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RELATIONSHIP BETWEEN INHIBITOR BINDING BY CHLOROPLASTS AND INHIBITION OF PHOTOSYNTHETIC ELECTRON TRANSPORT

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SUMMARY

The binding of radioactively labelled atrazin, metribuzin and phenmedipham by broken chloroplasts was studied. From the double-reciprocal plots (bound vs. free inhibitors) a high affinity binding reaction is graphically isolated which is related to the inhibition of photosynthetic electron transport. It is concluded that the specific binding sites correspond to the electron carrier molecules which are attacked by the inhibitors. The relative concentration of specific binding sites is 1 per 300–500 chlorophyll molecules.

The binding of the labelled substances is competitively inhibited by each of the indicated unlabelled substances, by DCMU and by several pyridazinone derivatives. These results suggest that triazines, triazinones, pyridazinones, biscarbamates and phenylureas interfere with the same electron carrier of the photosynthetic electron transport chain, according to the same molecular mechanism.

INTRODUCTION

Numerous herbicides are known to inhibit photosynthetic electron transport. Among those the phenylurea DCMU (diuron) [1] has widely been used in photosynthesis studies. DCMU inhibits electron transport in the region of Photosystem II. However, a precise localization of the inhibitory site seems to be impossible at present. The reducing side [2, 3] as well as the oxidizing side of Photosystem II [4] have been concluded to contain the DCMU sensitive component. Moreover the reaction center of the photosystem itself was believed to be affected by the inhibitor [5].

Abbreviations: Atrazin, 2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine; prometryn, 2-methylthio-4,6-*bis*-(isopropylamino)-1,3,5-triazine; metribuzin, 4-amino-6-isopropyl-3-methylthio-1,2,4-triazine-5-one; BAY 138992, 4-amino-6-(3-trifluoromethylphenyl)-3-ethyl-1,2,4-triazine-5-one; pyrazon, 4-chloro-5-amino-2-(phenyl)pyridazin-3-one; SAN 9774, 4-chloro-5-amino-2-(3-trifluoromethylphenyl)pyridazin-3-one; SAN 9789, 4-chloro-5-methylamino-2-(3-trifluoromethylphenyl)-pyridazin-3-one; SAN 6706, 4-chloro-5-(dimethylamino)-2-(3-trifluoromethylphenyl)-pyridazin-3-one: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; phenmedipham, 3-methoxycarbonylamino-phenyl-*N*-(3′-methylphenyl)-carbamate.

Several other classes of herbicides, although chemically unrelated to the phenylureas, seem to exhibit the same biological effect as DCMU, e.g. representatives of acylanilides [6], *N*-phenylcarbamates [7, 8], uracils [9], triazines [10], triazinones [11], pyridazinones [12], and benzimidazoles [13]. Therefore several attempts have been made to establish the relationships between chemical structure and biological activity [14–19]. Two substructures have been identified as features common to all these compounds:

(i) the group $>N-\overset{\overset{X}{\|}}{C}-$ (X = O or N, not S) and
(ii) a hydrophobic residue in close vicinity to the first group [17–19].

Accordingly, it has been concluded that this molecular arrangement plays an important role in the interaction of the herbicides with the receptor component of photosynthetic electron transport [17–19]. The considerations on structure-activity relationships assume that the different compounds actually react with the same receptor according to the same molecular mechanism. This has not yet been proved. This problem can be studied by competition experiments as carried out in the present paper [20].

Izawa and Good [21] reported that DCMU is reversibly bound by chloroplasts. By using different radioactively labelled inhibitors, we extended these investigations. Inhibitor binding is shown to be directly related to inhibition of electron transport. Therefore binding can be used as a relevant parameter for the study of inhibitor-receptor interaction. Provided the two inhibitors react with the same electron carrier according to the same mechanism, binding of the one should be competitively affected by the other. Using this method, it can be demonstrated that the investigated triazines, triazinones, biscarbamates and pyridazinones form similar complexes with the DCMU sensitive component of the photosynthetic electron transport chain.

