UNUSUAL EFFECTS OF A SUBSTITUTED THIOPHEN ON MITOCHONDRIAL SWELLING, CONTRACTION, AND OXIDATIVE PHOSPHORYLATION

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The cytotoxic immunosuppressive agent, 3-acetyl-5-(4-fluorobenzylidene)-2,5-dihydro-4-hydroxy-2oxothiophen, (ICI 47776 or ICI) inhibits succinate and NAD-linked oxidations by rat liver mitochondria but does not inhibit oxidation of ascorbate (Franklin et al., 1967 a,b). As reported herein this substituted thiophen is also a potent uncoupler of succinate and NAD-linked oxidations in concentrations less than those which cause respiratory inhibition. Simultaneous studies show that uncoupling concentrations of ICI cause contraction of mitochondria which subsequently swell as the ICI concentration is raised to levels which cause inhibition of respiration. These effects are noted with rat liver mitochondria and with rat skeletal muscle mitochondria even though previous investigators have failed to show swelling and contraction of rat skeletal muscle mitochondria under a variety of conditions. (Tata et al., 1963).

Rat skeletal muscle mitochondria were isolated by differential centrifugation after homogenization with glass beads in a CO_2-cooled mechanical shaker. The technique employed has been described in detail (Peter and Lee, 1967). Oxidative phosphorylation was studied at 26° in a Gilson oxygraph with simultaneous observation of swelling and contraction at 520 mμ, 26° in a Gilford recording spectrophotometer. Unless otherwise stated the oxygraph and spectrophotometric

studies utilized identical media containing substrate (15 mM pyruvate and 15 mM DL-malate or 45 mM succinate plus 0.02 mM rotenone), 25 mM TES, 30 mM P_i, 8.0 mM $MgSO_4$, 0.5 mM EDTA, 50 mM KCl, 0.17 bovine serum albumin/ml and about 0.5 mg mitochondrial protein/ml, pH 7.4. Other additions are noted in the figures. All reagents were highest purity commercially available. The substituted thiophen (ICI) was dissolved in dimethyl sulfoxide (DMSO) immediately before use. DMSO in concentrations less than 5% (volume/volume) had no effect on any of the parameters studied.

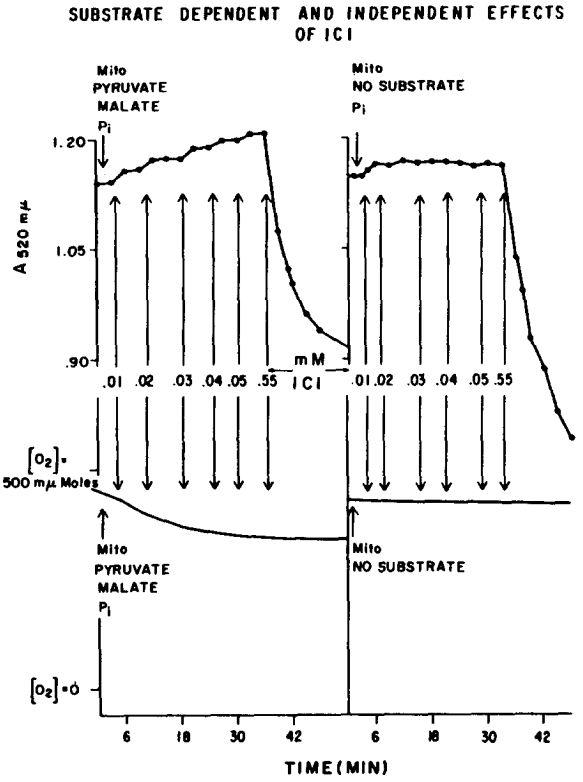

Figure 1.

Standard medium and conditions as described in the text were employed. Final volume in oxygraph was 2.0 ml and in spectro photometer was 3.0 ml. Protein concentration was 0.17 mg albumin/ml and 0.83 mg mitochondrial protein/ml.

