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THE INHIBITION OF OXYGEN PRODUCTION AND THE UNCOUPLING OF ELECTRON TRANSPORT IN PHOTOSYNTHESIS IN CHLOROPLASTS BY SUBSTITUTED THIOPHENS

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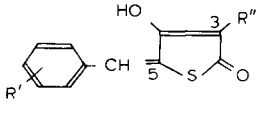
SUMMARY

3-Acyl-5-arylidene-4-hydroxy-2,5-dihydro-2-oxothiophens, known to inhibit mitochondrial oxidative phosphorylation, are potent inhibitors of electron transport, and uncouplers of photophosphorylation, in chloroplasts. The binding of these reagents to the chloroplast is reversible. The effects are increased by electron-withdrawing substituents in the aryl group, and the lowest inhibitor constant recorded was 0.3 μM. Kinetic studies showed that the sensitive site corresponded to some 300 chlorophyll molecules. In contrast to the action of 3-(3,4-dichlorophenyl)-1,1-dimethylurea, the inhibitor constant for the most potent oxothiophen was not increased when diphenylcarbazide was used as an alternative electron donor to System II in place of water. Comparisons were made with the weak-acid type of uncoupling reagent. It is suggested that the site of action of the oxothiophens lies between the point of action of diphenylcarbazide and the quinone pool in System II, and is distinct from its uncoupling action is System I.

INTRODUCTION

The materials under review, 3-acyl-5-arylidene-4-hydroxy-2,5-dihydro-2-oxothiophens, were first prepared by O'Mant [1]. Franklin et al. showed that one representative, ICI 47776 (VIII in Table I), inhibited electron transport in rat-liver mitochondria [2]. These authors also found that the P/O ratio was diminished by this



Abbreviations: ANT2s, 2-(3,4,5-trichloroanilino)-3,5-dinitrothiophen; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

reagent, indicating that there was an uncoupling action on oxidative phosphorylation. There was a difference between the oxothiophen and the ionophorous group of uncoupling agents in that the pK_a value of the former was found to be 2.58 [3] compared to 7 ± 1 for the latter. Attention has been drawn [4] to the close correlation between uncoupling activity and inhibition of the oxygen-evolving apparatus (System II) of the chloroplast in many reagents. Accordingly, when these oxothiophens became available, their effects on photosynthesis were a matter of some interest.

In this study the action of the oxothiophens is compared with those of other inhibitors and uncouplers. Differing patterns of inhibition should indicate different sites of action.

MATERIAL AND METHODS

Class B chloroplasts [5] were prepared from pea leaves (*Pisum sativum* L. 'Meteor') and suspended in a medium containing 0.02 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonate (HEPES), 5 mM KCl, 3 mM Na_2CO_3, 8 mM Na_2HPO_4, 1 mM $MgSO_4$, 0.3 M sorbitol, adjusted to pH 7.6 with NaOH.

Measurements of the rate of electron transport

The Hill reaction was measured in terms of the rate of oxygen production from a 3 ml chloroplast suspension in the above medium with the addition of 3 mM $K_3Fe(CN)_6$ at 25 °C, using a Clark-type oxygen electrode (Yellow Springs Instrument Corp., Ohio, U.S.A.). The light source was a 100 W quartz-iodine lamp focused by an f/1 condenser through a heat filter and an Ilford 203 red filter. The concentration of chloroplasts was 33 μg chlorophyll/ml. The light was of saturating intensity under these conditions.

System I was measured in terms of oxygen uptake in the Mehler [6] reaction in the same apparatus, according to Strotman and Gösseln [7]. The suspending medium in this case contained the additional components 1 mM methylviologen, 10 mM sodium isoascorbate, 0.1 mM 2,6-dichlorophenolindophenol, 10 M DCMU and 1 μg/ml catalase (Boehringer KAT 1). The concentration of chloroplasts used was 17 μg chlorophyll/ml.

