1,2,3-Thiadiazolyl-Phenyl-Ureas, New Inhibitors of Photosynthetic and Respiratory Energy Conservation

G. Hauska and A. Trebst

Lehrstuhl für Biochemie der Pflanzen, Abteilung Biologie, Ruhr-Universität Bochum

C. Kötter and H. Schulz

Pflanzenschutzforschung der Schering AG, Berlin-Bergkamen

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Thiadiazolyl-Phenyl-Urea, Inhibitor of ATP Formation, Photosynthesis, Respiration

Substituted 1,2,3-thiadiazolyl-phenyl-ureas were found to be inhibitors of energy conservation in respiration and photosynthesis. The most effective dichlorophenylderivative uncouples ATP formation in isolated chloroplasts or mitochondria, at a concentration of about 2 and 9 μM respectively. At a certain concentration range the compounds also appear to be energy transfer inhibitors, similar to the well known inhibition by carbodiimides. The significance of the chemical relation of carbodiimides to ureas in the mode of action on energy transfer is discussed. The thiadiazolyl-phenyl-ureas are inhibitors of electron flow only at relatively high concentrations, pointing out that sterig hindrance by two large aromatic rings at both nitrogens of the urea moiety abolishes the highly effective inhibition of photosynthetic electron flow by substituted urea derivatives, like DCMU.

Introduction

Substituted phenylureas are long known to be very effective inhibitors of photosynthetic electron flow [1–3]. Because they are also effective herbicides, their mode of action in chloroplasts has been extensively studied [1–3]. The comparison of inhibitory activity to chemical structure revealed that for optimal inhibition the urea backbone should be substituted at the N_1 position by a lipophilic aromatic ring for easy approach of the component to the active site in the chloroplast membrane and at the N_3 position with small aliphatic substituents to avoid shielding of the active structural element [1–3].

It might seem to come as a surprise therefore that a new group of substituted ureas comprises not electron flow inhibitors but rather uncouplers and inhibitors of energy transfer in photophosphorylation. As against the electron flow inhibitors these ureas carry ringsystems on both nitrogens.

The present study is to characterize their mode of action on photosynthetic chloroplast reactions and to compare their action with that of DCCD; a known energy transfer inhibitor [4]; derived from substituted ureas. Furthermore their effects on mitochondrial respiration is examined.

Requests for reprints should be sent to Prof. Dr. A. Trebst, Ruhr-Universität Bochum, Lehrstuhl für Biochemie der Pflanzen, *D-4630 Bochum-Querenburg*, Postfach 2148.

Abbreviations: DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; DCCD, dicyclohexyl carbodiimide; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethyl urea; DPIP, 2,6-dichlorophenolindophenyl; phenyl-TU, 1-(1,2,3-thiadiazol-(5)-yl)-3-phenyl urea; PMS, N-methylphenazonium-methosulfate.

Methods

Spinach chloroplasts were prepared according to McCarty and Racker [4].

Electron transport with anthraquinone-sulfonate as acceptor was measured as oxygen uptake as previously described [5]. The reaction mixture was kept in Warburg vessels in a final volume of 3 ml. It contained 30 mM Tris-HCl buffer (pH 8.0), 3 mM $MgCl_2$, 3 mM ADP, 3 mM Pi containing about 2×10^5 cpm ^{32}P, 3×10^{-5} M anthraquinone-2-sulfonate and chloroplasts corresponding to 200 μg chlorophyll. Further additions are specified in the legends to the tables. The samples were illuminated for 10 min at 15 °C with 35000 lx of white light. Cyclic phosphorylation was measured in nitrogen under the same conditions except for the presence of 5×10^{-5} M PMS plus 2×10^{-5} M DCMU instead of anthraquinone-2-sulfonate. Esterified $^{32}P_i$ was assayed according to ref. 4.

Proton uptake by illuminated chloroplasts was measured according to McCarty [6]. The reaction mixture contained in a final volume of 3 ml 50 mM NaCl, 5 mM $MgCl_2$, 2 mM tricine-NaOH (pH 8.5), 2×10^{-5} M DCMU, 10^{-5} PMS and chloroplasts (1 mg chlorophyll/ml in 10 mM NaCl) equivalent to 100 μg chlorophyll. The reaction vessel was cooled to 13 °C. The pH was adjusted to the value

of 8.5 by addition of dilute HCl or NaOH, the last small addition being recorded for evaluation of the buffer capacity. The intensity of the actinic white light was 20000 lx.