EXPERIMENTAL

Chloroplasts were isolated from freshly harvested spinach leaves in a medium containing 0.3 M sucrose and 10 mM sodium pyrophosphate, pH 7.4. After washing with the same medium, the chloroplasts were resuspended in 70 mM sucrose/2 mM Tricine buffer, pH 7.8. This treatment uncouples the chloroplasts by removal of the coupling factor CF_1 [22] and perforation of the thylakoid membranes [23]. In our experiments this type of uncoupled electron transport exhibits a broad pH optimum between 7 and 8. Finally the chloroplasts were taken up in a medium containing 70 mM sucrose, 1 mM $MgCl_2$ and 2 mM Tricine buffer, pH 7.8.

Electron transport was measured by photoreduction of the artificial electron acceptor ferricyanide, using a Zeiss PMQ II spectrophotometer with cross illumination equipment. The intensity of the red actinic light (Schott filter RG 630) was $8.7 \cdot 10^5$ ergs/cm^2 per s. The temperature was 20 °C. The reaction medium contained 25 mM Tricine buffer, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, and 1 mM $K_3(Fe(CN)_6)$. Inhibitors were added in methanol solutions; the final methanol concentration was constant in all experiments (2.5 %). The chlorophyll content in the cuvette was around 5 μg/ml. The total reaction volume was 2.0 ml. The reaction was started by the addi-

TABLE I

METRIBUZIN ADSORPTION BY CHLOROPLASTS

[^{14}C]Metribuzin concentration (μM)	Controls (cpm)	Supernatants of corresponding samples (cpm)
0.052	288	130
0.143	790	472
0.284	1564	1142
0.501	2759	2260

tion of ferricyanide after a short pre-illumination of the samples. Complete uncoupling of each chloroplast preparation was ascertained by the addition of uncoupler, which should not result in a stimulation of ferricyanide reduction.

Binding of labelled inhibitors by chloroplasts was carried out in the same reaction medium, except that the final methanol concentration was 5 % and the chlorophyll content about 50 μg/ml. Usually, in binding experiments, ferricyanide was omitted because it did not affect the results. The experiments were performed in small plastic tubes in diffuse daylight. The total reaction volume was 1.0 ml. After the addition of chloroplasts to the medium, the samples were thoroughly mixed and then centrifuged for 2 min at 15 000 $\times g$. The clear supernatants were carefully removed and stored for radioactivity measurements. Controls were carried out in the same way except that chloroplast medium was added instead of chloroplast suspension. Measurements of radioactivity were performed in Unisolve 1 scintillator (Koch-Light Laboratories Ltd.) using the liquid scintillation spectrometer Tricarb, model 3320 (Packard).

Adsorption of labelled inhibitors by the chloroplasts was calculated from the differences between the controls and the corresponding samples. In Table I raw cpm data for a typical binding experiment with [^{14}C] Metribuzin are shown. The radioactively labelled inhibitors were applied at the following specific activities: ^{14}C ring-labelled atrazin: 2.2 Ci/mol, ^{14}C ring-labelled metribuzin: 7.3 Ci/mol, [^{3}H]-phenyl-labelled Phenmedipham: 140 Ci/mol.

RESULTS

1. Inhibitor binding by broken chloroplasts and inhibition of uncoupled electron transport

The binding of the labelled triazine Atrazin, the triazinone Metribuzin, and the biscarbamate Phenmedipham as a function of inhibitor concentrations is shown in Fig. 1. The double reciprocal plots (bound inhibitor vs. free inhibitor) reveal that the inhibitors Atrazin and Phenmedipham exhibit biphasic binding curves in the applied concentration range. By extrapolation of the linear branch, a high affinity binding process may be separated. This process, which we call "specific binding", may be formulated by the following reaction equation:

$$A + X \rightleftharpoons XA$$

(A, inhibitor; X, specific binding site; XA, inhibitor-binding site complex).