The effects of the inhibitors on System II was also investigated using the Mehler reaction, and using diphenylcarbazide as an artificial electron donor. DCMU, isoascorbate and indophenol were omitted from the suspending medium in this case. The rate of electron transport under these conditions was 78 % of the rate of the Hill reaction with ferricyanide, hence the rate of donation from diphenylcarbazide to the (unknown) acceptor in System II was the rate-limiting step. The indophenol viologen reaction of System I was much faster than reactions in which System II was involved, so that System II contains the rate-limiting step in both Hill and diphenylcarbazide–Mehler reactions. The concentration of chloroplasts used was 33 μg/ml chlorophyll.

Photophosphorylation

In the Hill or Mehler systems above, the phosphate was labelled with $H_3{}^{32}PO_4$ (0.8 μCi/ml), and 3 mM ADP was added to the medium. Samples were withdrawn and assayed for ATP according to the method of Hagihara and Lardy [8]. Under the experimental conditions described, uncoupling of phosphorylation was accompanied

by an increase in the rate of oxygen output, or uptake, respectively, and quantitative measurements of uncoupling are presented in terms of this rate increase.

Treatment of results

The rates of the reactions were plotted against the $\log_{10}$ of the thiophen concentration. Sigmoid curves resulted, and the inhibitor or uncoupling constants taken as the concentrations giving 50 % response.

Reagents

A set of thiophen derivatives (see Table I) was kindly donated by Dr O'Mant. They were dissolved in dimethylsulphoxide to a concentration of approximately 20 mM, and immediately diluted 10-fold and made to volume with ice-cold reaction medium. The dimethylsulphoxide (final concentration 0.3 %) did not affect the measurements of reaction rates. At the conclusion of the work Dr O'Mant kindly verified the purity of the oxothiophens used. The anilinothiophen ANT2s was a gift from Dr Buchel.

In all the rate measurements, rates were proportional to chloroplast concentration up to at least 80 μg chlorophyll/ml.

RESULTS

Table I shows the effects of the thiophen derivatives on electron transport reactions in the chloroplasts, and includes for comparison data for DCMU and for two uncoupling agents of the weak-acid type: CCCP and ANT2s [9]. The most effective thiophen in inhibiting the Hill reaction is clearly VII. The form of the inhibition as a function of concentration is shown in Fig. 1, which shows that at low concentrations VII inhibits only that component of the reaction rate which is due to the presence of ethylamine. Thus the I_{50} value may be said to be ethylamine dependent.

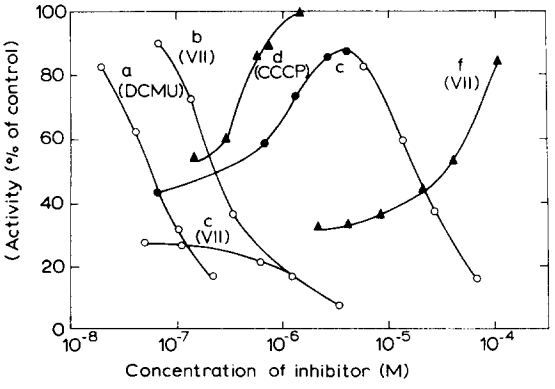


Fig. 1. Inhibition and uncoupling of electron transport in pea chloroplasts by oxothiophen VII compared with CCCP and DCMU. Activities are given as percentages of the rates obtained in the presence of 3 mM ethylamine · HCl and in the absence of inhibitor. Ethylamine · HCl was present in Expts a and b but was absent from c, d, e and f. ● or ○, the Hill reaction with $K_3Fe(CN)_6$: System II. ▲, the Mehler reaction with isoascorbate and indophenol: System I. ○, inhibition; ●, uncoupling. For conditions see the Methods section of the text.