The quench of 9-amino-acridine fluorescence [7] was measured as published previously [8], at an intensity of 20000 lx red light (RG 645 Schott, 2 mm) for illumination. The reaction mixture in this case contained in a final volume of 3 ml 50 mM tricine-NaOH (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, 10 mM PMS and chloroplasts corresponding to 20 μg chlorophyll.

Mitochondria were isolated from white potato tubers by Method II as outlined by Verleur [9]. The following modifications were made: Isolation medium was reduced (130 g tissue samples were homogenized in 250 ml isolation medium), mannitol molarity was decreased to 0.5 M from 0.7 M and the second washing of the mitochondrial suspension was omitted.

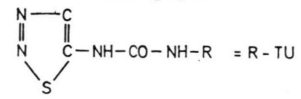
The basic reaction medium for respiration studies contained the following components in a total volume of 3 ml: 0.5 M mannitol, 0.01 M potassium phosphate (pH 6.5), 0.5 mM EDTA, 0.1% BSA, 18 μmol succinate, 0.5 ml aliquot of the final mitochondrial suspension, containing 1.8 mg protein. For studying state 3-respiration, 0.8 μmol of ADP were added (Table V and Fig. 3). To study the transition from state 3 to state 4, 0.4 μmol ADP were present (Table V).

The synthesis of the 1,2,3-thiadiazol(5)-γl-ureas has been accomplished by reaction of 5-amino-1,2,3-thiadiazole [10] with the respective arylisocyanates.

Results and Discussion

As Table I indicates, the substituted phenyl-TU are effective in inhibiting PMS-catalysed cyclic photophosphorylation in chloroplasts. As judged from the PI_{50} value the dichloroderivate is the most effective inhibitor, whereas an alkyl substitution of the phenyl ring diminishes inhibitory potency. Table II and Fig. 1 show that inhibition of photophosphorylation is due to an inhibition of energy transfer, since non-cyclic electron flow from H_2O is not inhibited beyond the basal rate, but coupled ATP formation is totally abolished. Furthermore the inhibition of electron flow by the phenyl-TU is reversed by the further addition of an uncoupler like NH_4Cl or gramicidin. From Fig. 1 it can be seen that this reversal is not complete when higher concentrations of the inhibitors are employed. We attribute this to the likeliness that the compounds

Table I. Inhibition of cyclic photophosphorylation by 1,2,3-thiadiazolyl-phenyl-ureas. The assay with PMS as cofactor is described under Methods. The 100% value of the phosphorylation rate was 8.3 μmol formed per 10 min and 0.2 mg chlorophyll.



= R-TU

R=	Concentrations for 50% inhibition [μM]	PI_{50}
phenyl-TU	24	4.64
p-chlorophenyl-TU	2.5	5.62
m-chlorophenyl-TU	3.3	5.50
3,4-dichlorophenyl-TU	2.2	5.67
p-tolyl-TU	135	3.89

Table II. Inhibition of pseudocyclic photophosphorylation by substituted ureas and reversal of electron flow inhibition by an uncoupler. The assay and the reaction mixture are described under Methods. Gramicidin D was added to 10^{-6} M.

	μM	μequival. O_2 taken up	ATP formed [μmol]	μequival. O_2 + gramicidin D
control	—	5.1	4.9	8.4
DCMU	20	0.1	0.1	0.1
p-chlorophenyl-TU	20	3.6	1.9	7.6
m-chlorophenyl-TU	40	3.6	2.0	7.2
3,4-dichlorophenyl-TU	6	4.5	1.5	7.2
phenyl-TU	80	3.5	1.8	7.5
p-tolyl-TU	40	4.0	3.0	7.5
N-(2,6-Dichlorobenzoyl)-N′-*p*-chlorophenyl-urea (DU 19892)	60	2.3	1.3	6.9
DCCD	20	3.8	2.7	8.7
DCCD	40	2.4	0.5	8.4
Dicyclohexyl-urea	120	5.0	3.8	8.7
N-phenyl-N′-(3,4-dichlorophenyl)-urea	12	4.5	2.5	8.1
basal rate (no ADP/Pi added)		3.3	—	—

as substituted ureas do have also some electron flow inhibitor potency in the photosystem II area like the well known ureas of the DCMU type. Similar results were obtained with ferricyanide or $NADP^+$ as terminal electron acceptor in photosynthetic electron flow from water. New insecticides of similar structure have recently been described by van Daalen *et al.* [11]. These are N-benzoyl-N-chlorophenyl-ureas which have no phytotoxic activity in spite of their chemical similarity to the urea herbicides [12].

According to Table II a compound of this class seems to behave like the thiadiazolylurea derivates described in this paper, *i. e.* as energy transfer inhibitor. However, at higher concentration it inhibits electron transport.

