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DIFFERENCE IN 2-THENOYLTRIFLUOROACETONE SENSITIVITY OF ELECTRON TRANSPORT WITH AND AGAINST THE REDOX POTENTIAL GRADIENT

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SUMMARY

The effect of 2-thenoyltrifluoroacetone on electron transport with and against the redox potential gradient, with succinate or ascorbate *plus* *N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine (TMPD) as electron donor, was studied in rat liver mitochondria. It was found that 2-thenoyltrifluoroacetone inhibited succinate-linked intramitochondrial pyridine nucleotide reduction at low concentrations, which neither affected succinate oxidation in the controlled state nor interfered with intramitochondrial pyridine nucleotide reduction in the ascorbate *plus* TMPD case. The effect of 2-thenoyltrifluoroacetone on succinate-linked intramitochondrial pyridine nucleotide reduction is not attributable either to blocking of the overall rate of electron flow in the succinate dehydrogenase branch of the respiratory chain or to interference with energy transformation. Transition from the controlled to the active state enhanced the inhibitory effect of 2-thenoyltrifluoroacetone on succinate-linked respiration, and it became as sensitive to 2-thenoyltrifluoroacetone as the succinate-linked intramitochondrial pyridine nucleotide reduction. In the light of the above findings, the possibility is discussed that electrons from succinate enter the main branch of the respiratory chain by different routes, according to whether the flow is with or against the potential gradient.

INTRODUCTION

2-Thenoyltrifluoroacetone, a powerful iron-chelating agent[1], is known as a specific inhibitor of succinate oxidation in intact mitochondria[2], submitochondrial preparations[3] and soluble succinate dehydrogenase[4]. Apart from this inhibitory capacity it acts as an uncoupling agent with regard to various mitochondrial functions associated with energy conservation[5,6]. While its inhibitory effect on succinate oxidation is generally attributed to interaction with nonheme iron[3,4], it is not clear whether a similar interaction is also responsible for its uncoupling activity[5,6]. LÖW AND VALLIN[7] described inhibition of succinate-dependent reduction of NAD^+ by 2-thenoyltrifluoroacetone. Since the succinate-linked reversal of electron transport

Abbreviations: $NAD(P)^+$, intramitochondrial pyridine nucleotide; TMPD, *N*, *N*, *N'*, *N'*-tetramethyl-*p*-phenylenediamine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

is energy dependent, its suppression by 2-thenoyltrifluoroacetone, as reported by the above authors, could have been the result of uncoupling and/or inhibition of the succinate dehydrogenase branch of the respiratory chain.

This paper describes and discusses experiments on the response of the electron transport with and against the potential gradient to 2-thenoyltrifluoroacetone. It is shown that when succinate is the substrate, the reversed electron transport is much more sensitive to 2-thenoyltrifluoroacetone than that descending with the potential gradient. This difference is not attributable either to depression of the overall rate of electron migration in the succinate branch of the respiratory chain or to interference with energy transformation.

MATERIALS AND METHODS

Materials

2-Thenoyltrifluoroacetone was obtained from Aldrich Chemical Co. (Milwaukee, Wisc., U.S.A.); carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was the generous gift of Dr. P. G. Heytler (E. I. Du Pont de Nemours and Co., Wilmington, Del., U.S.A.).

Rat liver mitochondria

Mitochondria were prepared according to ERNSTER AND LÖW[8], the pellet being resuspended in 0.25 M sucrose, to give 40–50 mg mitochondrial protein per ml.

Respiration and NAD(P)$^+$ reduction

Mitochondrial respiration was measured in a Gilson oxygraph (Model K) equipped with an oscillating platinum cathode. NAD(P)$^+$ reduction was assayed fluorimetrically in an Eppendorf photometer equipped with a fluorimetric attachment and coupled to a Varian graphic recorder (Model G-14A-1). An Hg 313–360-mμ filter was used for the excitation light and a 420–3000-mμ filter for the emitted light.

