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EFFECTS OF 2,4,3′,5′-TETRAHYDROXYSTILBENE ON OXIDATIVE PHOSPHORYLATION BY RAT LIVER MITOCHONDRIA*

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Abstract—The effects of an antifungal compound 2,4,3′,5′-tetrahydroxystilbene (THS)† on oxidative phosphorylation by rat liver mitochondria were studied. THS at low concentrations was found to inhibit state 3 respiration (I_{50} = 44 nmoles/mg protein) but had no effect on uncoupled respiration induced by 2,4-dinitrophenol. The inhibitory effect of THS on state 3 respiration was observed with either glutamate or succinate as respiratory substrates. However when rat liver mitochondria were respiring with glutamate as substrate and in the presence of calcium chloride and inorganic phosphate, THS produced a strong inhibition of 2,4-dinitrophenol-stimulated respiration. This inhibition was overcome by adding succinate. The tetramethoxy derivative of THS had no effect on the mitochondrial reactions mentioned above suggesting that the phenolic groups are essential for the action of THS. The sites at which THS inhibits mitochondrial oxidative phosphorylation are proposed.

2,4,3′,5′-Tetrahydroxystilbene† is a phenolic compound isolated from the aqueous extract of *Artocarpus lakoocha Roxb.* heartwood [1]. The substance was first isolated by Takaoka [2] from the root of the white hellebore (*Veratrum grandiflorum*) and since then it has been isolated from Osage orange (*Toxylon pomiferum*) [3], *Morus bambycis* [4], *Morus alba* [5], and *Chlorophora regia* [6]. THS and other hydroxystilbene derivatives from these plants have attracted attention because of their fungicidal and fungistatic properties [7], their effect on pulping under acid conditions [8], and the cause of coloration in pulps [9]. Barnes and Gerber [3] showed that THS is responsible for the antifungal action of the aqueous extract of Osage orange wood and that it inhibits the growth of five out of thirteen fungi tested. It has been suggested that the remarkable resistance of certain woods, for example, Osage orange and *Artocarpus lakoocha Roxb.*, is due to the toxicity of THS on the fungi which normally initiate the decomposition process. Moreover, this hydroxystilbene obtained from the aqueous extract of *Artocarpus lakoocha Roxb.* has been widely used in Thailand as an anthelmintic and is believed to be effective against tapeworm. The biochemical action of THS as well as the mechanism by which THS exerts its antifungal action have never been reported. In the present study, the effects of THS on mitochondria oxidative phosphorylation were investigated.

MATERIALS AND METHODS

Chemicals. *N*-Tris(hydroxymethyl) methyl-2 aminoethane sulfonic acid (TES), sucrose and L-glutamic acid were obtained from Sigma Chemical Company, Missouri. ADP was obtained from Calbiochem, California. Folin phenol reagent and succinic acid were obtained from British Drug Houses. THS was purified as described by Mongolsuk *et al.* [1] and its tetramethoxy derivative was prepared according to Barnes and Gerber [3].

Reagents were dissolved in double distilled water and if necessary the solutions were adjusted to pH 7.4 with dilute potassium hydroxide or hydrochloric acid. THS and the tetramethoxy derivative were dissolved in absolute ethanol. In the experiments in which alcoholic solutions of reagents were used, only small volumes (10–25 μl) were added to the reaction mixtures and equivalent amounts of pure alcohol were added to controls.

Preparation of rat liver mitochondria. Albino rats weighing 150–200 g were used. The liver mitochondria were prepared by the method of Hogeboom [10] as described by Myers and Slater [11]. Ice-cold 0.25 M sucrose was used throughout the procedure and all centrifugations were carried out in a Beckman model J-21B refrigerated centrifuge at 0°.

The concentration of protein in the mitochondrial preparation was determined with the folin phenol reagent according to Lowry *et al.* [12] as modified by Miller [13].

Measurement of oxygen uptake. The measurements of oxygen uptake were carried out at 26° in the chamber (about 2 ml) of a Gilson Oxygraph (Gilson Medical Electronics, Inc., Middleton, Wisconsin) with a Clark oxygen electrode. The rates of oxygen uptake under various conditions were expressed as μatoms O/ml/min.

The composition of the reaction mixture in the oxygraph chamber varied from experiment to experiment. However, in all cases the standard incubation medium (40 mM TES buffer, pH 7.4, 10 mM $MgCl_2$ and 80 mM KCl) was used. To this medium various cofactors were added.

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† Abbreviations used: THS, 2,4,3′,5-tetrahydroxystilbene; MeO-THS, 2,4,3′,5′-tetramethoxystilbene; DNP, 2,4 dinitrophenol; TES, *N*-Tris (hydroxymethyl) methyl-2 aminoethane sulfonic acid.