DCCD is an effective and well studied energy transfer inhibitor of electron flow in chloroplasts [4, 13] and mitochondria [14]. Carbodiimides are reactive compounds forming substituted ureas. The chemical relationship of ureas with cyclic or heterocyclic rings at both nitrogens to DCCD is obvious. One could visualize, that carbodiimides are hydrated by chloroplasts to ureas, which are the actual inhibiting compounds. However, dicyclohexyl-urea, the compound formed upon hydration of DCCD, is not a potent inhibitor of photophosphorylation (Table II).

Replacement of the cyclohexyl groups by substituted phenyl groups in N-phenyl-N′-3,4-dichlorophenyl urea does again yield a reasonable active energy transfer inhibitor (Table II). Another possibility is that DCCD is covalently bound to the membrane by addition of reactive groups forming substituted ureas. This is supported by experiments with mitochondria [15] and chloroplasts [16]. A urea derivative covalently bound to the site of its action would be much more efficient than the corresponding free urea.

The results reported here, that the phenyl-TU at low concentrations exhibit energy transfer inhibition like bound DCCD, might support such a hypothesis.

On the other hand energy transfer inhibition might be simulated by a combined activity of a DCMU-like electron transport inhibition and uncoupling. Table III demonstrates that this alternative is feasible indeed. While electron flow from water is slightly inhibited by the phenyl-TU, artificial electron flow with DAD through photosystem I is not inhibited and electron flow with DPIP is highly stimulated. This differential effect on different electron donor systems for photosystem I is typical for an uncoupler [17], as is the accompanying inhibition of photophosphorylation. However, the data of Fig. 1 can not be satisfactorily explained by such a double effect of the phenyl-TU; the reversal by an uncoupler of the inhibition of electron flow from water by the phenyl-TU employed in a low concentration range supports the notion that these compounds can also act as energy transfer inhibitors. At higher concentrations they indeed inhibit electron flow from water like DCMU. Electron transport inhibitions, but not uncoupling activity, has also been reported for DCCD [4].

Table III. Effect of 1,2,3-thiadiazolyl-phenyl-ureas on electron donor systems for photosystem I. The assay and the reaction mixture are described under Methods. In the case of artificial electron flow through photosystem I the mixture additionally contained 2×10^{-5} M DCMU, 3 mM ascorbate and 10^{-4} M DAD or DPIP. Asc stands for ascorbate.

Electron donor	*p*-Cl-phenyl-TU [μM]	μequival. O_2 taken up	ATP formed [μmol]
H_2O	—	3.6	3.2
H_2O	20	2.8	1.6
DAD/Asc	—	14.3	6.6
DAD/Asc	20	14.2	3.3
DPIP/Asc	—	3.2	1.5
DPIP/Asc	20	13.5	0.5

In the following the effects of phenyl-TU are compared with those of DCCD on two other reactions of chloroplasts which reflect the high energy state. One is the light-induced pH-rise [18], the other is the light-induced quenching of 9-amino acridine fluorescence. The latter is believed to indicate a pH-gradient across the thylakoid membrane during illumination [7].

Table IV. Effect of 1,2,3-thiadiazolyl-phenyl-ureas on light-induced pH-rise in untreated and EDTA-treated chloroplasts. The 100% value was 0.210 and 0.046 μequivalents H^+ taken up per mg chlorophyll in untreated and EDTA-treated chloroplasts, respectively. The pH of the reaction medium was 8.5; the assay and other details are described unter Methods.

Additions of phenyl TU [10 μM]	% Activity
Untreated:	
p-chlorophenyl-TU	71
3,4-dichlorophenyl-TU	50
DCCD	230
EDTA-treated:	
p-chlorophenyl-TU	134
3,4-dichlorophenyl-TU	134
DCCD	890

Table IV summarizes the results measuring the pH-rise. It can be seen that the extent of proton uptake in isolated, untreated chloroplasts is inhibited by phenyl-TU already at low concentrations. DCCD exhibits a stimulation at pH 8.5, as has been reported [16]. In EDTA-treated chloroplasts, which are deficient in coupling factor and show drastically decreased proton uptake [4], phenyl-TU, at low con-