The detailed composition of the reaction mixture used is indicated in the legend to the respective figures. Total volume: 2.0 ml in the respiratory assays and 3.0 ml in fluorimetric measurements. Temperature 25°. The mitochondria were preincubated for a few minutes to obtain partial reoxidation of NAD(P)$^+$ before the reaction was initiated by injecting the substrate into the mixture. In the course of this investigation, it was found that the most consistent results were obtained when inorganic phosphate and $MgCl_2$ were included in the basic mixture. The initial rate of increase in fluorescence following the injection of the electron donor was taken as a measure for the effectiveness of the energy-dependent NAD(P)$^+$ reduction. The initial rate and the maximum extent of NADP()$^+$ reduction were found to vary in a parallel manner under the influence of the various factors studied.

Pretreatment of mitochondria in experiments designed for the assessment of the reversibility of the action of 2-thenoyltrifluoroacetone

A sucrose homogenate of rat liver was prepared and the debris and nuclei were removed as described by ERNSTER AND LÖW[8]. Before separation of the mitochondrial fraction by centrifugation (8000 × *g*; 20 min), 2-thenoyltrifluoroacetone (0.5 mM) dissolved in ethanol (final concentration of ethanol 1%, v/v) was added

to one part of the supernatant, while to another part of the supernatant 1 % (v/v) ethanol was added. The mitochondrial fraction was washed by centrifugation (8000 × *g*; 20 min) with either 0.25 M sucrose or 0.25 M sucrose containing 0.50 mM EDTA. The washed mitochondrial pellets were resuspended in 0.25 M sucrose.

RESULTS

Effect of 2-thenoyltrifluoroacetone on the rate of succinate oxidation and on NAD(P)$^+$ reduction coupled with the oxidation

The 2-thenoyltrifluoroacetone sensitivities of succinate oxidation (State 4) and of the NAD(P)$^+$ reduction coupled with the oxidation were compared. From the results shown in Fig. 1, it can be seen that low concentrations of 2-thenoyltrifluoroacetone, which slightly stimulated succinate oxidation, suppressed NAD(P)$^+$ reduction. Significant inhibition of the respiration became apparent only when the concentration exceeded that required for almost complete inhibition of the reduction of NAD(P)$^+$.

The 2-thenoyltrifluoroacetone titration in Fig. 1 was carried out under State 4 conditions which are optimal for assay of reversed electron transport. It should be kept in mind, however, that under the above conditions the extent of inhibition of respiration does not reflect the 2-thenoyltrifluoroacetone sensitivity of succinate dehydrogenase, since the activity of the latter is not the rate-limiting step in electron transport[9]. One should also be aware of the possibility that under State 4 conditions inhibition of succinate oxidation by 2-thenoyltrifluoroacetone could be masked by the contribution to the overall rate of O_2 uptake made by endogenous substrates, insensitive to this inhibitor. Thus, in order to assess the true 2-thenoyltrifluoroacetone sensitivity of the dehydrogenase moiety, the titration was repeated under State 3 conditions (see Fig. 2), in which the dehydrogenase activity is known to limit the overall rate of electron flow[9]. Release of the respiratory control was effected either by ADP or by 2,4-dinitrophenol, yielding very similar titration curves. Comparison of the curves in Fig. 2 with those in Fig. 1 reveals that State 3 oxidation was several times more sensitive to 2-thenoyltrifluoroacetone than its State 4 counterpart. The low concentrations of 2-thenoyltrifluoroacetone which inhibited succinate oxidation (State 3) inhibited NAD(P)$^+$ reduction (measured under conditions of State 4) to a similar extent. At higher 2-thenoyltrifluoroacetone concentrations, however, the State 3 respiration was much more insensitive to inhibition than NAD(P)$^+$ reduction.