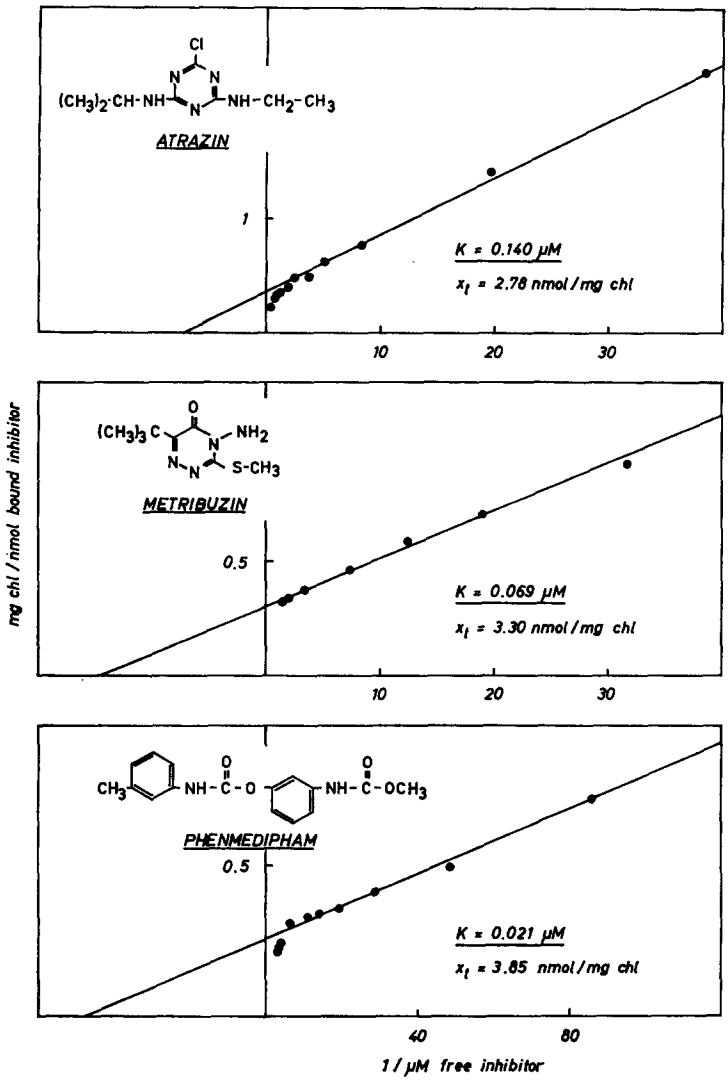


Fig. 1. Double-reciprocal plots of binding of the labelled inhibitors Atrazin, Metribuzin and Phenmedipham by broken chloroplasts.

From the double-reciprocal plots the specific binding constants (K)

$$K = \frac{a \cdot x}{xa} \tag{1}$$

and the total concentration of specific binding sites (x_t) are obtained. The process of specific inhibitor binding is described by the equation