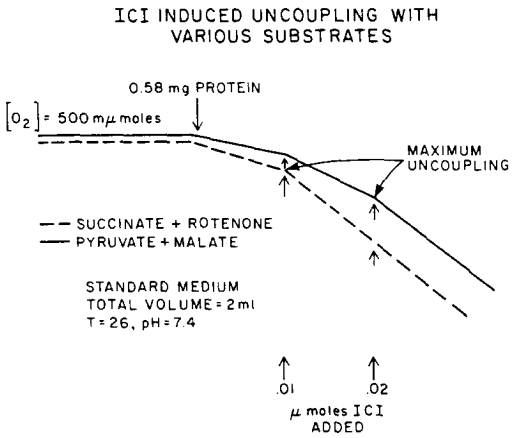

Figure 2.

Standard medium and conditions as described in text were employed.

ICI 47776 (ICI) in average concentration of 01 mM or .02 μmoles/mg total protein (mitochondrial protein plus added albumin) uncouples oxidative phosphorylation with pyruvate malate as substrate (Figure 1). At this concentration of ICI a substrate dependent contraction of the mitochondria is manifest by the increased absorbance at 520 mμ whereas concentrations approaching .045 μmoles/mg total protein inhibit respiration and cause substrate independent swelling of the mitochondria (Figure 1). Similar biphasic effects with increasing concentration of ICI are noted with succinate and rotenone present, but with this substrate uncoupling occurs with lower concentrations of ICI (Figure 2) The uncoupling of respiration and mitochondrial contraction induced by ICI is independent of the presence of added P_i, ADP and Mg^{++}. Low concentrations of ICI stimulate Mg^{++}-dependent ATPase of mitochondria (Franklin et al., 1967 a) but our data show that low concentrations of ICI release respiration in state 4 despite the presence of oligomycin (Figure 3). This indicates that the uncoupling is not an ATPase

effect and is not due to hydrolysis of any hypothetical high-energy phosphorylated compound.

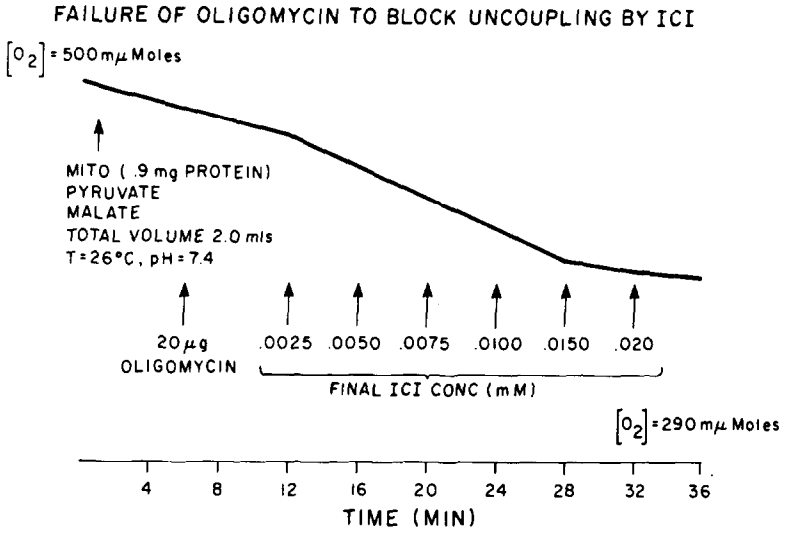

Figure 3.

Standard medium and conditions as described in text were employed.

Octylguanidine (0.2 mM) inhibits mitochondrial contraction (Figure 4) and uncoupling induced by low concentrations of ICI with pyruvate-malate. Although octylguanidine also completely inhibits mitochondrial contraction with succinate plus rotenone as substrate it only partially inhibits the ICI-induced uncoupling. This is compatible with the idea that the octylguanidine is not as effective in inhibiting succinate respiration as it is in inhibiting DPN-linked respiration (Pressman 1963). 3.0 mM phenethyl biguanidine (DBI) (Pressman, 1963) inhibits ICI-induced contraction in the presence of succinate plus rotenone or pyruvate plus malate; whereas, 0.2 mM DBI has no effect (Figures 5,6). These observations show that octylguanidine and DBI block the interaction of ICI with