Effect of 2-thenoyltrifluoroacetone on respriation and NAD(P)$^+$ reduction with ascorbate plus TMPD as electron donor

WARSHAW *et al.*[6] reported that 2-thenoyltrifluoroacetone can act not only as an inhibitor of succinate oxidase but also as an uncoupling agent and as such inhibits ATP-dependent reduction of NAD$^+$ by ascorbate *plus* TMPD in submitochondrial particles. Fig. 3 shows the effect of 2-thenoyltrifluoroacetone on ascorbate *plus* TMPD-linked functions in rat liver mitochondria. It is seen to suppress NAD(P)$^+$ reduction supported either by respiration or by ATP and to stimulate the rate of O_2 uptake under State 4 conditions. The above pattern of action is consistent with that expected for an uncoupling agent. Comparison of Fig. 3 with Fig. 1 shows that 2-thenoyltrifluoroacetone inhibited the succinate-linked reduction at a considerably

lower concentration than the ascorbate *plus* TMPD-linked one. An uncoupling agent would be expected to suppress ascorbate *plus* TMPD and succinate-linked NAD(P)$^+$ reduction at similar concentrations. This latter anticipation was verified in the case of 2,4-dinitrophenol and CCCP (Fig. 4). In some mitochondrial preparations of loose respiratory control, the ascorbate *plus* TMPD-linked variant proved to be the more sensitive function to uncoupling agents. Thus, the uncoupling activity observed with higher concentrations of 2-thenoyltrifluoroacetone cannot account for the inhibition of succinate-linked NAD(P)$^+$ reduction.

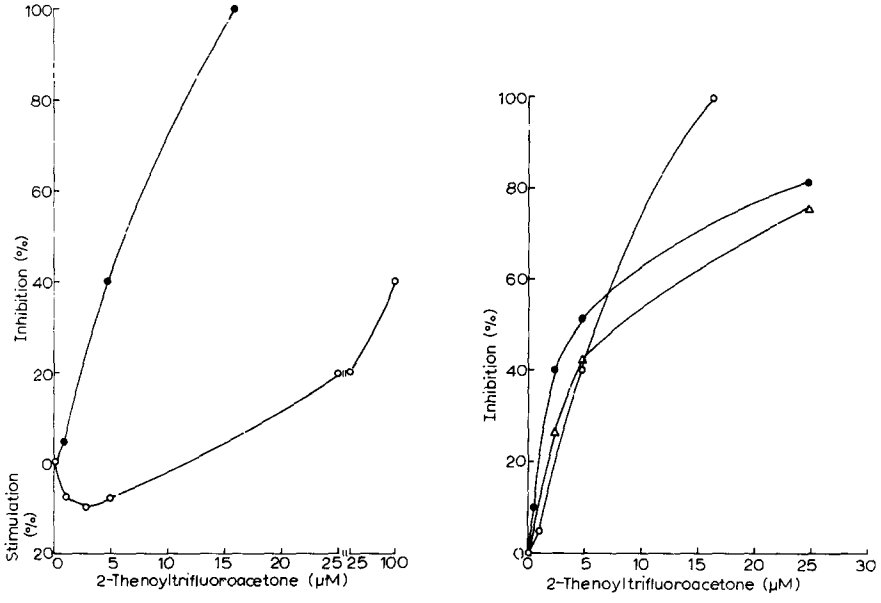

Fig. 1. Effect of 2-thenoyltrifluoroacetone on the rate of succinate ocidation in State 4 and on NAD(P)$^+$ reduction coupled to the oxidation. The same reaction mixture was used for measuring respiration and NAD(P)$^+$ reduction. It consisted of 20 mM Tris–HCl buffer (pH 7.4), 50 mM KCl, 0.33 mM EDTA, 8 mM $MgCl_2$, 3.3 mM potassium phosphate buffer (pH 7.4) and 2 mg protein/ml of rat liver mitochondria. 2-Thenoyltrifluoroacetone was added at the concentration indicated. The reaction was initiated by injecting 3.3 mM succinate into the assay medium. The rate of O_2 uptake (corrected for the endogenous rate) in the absence of 2-thenoyltrifluoroacetone was 5–7 nmoles/min per mg protein. The maximum rate of change in fluorescence in the absence of 2-thenoyltrifluoroacetone was 80–100 units/min/mg protein (unit = fluorescence of 0.1 μM aqueous NADH solution under the conditions of measurement). ○, respiration; ●, NADP(+) reduction.

