence of pyruvate and glutamate, but not in the presence of succinate as a substrate. Similar results had been reported by Fukami (1954) and Fukami and Tomizawa (1956) and Tomizawa and Fukami (1956).

Oberg (1961), using spectrophotographic techniques, demonstrated that rotenone inhibited the flow of electrons between diphosphopyridine nucleotide (DPN) and the cytochrome chain. Ernster (1957) reported that sodium amytal inhibited electron transfer between substrate and the cytochrome chain, and Chance (1958) indicated that the inhibition by amytal occurred between DPN and the cytochrome chain.

Since amytal and rotenone appear from the literature to have similar modes of action, the effect of amytal and rotenone on bluegill liver mitochondrial systems was investigated.

MATERIALS AND METHODS

Native, wild bluegill, *Lepomis macrochirus*, were maintained in aerated aquaria at 25° C. Fish were killed by severing the spinal cord; the livers were excised and placed in cold 0.15 M sucrose. Excess sucrose was removed by blotting with filter paper; livers were weighed quickly. One to ten homogenates were prepared by adding nine volumes of pH 7.5 phosphate buffered 0.15 M sucrose in a glass Potter-Elvejhem type homogenizer with Teflon Pestle. Mitochondria were obtained from the homogenates at -2° C by centrifugation (Schneider & Hogeboom, 1950) in a Serval refrigerated centrifuge for 10 min-

utes at 21,000 x g after a preliminary centrifugation at 800 x g for 5 minutes to remove debris. (Note—Early results indicated that all the oxidative ability were obtained under these conditions.) The mitochondria were washed once and resuspended in original volume of buffered 0.15 M sucrose. One ml of tissue preparation was used.

For maximum oxygen and phosphate uptake with alpha-ketoglutarate as substrate the mitochondria were prepared and resuspended in homogenizing medium containing 0.001 M ethylene diamine tetraacetic acid (EDTA).

Mitochondria used in the adenosinetriphosphatase (ATPase) investigation were prepared in unbuffered 0.15 M sucrose.

Oxidation of the substrates was estimated by conventional manometric techniques (1957) in a Warburg apparatus at 25° C (Umbreit, et al., 1951). The main compartment contained 0.5 μmoles of adenosinetriphosphate (ATP), 0.05 ml of 1% cytochrome C, 10 μmoles of magnesium chloride, 30 μmoles of substrate, 0.05 ml 1% bovine serum albumin, and 0.15 M Trishydroxymethylamino methane (Tris) buffer pH 7.5 to give a total volume of 3.0 ml. The single side arm contained 60 μmoles of glucose and 0.1 ml of 1% hexokinase, prepared prior to use. Fluted filter paper, and 0.2 ml of 20% potassium hydroxide were placed in the center well. Air was the gas phase. After 5 minutes of incubation, the contents of the side arm were tipped into the main compartment, and zero time readings recorded after a total of 10 minutes incubation. Oxygen uptake was estimated at 10-minute intervals for a total period of 30 minutes.

With alpha-ketoglutarate as substrate, the reaction medium also contained 0.5 μmoles of adenosinediphosphate (ADP) and CoA, and the preincubation period was 15 minutes.

The phosphate contents of the reaction media were estimated using essentially the procedures of Lowry and Lopez (1946), and color estimated using a Coleman Jr. spectrophotometer at 660 mμ. Phosphate uptake was determined by comparing the inorganic content of the reaction media with appropriate zero time samples prepared in 20 ml beakers.

The ATPase activity of freshly prepared mitochondria was investigated by estimating the release of inorganic phosphate from ATP in a reaction medium consisting of 0.05 ml of 0.1 M ATP, 0.02 or 0.05 ml of 0.1 M metal ion, all added as chloride salts, 0.2 ml of mitochondrial preparation, and Tris buffer pH 7.5, to give a total volume of 2 ml. The reaction media were incubated in 20 ml beakers for 15 minutes at 25° C in a Dubnoff metabolic incubator (1948). The enzyme reaction was stopped by transferring the contents of the reaction vessels into test tubes containing 1 ml of 5% trichloroacetic acid (TCA), and the phosphate content was estimated as indicated above.

The nitrogen content of all tissue preparations was estimated by direct Nesslerization of tissue digests (1955), and the color estimated at 425 mμ.

Oxygen data are expressed as Q_{O_2} values (μl O_2/hr/mg N), and phos-

phate data as μmoles of PO_4/hr/mg N, and all values have been corrected for endogenous activity. The data presented are average values from two or more experiments.

Results and Discussion

The effects of amytal and rotenone on oxygen and phosphate uptake are summarized in Table 1. Succinate oxidation was increased in the presence of amytal and rotenone, whereas the alpha-ketoglutarate oxidation was severely inhibited. As the concentration of amytal was reduced, the inhibition of alpha-ketoglutarate oxidation was reduced; and at an amytal concentration of 0.81×10^{-3} g per ml of reaction medium, no effect on either oxygen uptake or phosphate uptake was observed. Likewise, a reduction of the rotenone concentration also reduced the inhibition of the oxidation of alpha-ketoglutarate; but in contrast to amytal at a rotenone level which did not alter oxygen uptake, there was approximately a 20% reduction in phosphate. Therefore, rotenone appears to have an additional effect on phosphate uptake not exhibited by amytal under the conditions described.

Table 1.—Effect of Amytal and Rotenone on Oxygen and Phosphate Uptake in the Presence of Succinate and Alpha-ketoglutarate.

With Succinate

	g/ml	μliters O_2/hr/mg N		μmoles PO_4/hr/mg N	
Amytal	0.41×10^{-3} g	191	99	34	13
Rotenone	0.41×10^{-3} g	448	283	70	26

With Alpha-ketoglutarate

	g/ml	μliters O_2/hr/mg N		μmoles PO_4/hr/mg N	
Amytal	0.81×10^{-3} g	181	176	56	54
	0.41×10^{-3} g	0	176	4	54
Rotenone	0.66×10^{-3} g	102	201	38	48
	0.66×10^{-3} g	0	201	6	48

The total amount of phosphate in the reaction medium is the result of the total amount of inorganic phosphate incorporated altered by the quantity of inorganic phosphate which may be released from tissue organic phosphates, such as ATP, or by the inhibition of phosphate uptake. To determine if the net decrease in phosphate uptake might be due to the effect of rotenone on the hydrolysis of tissue phosphates, the effect of amytal and rotenone on the mitochondral ATPase was estimated. These results are summarized in Table 2. Neither amytal nor rotenone at the levels presented in Table 2 increased the hydrolysis of ATP; but higher concentrations of rotenone inhibited the hydrolysis of ATP. This would indicate that the effect of rotenone on phosphate uptake would be at some point or points during the conversion of inorganic phosphate into tissue organic phosphate. These data agree with the data presented by Chance and Hollunger (1963) and Ernster et al. (1963) whose data indicated that

TABLE 2.—Effect of Amytal and Rotenone on Mitochondria ATPase

metal ion	moles of metal added to reaction medium	Amytal 3.7×10^{-3} g/ml of reaction medium		Rotenone 3.9×10^{-5} g/ml of reaction medium	
		μmoles PO_4/hr/mg N		μmoles PO_4/hr/mg N	
Zinc	2	14.3	22.9	49.1	48.4
Cadmium	2	39.2	33.2	64.7	65.6
Manganese	2	47.7	41.6	35.1	36.0
Magnesium	2	42.0	45.2	18.9	20.3
Calcium	5	24.2	25.8	19.0	17.1

rotenone interfered at some point in the transfer of energy. Also, the point of inhibition or mechanism of action of rotenone has yet to be elucidated.

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