TABLE I

STRUCTURE AND ACTIVITY OF THIOPHEN DERIVATIVES IN PHOTOSYNTHETIC REACTIONS

I_{50}, U_{50} are the mid-point concentrations for inhibition and uncoupling, respectively. [a]Numbers supplied by Imperial Chemical Industries, Ltd, Pharmaceutical Division. [b]Refer to the structural formula. [c]The Hill reaction with ferricyanide. [d]The Mehler reaction with diphenylcarbazide as donor, [e]with ascorbate-indophenol as donor. [f]No uncoupling up to the limit of solubility. [g]Values are K_{eq} (see text). n.d., not determined.

No.	ICI No.[a]	3-Substituent[b]	Arylidene substituent[b]	System II			System I
				$I_{50}(H_2O)$[c] (μM)	I_{50}(donor)[d] (μM)	$U_{50}(H_2O)$ (μM)	U_{50}[e] (μM)
I	3965	$-CO.O.C_2H_5$	4′-dimethylamino	54	n.d.	—	n.d.
II	41141	$-CO.CH_3$	4′-nitro	3	4	—	29
III	51001	$-CO.O.C_2H_5$	2′,4′,6′-trichloro	3	4	—	20
V	3970	$-CO.O.C_2H_5$	4′-methoxy	6.1	n.d.	—	250
VI	4032	$-CO.O.C_2H_5$	4′-methyl	10	n.d.	—	175
VII	48161	$-CO.O.CH(CH_3)_2$	4′-chloro	0.5[g]	0.3[g]	—	26
VIII	47776	$-CO.CH_3$	4′-fluoro	0.8	12	—	36
ANT2s	—	—	—	1.7	5	0.02	0.3
CCCP	—	—	—	12	10^3	1.4	0.3
DCMU	—	—	—	0.02[g]	0.08[g]	—	None[f]

No uncoupling of the Hill reaction could be observed with thiophens I to VII, and the uncoupling of the System I reaction occurred at concentrations of an order of magnitude greater than those which inhibited System II.

Reversibility

Chloroplasts were treated with 3.2 μM of VII, which reduced their Hill activity to approximately 5 %. The chloroplasts were sedimented by centrifugation and the supernatant discarded. After suspension in fresh reaction medium, the chloroplasts regained 80 % of their previous activity, and stimulation of the Hill reaction rate could again be demonstrated, showing that neither the electron transport nor the coupling of System II was destroyed by VII.

In a similar experiment the chloroplasts were discarded from the digest and fresh chloroplasts added to the supernatant liquid. It was found that the activity of the second digest was greater than that of the first. When the chloroplasts were removed again and fresh ones added, the degree of inhibition was found to have declined again, indicating an equilibrium of the type found by Izawa and Good [10]. The precision was insufficient in this case for determination of the size of the target site, and a kinetic analysis was made for this purpose.

If one molecule of the inhibitor combines with one active site rendering it inactive, and if C is the total concentration of such sites, b the concentration of inhibited sites and x the total concentration of inhibitor,

$$K_{eq} = (C-b)\,(x-b)/b$$

When $x = I_{50}$, $b = \frac{1}{2}C$, so that

$$K_{eq} = I_{50} - \tfrac{1}{2}C$$

$$I_{50} = K_{eq} + \tfrac{1}{2}C,$$

a linear relationship to which the data of Fig. 2, which shows the dependence of I_{50} on chloroplast concentration, may be fitted. K_{eq} is obtained from the graph and repre-

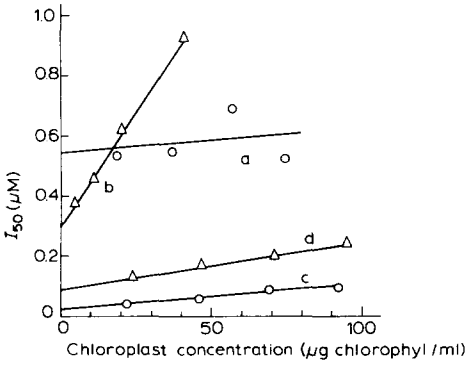


Fig. 2. The dependence of the inhibitor constants of DCMU and oxothiophen VII on chloroplast concentration and on the electron donor to System II. The ordinate is the apparent inhibitor constant determined graphically from sets of four observations each. ○, the Hill reaction with $K_3Fe(CN)_6$, water being the donor. △, the Mehler reaction with diphenylcarbazide as the donor. In Expts a and b, oxothiophen VII was the inhibitor, and DCMU was used in c and d. For conditions see text.