Fig. 2. Effect of 2-thenoyltrifluoroacetone on the rate of succinate oxidation in State 3 compared with its effect on NAD(P)$^+$ reduction. Conditions as in Fig. 1. ○, NAD(P)$^+$ reduction; ●, respiration in the presence of 0.4 mM ADP; △, respiration in the presence of 5.0 μM 2,4-dinitrophenol. Rate of respiration in the presence of ADP or 2,4-dinitrophenol and in the absence of any inhibitor, 40–60 nmoles O_2 per min/mg protein.

Succinate oxidation and energy-linked NAD(P)$^+$ reduction under conditions by which the flow of electrons from succinate into the respiratory chain is restricted

In the experiment presented in Table I the rate of the flow of electrons from succinate into the respiratory chain was restricted either by limiting the succinate concentration or by adding malonate. It can be seen from the results shown in Table I that, unlike the case of 2-thenoyltrifluoroacetone (Fig. 1), the rate of respiration

was somewhat more impaired than that of the reversed electron transport. It is also apparent from Table I that a low concentration of malonate which slightly inhibited the rate of succinate oxidation stimulated the rate of reversed electron transport. The reason for the latter effect is not clear. One possibility is that the stimulation is due to the more oxidized state of the endogenous NAD(P)$^+$ when malonate is present before the addition of the electron donor. Only when malonate is present in low concentration, its inhibition of succinate-dependent NAD(P)$^+$ reduction does not mask the increased reduction rate.

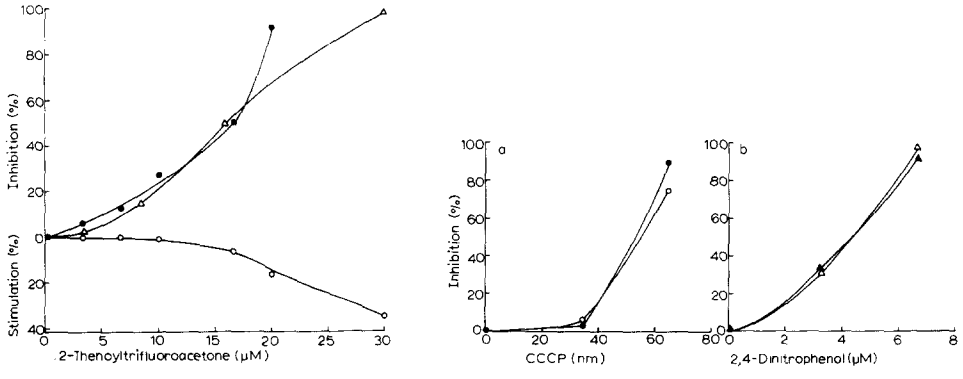

Fig. 3. Effect of 2-thenoyltrifluoroacetone on ascorbate *plus* TMPD-linked reactions. Conditions as in Fig. 1. 2.5 mM ascorbate *plus* 0.25 mM TMPD were injected into the incubation mixture to initiate the reaction. ○, respiration in State 4; ●, NAD(P)$^+$ reduction coupled to respiration; △, NAD(P)$^+$ reduction in the presence of 30 μM ATP and 2 mM KCN.

Fig. 4. Inhibition of NAD(P)$^+$ reduction by uncouplers. a. CCCP dissolved in ethanol added at the concentration indicated. ○, substrate: 3.3 mM succinate; ●, substrate: 2.5 mM ascorbate *plus* 0.25 mM TMPD. b. 2,4-Dinitrophenol added at the concentration indicated. △, substrate: 3.3 mM succinate; ▲, substrate: 2.5 mM ascorbate *plus* 0.25 mM TMPD. Other conditions as in Fig. 1.

TABLE I

ENERGY-DEPENDENT REDUCTION OF NAD(P)$^+$ UNDER CONDITION OF RESTRICTED ELECTRON FLOW FROM SUCCINATE INTO THE RESPIRATORY CHAIN

For experimental details and the units in which the rate of NAD(P)$^+$ reduction is expressed, see legend to Fig. 1.

