Some Effects of Tetrazolium Salt on the Metabolism of Mitochondria Isolated from Jerusalem Artichoke Tubers

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Summary. A series of tetrazolium salts were found to accept electrons more readily from succinate than malate even though the rate of oxygen uptake was similar with both substrates. This difference was explained by showing that all the tetrazolium salts tested caused a reduction in electron flow between NAD$^+$ and Cyt.b. The tetrazolium salts were also found to be able to uncouple phosphorylation from electron transport. The monotetrazolium salts causing complete uncoupling around 100 μmoles/litre and the ditetrazolium salts causing complete uncoupling around 20 μmoles/litre.

Introduction

While investigating the ability of tetrazolium salts to accept electrons during the oxidation of citric acid cycle intermediate by mitochondria isolated from tubers of Jerusalem artichoke (Helianthus tuberosus) it was found that several of the salts were not reduced with malate as the substrate. The same salts were rapidly reduced when succinate was supplied as the substrate. This observation led to a study of the inhibitory effects of tetrazolium salts on respiratory metabolism.

Tetrazolium salts are commonly used for the histochemical assay of dehydrogenase enzymes in biological tissue (SELIGMAN, 1963); however there have been several references to the toxic effect of tetrazolium in various biological systems (LETTRE and ALBRECHT, 1943; RUTENBURG, GOFSTEIN and SELIGMAN, 1950; PEARSE, SCARPPELLI and HESS, 1960) suggesting that the salts may inhibit electron transport. Little definitive evidence exists to indicate at what point tetrazolium salts inhibit respiratory metabolism. During the last year data has been presented to show that in rat liver mitochondria tetrazolium salts can uncouple electron transport from phosphorylation (CLARK, GREENBAUM and SLATER, 1965) and that triphenyl tetrazolium can inhibit electron transport between flavoprotein and cytochrome b (SATO and SATO, 1965).

It was therefore of interest to investigate whether the failure of malate to reduce some of the tetrazolium salts in plant mitochondria was due to an inhibition of electron transport and by using a compre-
hensive series of tetrazolium salts to see if there was any correlation between the standard redox potential or molecular structure of the tetrazolium salts and their inhibitory activity.

**Materials and Methods**

The tetrazolium salts\(^1\) (TTC, BT, NT, MTT, INT, NBT, TNBT) and ADP, ATP were obtained from Sigma Chemicals.

The Jerusalem artichoke tubers were supplied by the Biological Supply Unit of the University of London and were washed and then stored at 4°C before use.

**Isolation of the Mitochondria.** The method used was similar to that described by **Wedding and Black** (1962). 250 g of peeled Jerusalem artichokes were ground in 200 ml of a solution containing 0.6 M sucrose, 0.05 M tris and 0.005 M EDTA at pH 8.0. The homogenate was then passed through 4 layers of muslin and the resulting solution was centrifuged at 3000 × g for 10 minutes to remove nuclei and cell wall debris. The supernatant was re-centrifuged at 15,000 × g for 20 minutes and the pellet of mitochondria produced by this centrifuging was resuspended in 50 ml of a solution containing 0.6 M sucrose and 0.05 M tris at pH 7.0 and again centrifuged at 15,000 × g for 20 minutes; the mitochondria were finally suspended in 4.0 ml of solution containing 0.4 M sucrose and 0.05 M tris at pH 7.0 before use. All procedures were carried out at between 1 and 4°C. The nitrogen content of the mitochondria was measured using the method described by **Johnson** (1941).

**Measurement of the Rate of Respiration and Oxidative Phosphorylation.** A polarographic method, using a vibrating platinum electrode, covered with a collodion film (GME Oxygraph), was used to measure the rate of respiration in all the experiments other than those in which the P/O ratio was measured. The polarographic measurements were carried out at 30°C in 1.6 ml of medium containing 500 μmoles sucrose, 40 μmoles tris, 25 μmoles KH₂PO₄, 50 μmoles ADP, 50 μmoles ATP, 50 μmoles substrate and 150 μg of mitochondrial nitrogen; the final pH of the medium was 7.0.

When measuring the P/O ratio the rate of respiration was measured using conventional Warburg techniques (**Umber et al.**, 1964). The final volume of medium in the flask was 3.0 ml and contained the following, in μmoles: Sucrose 750, tris 200, cytochrome c 0.001, ADP 1.0, MgCl₂ 5.0, glucose 50, NaF 30, KH₂PO₄ 50 (containing 2--5 μCi P³²); succinate 60 together with hexokinase 50 KM units/flask. The mitochondria (containing 400 μg nitrogen) were placed in the side arm of the flask and allowed to equilibrate for 5 min. before the mitochondria were tipped into the main body of the flask. Readings of oxygen uptake were then taken for a period of 30 minutes at a temperature of 30°C.

Esterification of inorganic phosphate to glucose-6-phosphate was measured using the method of **Hayakawa and Lardy** (1960) on an aliquot of the reaction mixture at the termination of the experiment.

**Reduction of the Tetrazolium Salts.** The mitochondria (50 μg nitrogen) were incubated for 20 mins. at 30°C in 1.0 ml of medium at pH 7.0 containing the following, in μmoles: sucrose 250; tris 20; KH₂PO₄ 10; ADP 2.5; substrate 25;

\(^1\) Abbreviations. TTC, 2,3,5-triphenyl-2,1,3,4-tetrazolium chloride; BT, 5,5'-di-phenyl-3,3'-(3,3'-dimethoxy-4,4'diphenylene)-ditetrazolium chloride; NT, 2,2,5,5'-tetraphenyl-3,3'(p-diphenylene)-ditetrazolium chloride; MTT, 3-(4,5'dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide; INT, 2-(p-iodophenyl)-3-p-dinitrophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride; NBT, 2,2'-di-nitrophenyl-5,5'diphenyl-3,3'dimethoxy-4,4'diphenylene)-ditetrazolium chloride; TNBT, 2,2',5,5'tetra-p-nitrophenyl-3,3'dimethoxy-4,4'diphenylene) ditetrazolium chloride.