RESULTS

The effects of THS and MeO-THS on oxidative phosphorylation. The effects of THS and MeO-THS on oxidative phosphorylation by rat liver mitochondria are shown in Fig. 1. In these experiments glutamate was present as respiratory substrate. Curve A shows the control response of rat liver mitochondria to the addition of ADP + Pi and DNP. When mitochondria were pretreated with 0.28 mM THS for about 3 min the respiratory response of rat liver mitochondria at the addition of ADP + Pi was severely depressed whereas DNP-stimulated respiration was much less inhibited (curve B). Curve C shows that the tetramethoxy derivative of THS (MeO-THS) had almost no effect on both the ADP + Pi and DNP-stimulated respirations. Similar results were obtained when rat liver mitochondria were respiring in the presence of succinate as substrate. In other experiments it was observed that the inhibitory effect of THS on mitochondrial oxidative phosphorylation had a rapid onset of action since addition of THS shortly after the addition of excess ADP + Pi caused almost immediate inhibition of ADP + Pi induced increase in respiratory rate and the degree of inhibition did not increase with time. It was also observed that two times washing of rat liver mitochondria which were pretreated with THS did not restore the respiratory response of rat liver mitochondria to the addition of ADP + Pi. This suggests that THS binds strongly or produced irreversible damage to the mitochondrial inner membrane.

Figure 2 shows the degree of inhibition of oxidative phosphorylation and DNP-stimulated respiration at various concentrations of THS. Significant inhibition of the respiratory response of rat liver mitochondria to ADP was observed at concentrations of THS as low as 0.01 mM whereas DNP-stimulated respiration remained unaffected up to 0.2 mM THS. At about 0.55 mM THS, 90 per cent of ADP-stimulated respiration was inhibited while the uncoupling effect of DNP was only 20 per cent depressed. The I_{50} of THS on oxidative phosphorylation was found to be 44 nmoles/mg protein. It is clear from these experiments that oxidative phosphorylation was more sensitive

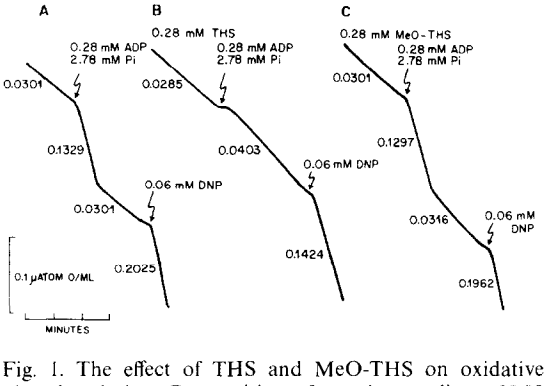

Fig. 1. The effect of THS and MeO-THS on oxidative phosphorylation. Composition of reaction medium: 28.89 mM TES (pH 7.4), 7.22 mM $MgCl_2$, 16.67 mM potassium glutamate, 27.78 mM sucrose and KCl to 250 mOsM; THS, MeO-THS, ADP, Pi and DNP as indicated. The figures denote the rate of oxygen uptake in μatom O/ml/min. Mitochondrial protein was 2.6 mg/ml.

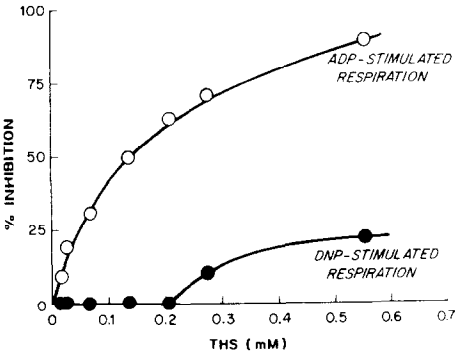

Fig. 2. Inhibition of ADP + Pi- and DNP-stimulated respirations by various concentrations of THS. Composition of reaction medium: 28.89 mM TES (pH 7.4), 7.22 mM $MgCl_2$, 16.67 mM potassium glutamate, 27.78 mM sucrose, and KCl to 250 mOsM. Various concentrations of THS were added as indicated; 0.28 mM ADP and 2.78 mM Pi were added after 4 min of preincubation with THS. DNP (0.06 mM) was added 3 min after the additions of ADP and Pi. Mitochondrial protein was 3.17 mg/ml.

than the DNP-induced uncoupling to the inhibitory action of THS.