Expt. No.	*[Succinate] (mM)*	*[Malonate] (mM)*	*O_2 uptake (nmoles/min per mg protein)*	*Rate of NAD(P)$^+$ reduction (units/mg protein)*
1	0	0	4.4	0
	0.17	0	5.5	98
	3.0	0	9.5	92
2	3.0	0	8.0	98
	3.0	0.5	7.1	130
	3.0	2.0	5.0	32
	3.0	3.0	4.0	0

Reversibility of 2-thenoyltrifluoroacetone-induced inhibition of mitochondrial functions

BOYER *et al.*[5] reported that, in submitochondrial particles, 2-thenoyltrifluoroacetone-induced inhibition of respiration and of [^{32}P]ATP-exchange is readily

TABLE II

REVERSIBILITY OF THE EFFECT OF 2-THENOYLTRIFLUOROACETONE ON RESPIRATION AND ON $NAD(P)^+$ REDUCTION

Procedure used for preincubation and washing as described under MATERIALS AND METHODS; see legend to Fig. 1 for experimental details and definition of units in which the rate of $NAD(P)^+$ reduction is expressed.

Preincubation of mitochondria	*Washing of mitochondria*	*O_2 uptake (nmoles O_2/min per mg protein)*				*Rate of change in fluorescence (units/min per mg protein)*	
		Succinate (3.3 mM)		*Ascorbate (2.5 mM) + TMPD (0.25 mM)*		*Succinate (3.3 mM)*	*Ascorbate (2.5 mM) + TMPD (0.25 mM)*
		−ADP	*+ADP (0.4 mM)*	*−ADP*	*+ADP (0.4 mM)*		
Ethanol	Sucrose	10.2	40.0	35.0	55.0	65	68
	Sucrose + EDTA	13.5	47.0	47.0	77.0	112	102
2-Thenoyltrifluoroacetone	Sucrose	7.6	9.8	39.0	51.0	0	37
	Sucrose + EDTA	10.9	41.5	56.0	71.0	50	67

reversed, even by dilution. In an attempt to detect reversibility differences between the various functions studied in the present work, we preincubated rat liver mitochondria with 2-thenoyltrifluoroacetone and washed them with either sucrose or sucrose *plus* EDTA. The effectiveness of respiration and respiration-linked $NAD(P)^+$ reduction in the 2-thenoyltrifluoroacetone-pretreated particles with that in the controls was then compared. The underlying assumption was that, while washing with pure sucrose removes the loosely bound 2-thenoyltrifluoroacetone, EDTA may decompose the more stable 2-thenoyltrifluoroacetone complex of respiratory carriers by displacement. Results are summarized in Table II. It can be seen that pretreatment of mitochondria with 2-thenoyltrifluoroacetone in the cold followed by washing with sucrose greatly diminished their ability to oxidize succinate and to reduce $NAD(P)^+$ with succinate as the electron donor. Under the above conditions the rate of ascorbate *plus* TMPD oxidation remained as in the control and ascorbate *plus* TMPD-linked $NAD(P)^+$ reduction was only moderately inhibited. The inhibited functions were significantly improved when the sucrose used for washing the mitochondria pretreated with 2-thenoyltrifluoroacetone also contained EDTA. It is also noteworthy that in the EDTA-washed controls almost all the functions studied showed higher activity than in the sucrose-washed controls.