$$xa = \frac{x_t \cdot a}{K + a} \tag{2}$$

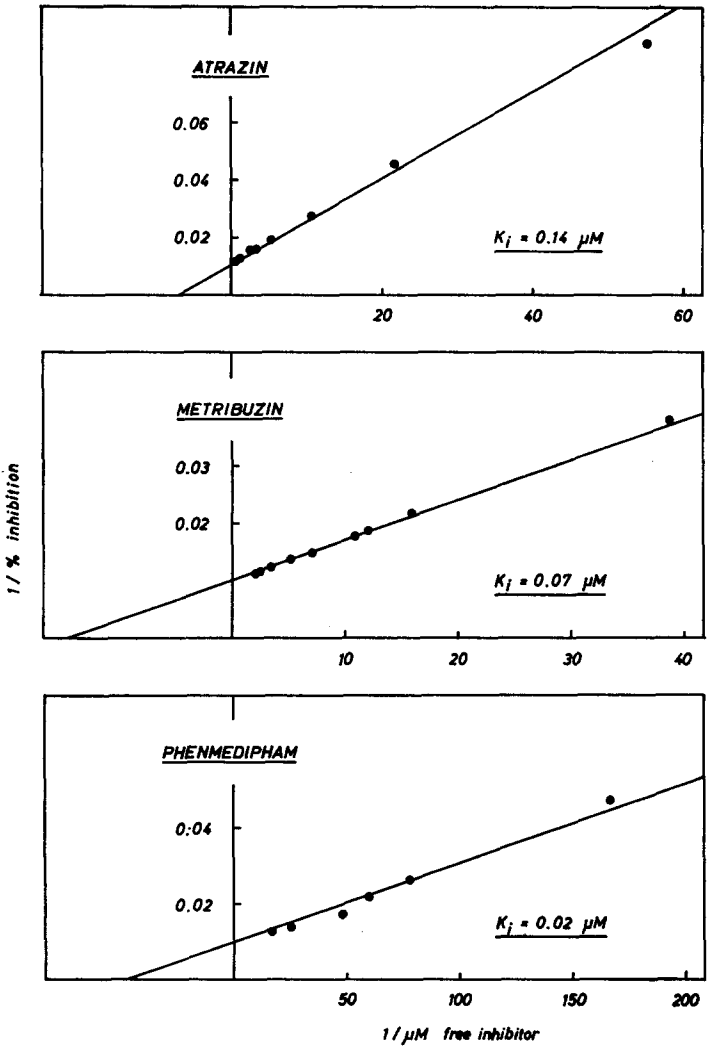


Fig. 2. Double-reciprocal plots of inhibition of uncoupled photosynthetic electron transport by Atrazin, Metribuzin and Phenmedipham. The control rates of ferricyanide reduction were 1250 µmol/mg chlorophyll · h (Atrazin curve), 1103 µmol/mg chlorophyll · h (Metribuzin curve) and 1330 µmol/mg chlorophyll · h (Phenmedipham curve), respectively.

(*xa*, concentration of inhibitor-binding site complex; *x*, concentration of unoccupied binding sites, *a*, concentration of free inhibitor). In Fig. 2, the inhibition of uncoupled electron transport by the same herbicides is shown. Provided that specific inhibitor binding is directly related to inhibition (I) of electron transport, the inhibition curve is described by the equation

$$I = \frac{a}{K_i + a} \tag{3}$$

where the inhibition constant K_i should be identical with the specific binding constant K. K_i can be obtained from the inhibition curve, if $1/I$ is plotted vs. $1/a$. It is noteworthy that for the calculation of K_i, equilibrium concentrations of free inhibitors are required rather than total inhibitor concentrations. In the experiments shown in Fig. 2, the free inhibitor concentrations were determined in parallel binding studies with labelled inhibitors.

The reciprocal plots reveal that the inhibition curves for the three substances are monophasic. The inhibition constants correspond well to the binding constants as shown in Fig. 1. Most probably, the specific binding sites of the inhibitors are identical with the inhibitor-sensitive components of the electron transport chain. This is supported by the fact that the total concentrations of specific binding sites (see Fig. 1) exhibit magnitudes similar to those of most of the electron carriers in photosynthesis (see Discussion).

2. Competitive binding of inhibitors by chloroplasts

If two inhibitors A and B compete for the same binding site X, the competitive character of binding can be theoretically simulated, provided that the binding constants K_a and K_b and the total concentration of binding sites x_t are known. For the mathematical simulation the following equations are required:

$$K_a = \frac{x \cdot a}{xa} \tag{4}$$

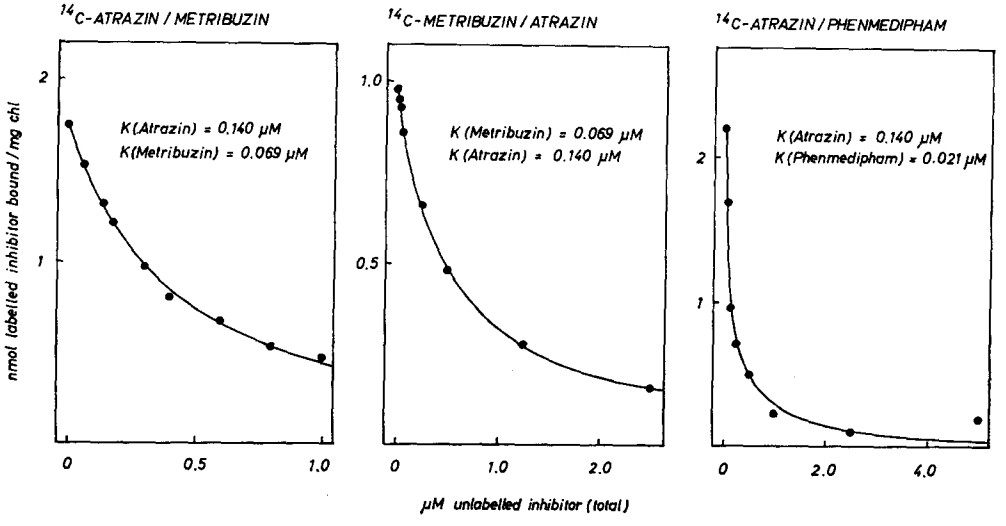


Fig. 3. Competitive binding of the inhibitor couples [^{14}C]Atrazin/Metribuzin, [^{14}C]Metribuzin/Atrazin and [^{14}C]-Atrazin/Phenmedipham by broken chloroplasts. The applied concentrations of labelled inhibitors were: 0.5 µM [^{14}C]-Atrazin and 0.125 µM [^{14}C]Metribuzin. The unlabelled inhibitors were added at the concentrations indicated in the figures. Theoretical competition curves (solid lines) were computed according to Eqn. 8 using the indicated K values, which were obtained from Fig. 1. The x_t values were calculated from the control experiments (absence of unlabelled inhibitors) according to $x_t = (K/a+1) \cdot xa$.

$$K_b = \frac{x \cdot b}{xb} \tag{5}$$

$$x_t = xa + xb + x \tag{6}$$

$$b_t = xb + b \tag{7}$$

The concentration of bound inhibitor A (xa) in dependence of total concentration of inhibitor B (b_t) is described by the quadratic equation

$$xa^2 + \left(\frac{a \cdot K_a(K_b + b_t - x_t) + a^2 \cdot K_b}{K_a^2 + a \cdot K_a}\right) xa - \frac{a^2 \cdot K_b \cdot x_t}{K_a^2 + a \cdot K_a} = 0 \tag{8}$$

which has the common form

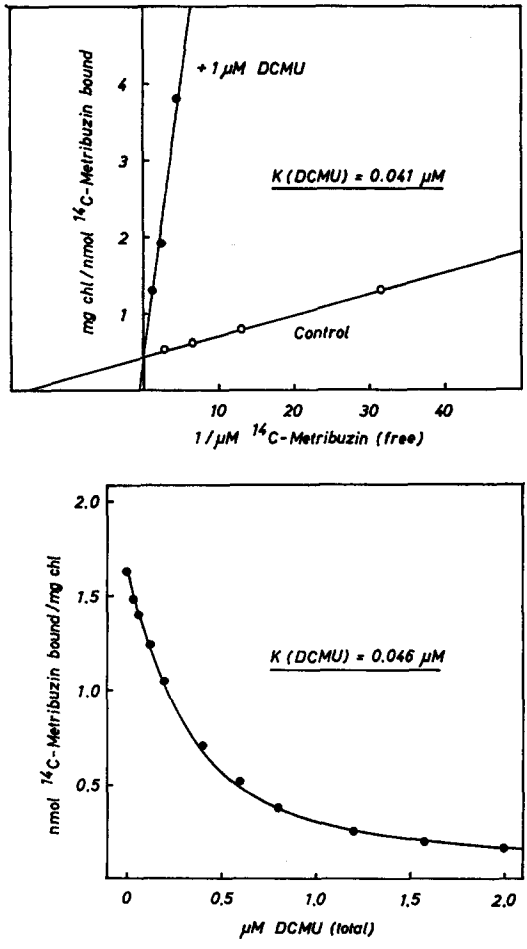


Fig. 4. Binding of [^{14}C]Metribuzin as affected by unlabelled DCMU. In the upper curve, the concentration of [^{14}C]Metribuzin was varied in the absence and presence of 1 µM DCMU. In the lower curve the DCMU concentration was varied at constant [^{14}C]Metribuzin concentration (0.125 µM). The indicated K values were computed from the curves.