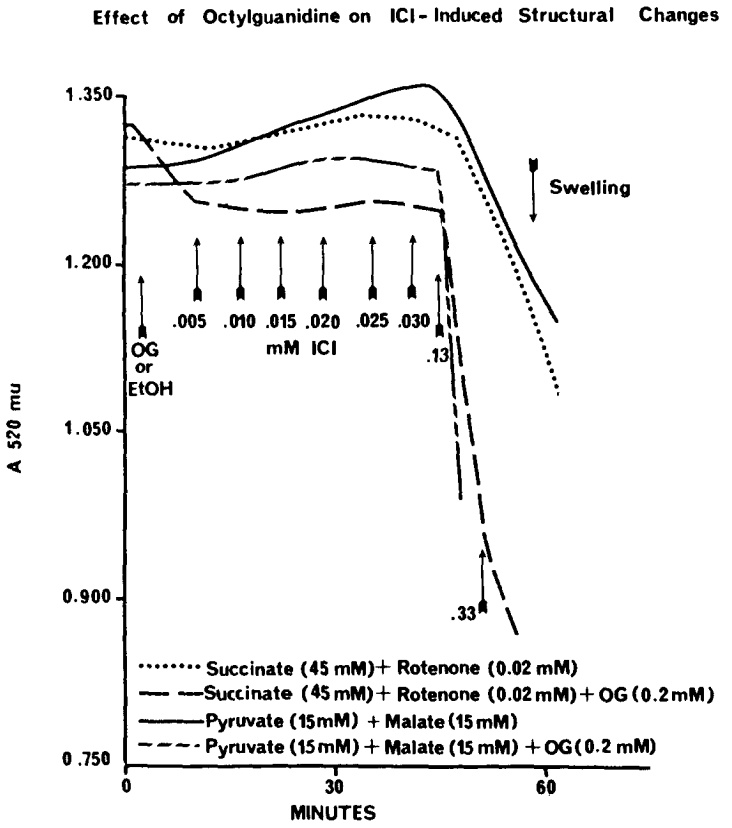

Figure 4.

Standard medium and conditions as described in the text were employed. Final volume in spectrophotometer was 3.0 mls. Protein concentration was .17 mg albumin/ml and 0.58 mg mitochondrial protein —— ml.

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the energy transfer system which causes contraction. These data do not conclusively localize the site of interaction of ICI but show that at least part of the energy required for contraction comes from site 2. DBI (3.0 mM) does not completely block the ICI-induced uncoupling even though it completely blocks ICI-induced contraction under identical conditions. The difference in susceptibility of ICI-induced uncoupling and of ICI-induced contraction to inhibition by DBI presumably reflects

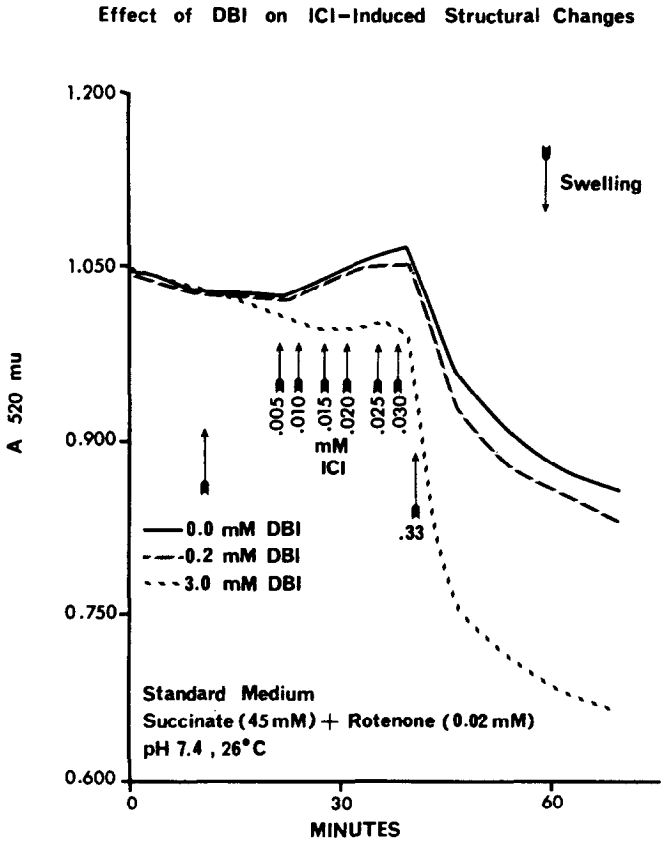

Figure 5.