The effect of THS on calcium-stimulated respiration. The effect of THS on calcium-stimulated respiration by rat liver mitochondria is shown in Fig. 3. Like the preceding experiments, glutamate was used as substrate and inorganic phosphate was present initially in excess. Curve A shows the control response of the mitochondria to two additions of 0.2 mM $CaCl_2$. In the presence of 0.28 mM THS the respiratory response of the mitochondria to the first addition of $CaCl_2$ was significantly depressed as evidenced by a slower rate of respiration; however, the mitochondrial response to the second $CaCl_2$ addition was completely abolished (curve B). A somewhat more striking result was obtained with higher amounts of $CaCl_2$. As shown in curve D, THS at the same concentration caused more inhibition of the initial respiratory response to $CaCl_2$ when higher amounts of $CaCl_2$ was added to the mitochondria. This initial, brief calcium-stimulated respiration was followed by inhibition as compared to control experiment without THS (curve C). The finding that there was some inhibition of the

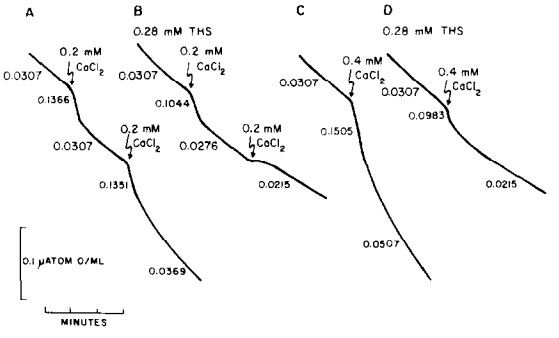

Fig. 3. The effect of THS on calcium-stimulated respiration. Composition of reaction medium: 33.33 mM TES, 8.33 mM $MgCl_2$, 15.79 mM potassium glutamate, 13.89 mM sucrose, 2.63 mM P_i and KCl to 250 mOsM; THS and $CaCl_2$ as indicated. Mitochondrial protein was 1.28 mg/ml.

initial mitochondrial response to $CaCl_2$ is consistent with the earlier observation that THS at relatively high concentrations also depressed DNP-stimulated respiration.

The above results suggest that when mitochondria have accumulated some $CaCl_2$, THS interferes with the process of energy conservation associated with the respiratory chain so that further respiratory stimulation by $CaCl_2$ is inhibited. This hypothesis is supported by results of the experiments reported in Fig. 4. Curve A shows the control response of rat liver mitochondria to $CaCl_2$ and DNP. With curve B, it can be seen that THS completely inhibited the DNP-stimulated respiration when DNP was added after the respiratory response of mitochondria to $CaCl_2$ had stopped. This inhibition, observed with glutamate as substrate, was relieved by adding succinate. Identical results were obtained when THS was added after $CaCl_2$ (curve C). It is interesting to note that when THS was added after $CaCl_2$ there was a small and brief stimulation of mitochondria respiration (curve C). The tetramethoxy derivative of THS at the same concentration as THS was found to be ineffective. Similar results were also obtained when malate plus pyruvate were used as respiratory substrates.

In order to determine the role of $CaCl_2$ and Pi in the inhibitory effect of THS on mitochondrial response to DNP, similar experiments in the presence and absence of Pi were carried out. The results are shown in Fig. 5. It can be seen that only a small inhibition of DNP-stimulated respiration was observed when $CaCl_2$ was added, in the absence of Pi, to the mitochondria respiring in the presence of glutamate and THS (curve B), as compared to the control (curve A). When Pi was present initially together with THS, DNP-stimulated respiration was totally inhibited when DNP was added after $CaCl_2$ (curve C). Again, this inhibition was completely reversed by the addition of succinate. It was also observed in other experiments that, in the absence of $CaCl_2$, the small inhibition of DNP-stimulated respiration produced by THS was not altered by the presence of excess Pi. These findings indicated that rat liver mitochondria had to accumulate both $CaCl_2$ and Pi before THS was able to block the respiratory response to DNP. The results of the experiments reported in Fig. 6 showed that, with glutamate as substrate and in the presence of excess Pi, rat liver mitochondria had to accumulate a certain amount of calcium ion before the DNP-stimulated respiration was further depressed by THS. It should be pointed out that a small inhibition of DNP-stimulated respiration by THS was also found in the absence or in the presence of a small amount of $CaCl_2$. This is in agreement with the earlier observations that THS at relatively high concentrations, besides strongly inhibiting oxidative phosphorylation, also caused some depression of the respiratory response of rat liver mitochondria to DNP.