$$xa^2 + p \cdot xa - q = 0$$

and can be resolved to

$$xa = -\frac{p}{2} + \sqrt{\left(\frac{p}{2}\right)^2 + q}$$

In Fig. 3 the binding curves of a labelled inhibitor in the presence of increasing concentrations of an unlabelled inhibitor are shown. Moreover the curves were simulated using Eqn. 8 and the K values as determined in Fig. 1. For all three inhibitor couples the experimental values fit the theoretical competition curves. Therefore we may conclude: (i) that the three herbicides Atrazin, Metribuzin and Phenmedipham bind to the same specific site of the thylakoid membrane, and (ii) that the binding competition method generally can be used for the identification of a common binding site and mechanism of two different inhibitors.

In Fig. 4, the binding of [^{14}C]Metribuzin was studied in the presence of DCMU. In the upper part, [^{14}C]Metribuzin concentration was varied in the absence and presence of 1 μM DCMU. In the double-reciprocal plot, both curves show the same intercept with the y-axis, indicating that DCMU is competitive to Metribuzin with respect to binding by thylakoid membranes. In the lower part of Fig. 4, binding of labelled Metribuzin in the presence of increasing DCMU concentrations is shown. From the competition curves the binding constant for DCMU can be computed using another expression of Eqn. 8:

$$K_b = \frac{K_a \cdot xa}{a} \cdot \left(\frac{b_t}{x_t - xa - \frac{K_a \cdot xa}{a}} \right) - 1 \qquad (9)$$

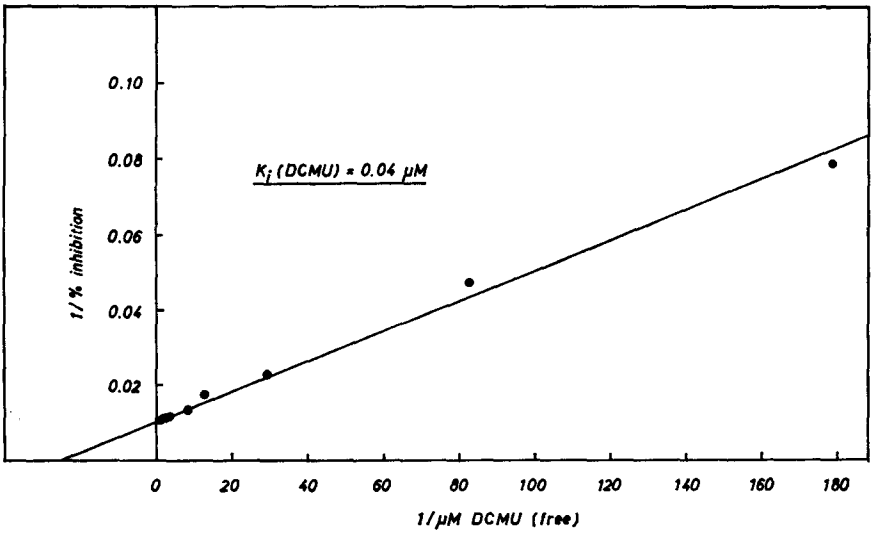


Fig. 5. Double-reciprocal plot of inhibition of uncoupled electron transport by DCMU. The control rate of ferricyanide reduction was 1230 μmol/mg chlorophyll · h.

From the two different experiments K values were computed. They were found to correspond well.

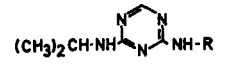
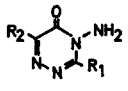
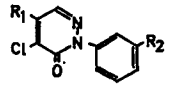
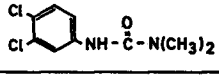
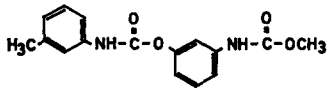
In Fig. 5, DCMU inhibition of electron transport is shown. For this experiment the equilibrium concentrations of free DCMU were calculated under the reasonable assumption that the concentrations of specific binding sites for DCMU and Metribuzin are equal.