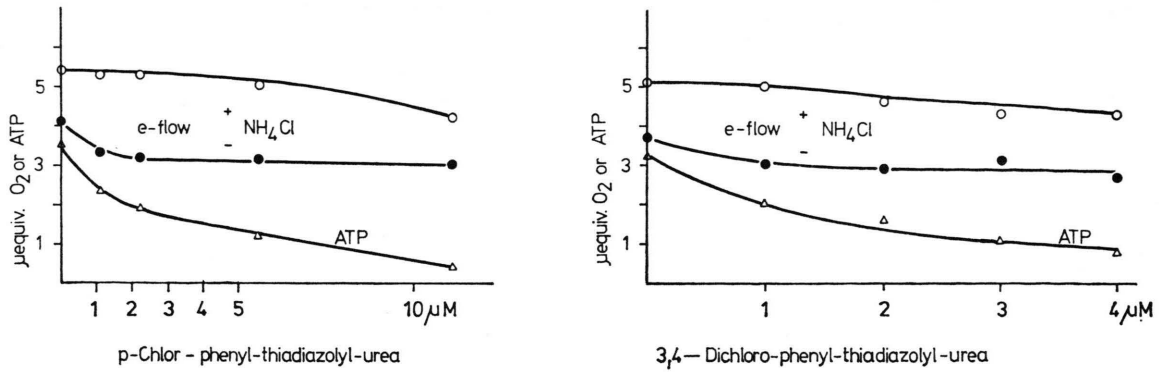


Fig. 1. Titration of pseudocyclic electron flow with 1,2,3-thiadiazolylureas. The assay and the reaction mixture are described under Methods. NH_4Cl was added to 5 mM.

centration, have a small reconstitutive effect, which is not always found. The effect of DCCD, which has been reported before [4], is much larger.

The results from measurement of fluorescence quench show a similar pattern. In untreated chloroplasts (Fig. 2) the % quenching is stimulated slightly by DCCD, suggesting that the pH gradient is increased, but phenyl-TU inhibit. The dichloro-derivative was the more potent inhibitor. It should be noted that the rate of the back reaction reflecting proton efflux is markedly increased in the presence of the phenyl-TU, which is typical for uncouplers. DCCD, in the other hand, decreases the rate of efflux as well as that of influx. The fluorescence