Standard medium and conditions as described in the text were employed. Final volume in spectrophotometer was 3.0 ml. Protein concentration was 0.17 mg/ml albumin and .55 mg/ml mitochondrial protein.

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the action of DBI as an inhibitor of energy transfer as opposed to electron transport per se.

The small amplitude contraction of mitochondria seen with low concentrations of ICI is substrate-dependent and to a less extent Mg^{++}-dependent, occurs simultaneously with uncoupling of oxidative phosphorylation, is inhibited by octylguanidine or DBI and does not

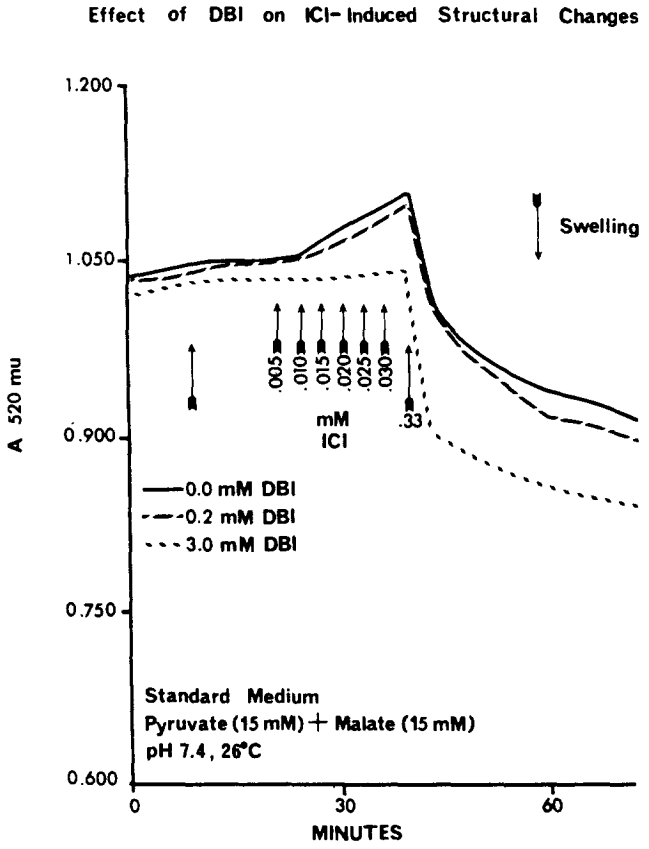

Figure 6.

Standard medium and conditions as described in the text were employed. Final volume in spectrophotometer was 3.0 ml. Protein concentration was 0.17 mg/ml albumin and .55 mg/ml mitochondrial protein.

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depend on substrate level phosphorylation (occurs with succinate and rotenone). This contraction resembles that described as characteristic of a high energy state of mitochondria (Azzone and Azzi, 1965), but actual volume change or extrusion of water from mitochondria has not been demonstrated comcomitant with small amplitude contraction (Lehninger, 1962; Chappel and Greville, 1963; Azzone and Azzi, 1965).

Therefore such contraction need not be analogous to the large amplitude contraction seen after ATP addition to swollen mitochondria.

Mitochondrial swelling occurring at high ICI concentration is associated with inhibition of respiration by ICI and is blocked but not reversed by 4 mM ATP even in the presence of oligomycin. The swelling is not influenced by 4 mM ITP or GTP, occurs at lower concentrations of ICI when both Mg^{++} and substrate are omitted from the medium, occurs in the absence of substrate and P_i, and is not blocked by oligomycin, octylguanidine, DBI or Na_2S. These characteristics are dissimilar from those of large-amplitude, P_i-induced swelling (Chappell and Greville, 1963). Current evidence suggests that high concentrations of ICI induce irreversible swelling of mitochondria and that this swelling is facilitated by a low energy state of the mitochondria.

Studies are in progress to characterize further the apparently opposing actions of different concentrations of ICI on structural changes in mitochondria. The titration approach illustrated in this study should provide a good basis for comparison of the effects of increasing concentrations of ICI and other compounds on swelling, contraction and oxidative phosphorylation and may provide an explanation for discrepancies noted in previous studies of uncouplers on structural states of mitochondria (Lehninger, 1962).

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