From the double reciprocal inhibition curve, the inhibition constant for DCMU was obtained. This value satisfactorily corresponds to the binding constants as determined in Fig. 4. The results demonstrate that the sites of binding and inhibi-

TABLE II

BINDING CONSTANTS K AND INHIBITION CONSTANTS K_i OF DIFFERENT DCMU-TYPE INHIBITORS AS OBTAINED BY DIRECT MEASUREMENTS OR BY COMPETITION EXPERIMENTS

A and M indicate the K values when they were computed from competition experiments with [^{14}C]Atrazin and [^{14}C]Metribuzin, respectively, as carried out in Fig. 3. The theoretical competition curves were approximated to the experimental values by the mathematical method of least squares by using a computer program.

Formula		Name	K (μM)	K_i (μM)
	R: $-CH_2CH_3$	Atrazin	0.140	0.14
	R: $-CH(CH_3)_2$	Prometryn	0.033 A	0.03
	R_1: $-SCH_3$ R_2: $-C(CH_3)_3$	Metribuzin	0.069	0.07
	R_1: $-CH_2CH_3$ R_2: 	BAY 138992	0.085 M 0.090 A	0.09
	R_1: $-NH_2$ R_2: $-H$	Pyrazon	8.2 A	8.2
	R_1: $-NH_2$ R_2: $-CF_3$	SAN 9774	3.4 A	3.9
	R_1: $-NHCH_3$ R_2: $-CF_3$	SAN 9789	24.4 M 26.0 A	24.0
	R_1: $-N(CH_3)_2$ R_2: $-CF_3$	SAN 6706	3.2 A	3.8
		Diuron (DCMU)	0.041 M 0.046 M 0.037 A	0.04
		Phenmedipham	0.021	0.02

tion of the herbicides DCMU, Metribuzin, Atrazin and Phenmedipham are identical.

In a similar way, binding and inhibition constants for a series of pyridazinone derivatives were obtained. Although these substances are weak inhibitors of photosynthetic electron transport, they seem to react at the same site as DCMU. The binding and inhibition constants of the various investigated herbicides are summarized in Table II.

DISCUSSION

Binding of Metribuzin seems to be a monophasic process, while Atrazin and Phenmedipham exhibit biphasic binding curves. In the double-reciprocal plots, a high affinity reaction ("specific binding") can be separated from a low affinity binding process ("unspecific binding"). Our results in some respect seem to be different from those which have been reported by Izawa and Good [21]. These authors distinguished three different processes of interaction of the inhibitors DCMU, CMU and Atrazin with chloroplasts: (a) a partitioning of the inhibitors between the aqueous solvent and the chloroplast phase, (b) an irreversible binding of about 1 inhibitor molecule per 1000 chlorophyll molecules, which is saturated at low concentrations and not associated with inhibition, and (c) a reversible binding closely related to inhibition of electron transport.

In our experimental process, (a) is irrelevant, because the chloroplasts we used were naked disrupted thylakoid membranes. Therefore, inhibitor retention must be referred exclusively to the thylakoid membrane itself. Process (b) does not correspond to the unspecific binding reaction in our experiments, because of the completely different concentration dependencies. However, process (c) obviously coincides with the specific binding reaction as suggested by us. It is highly probable that the specific binding site is identical with the electron carrier of the electron transport chain which is blocked by the inhibitors.

Maximum binding of the three used labelled inhibitors by the specific binding sites ranges between 2.4 and 4.0 nmol/mg chlorophyll. Assuming one binding site per electron carrier molecule, this would mean a ratio of 1 carrier molecule per 300–500 chlorophyll molecules. This ratio corresponds well to the relative concentrations of other known electron carriers in photosynthesis, except for plastoquinone. The molar ratio of *P*-700 is 1 per 400 chlorophylls [24, 25]. The ratio between *P*-700 on one hand and ferredoxin, cytochrome *f* and plastocyanin on the other hand is 1 [26, 27]. For chlorophyll a_{II} and *c*-550, a ratio of 1 per 300 chlorophyll molecules has been determined [28]. According to Siggel et al. [29] the concentration of reaction center II is 1 : 660 chlorophyll molecules. In contrast to our data, which agree well with these numbers, the determination of specific DCMU binding sites by indirect methods yielded much lower concentrations [21, 29].