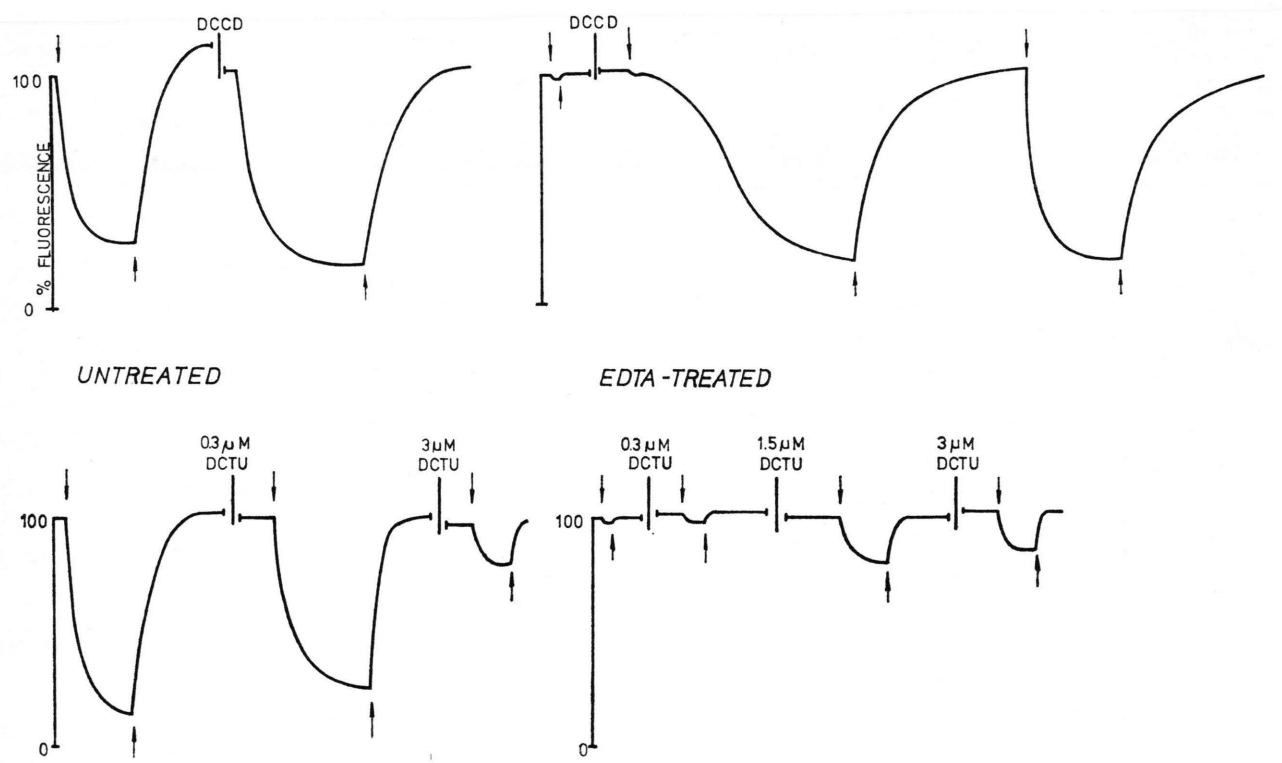


Fig. 2. Effect of DCCD and 1,2,3-thiadiazolyl-3,4-dichloro-phenyl-urea on light induced quenching of 9-amino-acridine fluorescence in untreated and EDTA-treated chloroplasts. The assay and the reaction mixture are described under Methods. DCCD was added to 10^{-5} M. DCTU stands for 3,4-dichlorophenyl-TU. Downward arrows mark "light on", upward arrows "light off".

quench is almost totally inhibited by EDTA treatment and is fully restored by DCCD (Fig. 2).

The restoration shows an interesting time lag, which is shortened by illumination. This time dependence of the reconstitutive effect of DCCD has been reported before for measurements of the light-induced pH rise in chloroplasts [16]. In the light groups reacting with DCCD might be more exposed reflecting a conformational change in the membrane [19]. Phenyl-TU, at low concentrations, showed a small reconstitutive effect, similar to that for the pH-rise. At higher concentrations the effect is reversed. However, no time dependence of restoration as with DCCD was observed.

The rates of the back reaction stayed much faster in the presence of phenyl-TU compared to DCCD.

Reconstitution of photophosphorylation in EDTA-treated chloroplasts as reported for DCCD [4] could not be demonstrated with phenyl-TU, presumably because the inhibitory effects dominated already at low concentrations.

Table V. Effects of 1,2,3-thiadiazolyl-phenyl-ureas and uncouplers on the activity of mitochondria from potato tubers. The assay is described under Methods. A separate experiment was run for each compound.

Compound	μM	nmol O_2/min/3 ml Controls without compound		Plus compound State 4
		State 3	State 4	
phenyl-TU	120	38.76	12.86	30.49
p-chlorophenyl-TU	10	43.72	12.11	27.44
m-chlorophenyl-TU	21	43.46	12.60	37.41
p-tolyl-TU	42	46.54	17.45	30.18
3,4-dichlorophenyl-TU	9	24.43	9.83	22.52
N-phenyl-N'-3,4-dichlorophenylurea	20	48.97	13.99	43.44
2,4-DNP	27	43.97	7.60	37.46

Table V shows that the phenyl-TU act as uncouplers in mitochondrial respiration, with similar efficiency as dinitrophenol. The rate of respiration in state 3 were not affected by the compounds. The uncoupling activity of phenyl-TU is also demonstrated by the release of the inhibition of respiration by the energy transfer inhibitor oligomycin (Fig. 3).

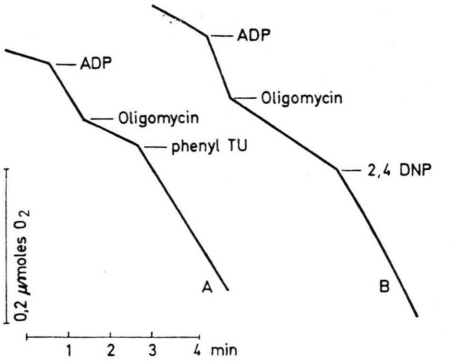


Fig. 3. Stimulation of oligomycin-inhibited respiration by phenyl-TU and DNP. The assay and the reaction mixture are described under Methods. Where indicated, ADP was added to 0.27 mM, oligomycin to 1 μg/ml, phenyl-TU to 120 μM and DNP to 27 μM.

In conclusion the main inhibitory action of phenyl-TU in respiratory as well as photosynthetic electron transport is uncoupling. In photosynthetic electron flow from water at higher concentrations they also act as electron transport inhibitors like DCMU. From Fig. 1 and also from the results shown in Fig. 2 we derive that at a certain concentration range these compounds show effects of energy transfer inhibitors.

This is particularly interesting, since DCCD might also exert its action as an energy transfer inhibitor as a urea derivative, which is formed by reaction with special groups of a membrane component. Its higher efficiency might be explained by the binding close to its site of action. Also the lack of uncoupling is expected if the urea derivative of DCCD was immobilized, following the views of the chemiosmotic hypothesis [20].

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