DISCUSSION

The results of the present investigation show discrepancies between the effect of 2-thenoyltrifluoroacetone on the rate of oxidation (State 4) of succinate on the one hand and on the succinate-linked reversal of the electron transport on the other. The most conspicuous is the discrepancy in the low range of concentrations of 2-thenoyltrifluoroacetone in which the latter accelerated the O_2 uptake but inhibited the reversal of electron transport. An increased contribution by the endogenous substrate to the overall rate of O_2 uptake in the presence of 2-thenoyltrifluoroacetone could not account for the above pattern of electron flow. Furthermore, when restriction of the electron flow from succinate into the respiratory chain was achieved by malonate or by lowering the succinate concentration, then the respiration was more affected than the reversed electron transport, in contrast to the changes observed in the presence of 2-thenoyltrifluoroacetone. Thus, it is apparent that the suppression of succinate-linked $NAD(P)^+$ reduction induced by 2-thenoyltrifluoroacetone cannot be unambiguously related to inhibition of succinate oxidation.

An alternative possibility that the effect of 2-thenoyltrifluoroacetone on succinate-linked $NAD(P)^+$ reduction would be due to an interference with energy transformation is also counterindicated by the results of the experiments presented in Figs. 3 and 4. In its preferential effect on the reversed electron transport, 2-thenoyltrifluoroacetone most closely resembles piericidin A, which according to VALLIN AND LÖW[10] also suppresses the energy-dependent reduction of NAD^+ catalyzed by submitochondrial particles at a lower concentration compared with its inhibition of NADH oxidation. However, the analogy here is incomplete since piericidin A, unlike 2-thenoyltrifluoroacetone, was reported to suppress reversed transport to the same extent, irrespective of the electron donor; in this latter respect its effect is more analogous to that of the uncoupling agents 2,4-dinitrophenol and CCCP (see Fig. 4). The pattern of action of 2-thenoyltrifluoroacetone cannot thus be compared directly with that of respiratory inhibitors or of uncoupling agents, and one

is tempted to assume that some unorthodox mechanism is involved. An admittedly very speculative explanation which we could offer in the present state of investigation in order to account for our experimental findings is as follows: let us visualize the 2-thenoyltrifluoroacetone-sensitive component (probably nonheme iron; *cf.* refs. 1 and 3) as a branching point in the succinate dehydrogenase side-chain, *i.e.* the carrier to which it transfers electrons during energy-dependent $NAD(P)^+$ reduction is not the same as when the final electron acceptor is O_2. Let us assume, furthermore, the existence of a 'shunt' (or a deactivated form of the 2-thenoyltrifluoroacetone-sensitive component) through which electrons can migrate to the second but not to the first carrier when the sensitive component is blocked by 2-thenoyltrifluoroacetone. At slow respiration rates, as under conditions of State 4, enough electrons are available to support it at the maximum level attainable in this condition. In these circumstances reversed electron transport may be completely inhibited before any effect of 2-thenoyltrifluoroacetone on succinate oxidation (State 4) becomes manifest. When, however, the overall rate of electron transport towards O_2 is increased by release of the respiratory control (ADP, uncoupler) and the succinate dehydrogenase becomes rate-limiting with regard to the respiration[9], then blocking of the sensitive component by 2-thenoyltrifluoroacetone results in depression of both the respiratory rate and that of $NAD(P)^+$ reduction. In that case an apparent correlation exists between the inhibitory effect of 2-thenoyltrifluoroacetone on succinate-linked $NAD(P)^+$ reduction and on State 3 succinate oxidation (see Fig. 2), although the reduction occurs in the controlled state rather than in State 3. It is noteworthy that VALLIN AND LÖW[10] (see p. 268) considered the possibility of alternative routes for the electrons ascending and descending with the potential gradient but in the end preferred to attribute the differences in piericidin A sensitivity to different states of reduction of the respiratory chain in the coenzyme Q region.

The reconstitution experiments also deserve some comment. It is likely that in the range of low concentrations, in which 2-thenoyltrifluoroacetone titrated in a parallel manner succinate oxidation (State 3) and succinate-linked $NAD(P)^+$ reduction, it interacted with the highly 2-thenoyltrifluoracetone-affinitive component of succinate dehydrogenase and the complex formed may be the one which withstood washing with pure sucrose but was decomposed in the presence of EDTA. The complex formed between 2-thenoyltrifluoroacetone and the component of low 2-thenoyltrifluoroacetone affinity would then be the one which decomposed by washing with pure sucrose alone.

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