To obtain maximum sensitivity of steady state electron transport to an inhibitor, other rate limiting factors have to be excluded. Under these conditions, a hyperbolic inhibition curve in dependence of inhibitor concentration can be expected. Fig. 2 shows that this is the case under our experimental conditions (uncoupling and saturating light intensities). It should be noticed that uncoupling by CF_1 stripping yielded the most reliable results. Inhibition curves of coupled or incompletely uncoupled chloroplasts exhibited a sigmoidal shape. On the other hand, at limiting light

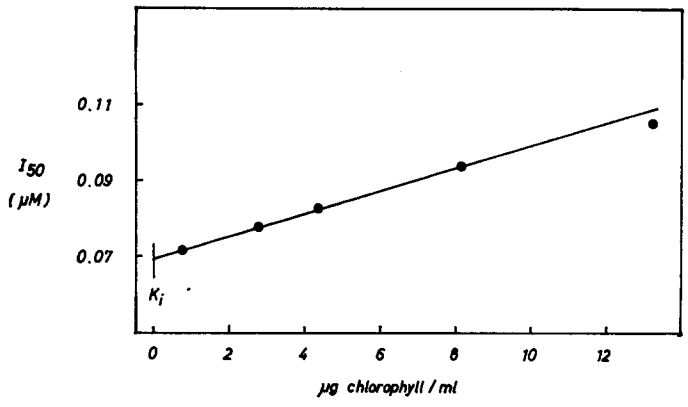


Fig. 6. I_{50} values of inhibition of uncoupled electron transport by Metribuzin in dependence of chlorophyll concentrations used in the assay.

intensities the effect of a given inhibitor concentration on uncoupled electron transport was lower than in saturating light. Inhibitor constants were computed by the inhibition of uncoupled electron transport and simultaneous determination of equilibrium concentration of free inhibitor. The fact that inhibition constants correspond well to the binding constants may be taken as a direct proof for the identity of specific binding site and inhibitor-sensitive receptor component of electron transport.

Usually the effectiveness of an inhibitor is expressed by its I_{50} value, i.e. the concentration which produces 50 % inhibition. In contrast to K_i which is an actual equilibrium constant, I_{50} values depend on the chlorophyll contents used in the assay (Fig. 6). The largest deviation between I_{50} and K_i can be observed in the case where a very effective inhibitor is used at high chlorophyll concentrations. However, for a given inhibitor the relationship between I_{50} and chlorophyll concentration is linear. This can be deduced from a theoretical consideration.

At 50 % inhibition, the concentrations of unoccupied and occupied specific binding sites are equal and K_i corresponds to the concentration of free inhibitor:

$$x = xa = \tfrac{1}{2}x_t \tag{10}$$

$$K_i = a \tag{11}$$

Under the same conditions

$$I_{50} = a_t = a + xa \tag{12}$$

By combination of the equations, the following relationship between I_{50} and K_i is obtained:

$$I_{50} = K_i + \tfrac{1}{2}x_t \tag{13}$$

Though x_t is directly related to chlorophyll concentration, it cannot be determined in an inhibition experiment. Eqn. 13 indicates that K_i can be easily ascertained by measurement of I_{50} values at two different chlorophyll concentrations and extrapolation to zero chlorophyll. The validity of this theoretical approach is demonstrated in the experiment shown in Fig. 6.

Inhibitor binding and inhibition are probably more complex processes. However the results show that they can be satisfactorily described by simple adsorption isotherms. The consistency of this approach is also confirmed by the results of our competition experiments, which agree with the theoretical expectations.

The competition experiments show that phenylureas, biscarbamates, triazines, triazinones and pyridazinones inhibit electron transport by interaction with the same component according to the same mode of action. Therefore the search for a common substructure is a legitimate way to draw conclusions on the mechanism of their interaction with the biological component.

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