sents I_{50} at infinite dilution of chloroplasts, and is entered as such in Table I. In the case of the other oxothiophens, this procedure made no difference. Since the slope of the lines of Fig. 2 relate $\frac{1}{2}C$ to the concentration of chlorophyll, the relative abundance of the target site could be calculated. With the Hill reaction and VII (which consistently showed a much greater degree of experimental scatter, see line a, Fig. 2) the statistically fitted line indicated one site per 340 chlorophyll molecules. The corresponding value for the inhibitor DCMU was 290. The results for the diphenylcarbazide–Mehler reaction were 330 for DCMU and 35 for VII. The first three figures are similar, considering the precision of the measurements; the fourth is clearly inconsistent with the others, and this is not explained.

DISCUSSION

From the data presented in Table I, the most effective oxothiophens are II, III, VIII and VII. These all contain a halogenated arylidene group. The table also shows that these reagents do not uncouple electron transport at concentrations as low as those which inhibit the Hill reaction. This is in striking contrast to CCCP and ANT2s in which the uncoupling effect appears at lower concentrations. CCCP and ANT2s have been grouped by Renger [11] in a class which "accelerate the deactivation reactions of system Y" (ADRY). This group is characterised by, among other features, pK_a values in the range 6–8. It may be noted that when diphenylcarbazide is used as an artificial donor to System II, CCCP has no inhibitory action, while ANT2s inhibits, but at a significantly higher concentration. Haveman and Donze [12] showed that inhibition by DCMU was diminished in the presence of diphenylcarbazide (a result confirmed in Table I). DCMU is not an ADRY reagent, and does not uncouple at concentrations up to 0.1 mM. ANT2s appears to have two types of action, one of the ADRY type and one resembling DCMU.

Thiophen VII has a lower K_{eq} in the presence of diphenylcarbazide, and therefore differs from DCMU in addition to the uncoupling action which DCMU lacks. Since the uncoupling effect of the oxothiophens is only observed at higher concentrations, however, it may not be obligately connected with inhibition of System II by this type of compound.

The calculation from kinetic data of one target site for compound VII per 300 chlorophyll molecules is in good agreement with the first estimate made by Izawa and Good [10] for the target site for DCMU. These authors, however, corrected their result to allow for binding of DCMU to non-active sites in the chloroplast, and their final value for the abundance of DCMU-sensitive, photosynthetically active sites was 1 per 2500 chlorophyll molecules [10]. The data at present available for VII does not permit a similar correction to be attempted.

Since there are four reaction centres of each photosystem in a photosynthetic unit of approx. 2500 chlorophyll molecules, there are not more than two (and probably less) target sites for VII per reaction centre of System II. One component of System II which might have such an abundance is that which accepts electrons from diphenylcarbazide. The finding that K_{eq} for VII is lower in the presence of diphenylcarbazide is consistent with an attack of VII on that component.

Thiophen VIII appears to resemble DCMU in that there is appreciable relief of the inhibition in the presence of diphenylcarbazide. This is an unlikely result since

the structures of VII and VIII are very similar, differing mainly in the 3-substituent. The reactivity of the 3-acetyl group in the Mehler reaction system is under investigation. However there is no reason why any one inhibitor should be restricted to one only of several similar sites.

Of the inhibitors discussed, the ADRY reagents act nearest to the point of production of molecular oxygen [11]. On the reducing side of that site is that at which diphenylcarbazide donates electrons, which also has been argued to be the site of action of DCMU [12, 13] (in contrast to the traditional view originally proposed by Duysens [14]). The present study provides some evidence for a third site on the reducing side of the other two.

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