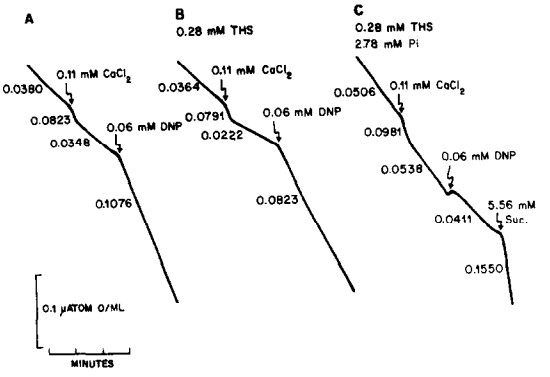

Fig. 5. The effect of THS on DNP-stimulated respiration after the addition of calcium chloride in the presence or absence of inorganic phosphate. Composition of reaction medium: 30 mM TES, 7.5 mM $MgCl_2$, 16.67 mM potassium glutamate, 20.83 mM sucrose and KCl to 250 mOsM; THS, $CaCl_2$, Pi, DNP and succinate as indicated. Mitochondrial protein was 2.21 mg/ml.

DISCUSSION

The results of these experiments indicate that THS has profound effects on energy-linked reactions of rat liver mitochondria. The finding that THS at low concentrations selectively inhibited the respiratory response of mitochondria to ADP (+Pi) but had no effect on DNP-stimulated respiration indicates that

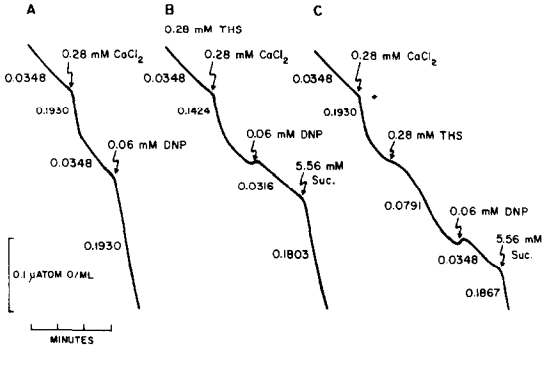

Fig. 4. The effect of THS on DNP-stimulated respiration after the addition of calcium chloride and inorganic phosphate. Composition of reaction medium: 30 mM TES, 7.5 mM $MgCl_2$, 16.67 mM potassium glutamate, 2.78 mM Pi, 20.83 mM sucrose and KCl to 250 mOsM; $CaCl_2$, THS, DNP and succinate as indicated. Mitochondrial protein was 2.49 mg/ml.

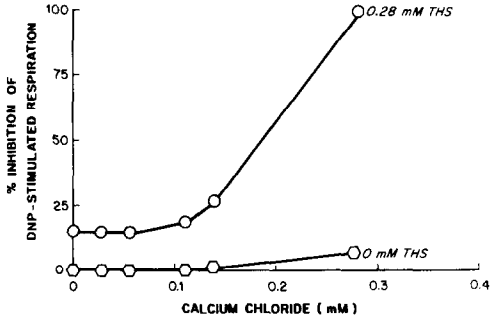

Fig. 6. The effect of THS on DNP-stimulated respiration at different calcium chloride concentrations. Composition of reaction medium: 31.11 mM TES (pH 7.4), 7.78 mM $MgCl_2$, 16.67 mM potassium glutamate, 2.78 mM potassium phosphate, 12.89 mM sucrose and KCl to 250 mOsM; THS and $CaCl_2$ as indicated. DNP (0.06 mM) was added 2 min after the addition of $CaCl_2$. Mitochondrial protein was 1.81 mg/ml.

THS acts beyond the site of action of DNP, presumably at the phosphorylation process itself. However, the exact point and mechanism of action are not known at present. Since the tetramethoxy derivative of THS was found to be inactive, it appears that phenolic groups are essential for the action of THS.

Of particular interest is the observation with rat liver mitochondria respiring in the presence of NAD-linked substrates accumulate certain amounts of calcium and inorganic phosphate. Under these conditions, THS produced a strong inhibitory effect on the respiratory response of mitochondria to DNP. It is known that when rat liver mitochondria accumulate calcium in the absence of phosphate or similar anions, calcium becomes bound to the inner mitochondrial membrane. Only when matching permeant anions are present does the accumulated calcium appear in the matrix, together with the anions [14]. This suggests that THS produces an inhibitory effect only when calcium and Pi are in the mitochondrial matrix. Again, the tetramethoxy derivative of THS was also found to be inactive in this respect. Since addition of succinate completely relieved the inhibition of DNP-stimulated respiration induced by THS in the presence of calcium and inorganic phosphate, it is likely that the site of action of THS involves the energy-conservation reactions associated with the oxidation of reduced nicotinamide adenine nucleotides by the mitochondrial respiratory chain.

From these studies, the concentrations of THS for complete inhibitions of respiration stimulated by ADP and by DNP are 0.6 mM (Fig. 2) and 0.28 mM (at 0.3 mM $CaCl_2$, Fig. 6), respectively. These concentrations of THS are comparable with that required for antifungal activity (0.815 mM) as reported by Barnes and Gerber [3]. It is not known at present whether inhibition of mitochondrial oxidative phosphorylation by THS is responsible for its antifungal action.

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