Phytochemistry, 1972, Vol. 11, pp. 2693 to 2699. Pergamon Press. Printed in England.

ASSOCIATION OF MONURON IN CHLOROPLASTS IN RELATION TO INHIBITION OF HILL REACTION*

S. S. LEE and S. C. FANG

Department of Agricultural Chemistry, Oregon State University, Corvallis, OR 97331, U.S.A.

(*Received* 10 *February* 1972)

Key Word Index—*Spinacea oleracea*; Chenopodiaceae; monuron; chloroplast binding sites; Hill reaction.

Abstract—The absorption of monuron by spinach chloroplasts from aqueous suspension medium and its relation to the inhibition of Hill reaction has been investigated using ^{14}C-labeled monuron. Attempts were made to estimate the distribution of monuron within the chloroplast. Two compartments were found to exist for monuron absorption. The association of monuron in compartment 1 was responsible for the inhibition of Hill reaction; the compound could be removed from the chloroplast by washing, with concomitant restoration of activity. The amount of monuron absorbed by the chloroplasts correlated with the degree of inhibition regardless of oxidant ($Fe(CN)_6^{3-}$, DPIP, or $NADP^+$) used, pH of the reaction mixture, light intensity, or chloroplast integrity. Monuron absorption by the chloroplast increased with a shift in pH of the reaction medium from 7·8 to 6·8. However, this increase of uptake was mainly due to an increase of inactive binding site. A study of the activation energy between the monuron molecule and the binding sites of the chloroplast revealed that the association was due to some very weak physical adsorption.

INTRODUCTION

NUMEROUS reports have been presented during the last two decades to indicate that the substituted ureas inhibit the Hill reaction of isolated chloroplasts[1-4] and the degree of inhibition by these herbicides was found to be associated with their potency of killing plants. From the work of Bishop,[5] Jagendorf and Avron,[6] and Vernon and Zaugg,[7] the action of the substituted ureas was further narrowed down to the oxygen-evolving systems (PS II).

Wessels and Van der Veen[1] suggested that the phenylureas might associate with the cyclopentanone ring of chlorophyll through hydrogen bonds and thus prevent the energy transfer reaction. A different view was postulated by Izawa and Good[8] who considered that the substituted ureas inhibit the Hill reaction by reacting with the unidentified catalytic center responsible for oxygen evolution.

The inhibitory effect of monuron on the Hill reaction of spinach chloroplasts with three of the most commonly used oxidants (ferricyanide, dichlorophenol indophenol, and $NADP^+$) has been reported by Jagendorf and Avron,[6] Jagendorf and Margulies,[9] Izawa

* Technical Paper No. 3306 Oregon Agricultural Experiment Station. This work was supported in part by a research grant ES-00141-10 from U.S. Public Health Service, Institute of Environmental Health Science.

[1] J. S. C. WESSELS and R. VAN DER VEEN, *Biochem. Biophys. Acta* **19,** 548 (1956).
[2] J. D. SPIKES, *Plant Physiol.* **31,** xxxii (1956).
[3] A. R. COOKE, *Weeds* **4,** 397 (1956).
[4] D. E. MORELAND, *Proc SWC* **10,** 146 (1957).
[5] N. I. BISHOP, *Biochem. Biophys. Acta.* **27,** 205 (1958).
[6] A. T. JAGENDORF and M. AVRON, *Arch. Biochem. Biophys.* **80,** 246 (1959).
[7] L. P. VERNON and W. S. ZAUGG, *J. Biol. Chem.* **235,** 2728 (1960).
[8] S. IZAWA and N. E. GOOD, *Biochim. Biophys. Acta* **102,** 20 (1965).
[9] A. T. JAGENDORF and M. M. MARGULIES, *Arch. Biochem. Biophys.* **90,** 184 (1960).

and Good,[8] and Bamberger *et al.*[10] At low light intensity the I_{50} was found to be 3×10^{-7} M for both $Fe(CN)_6^{3-}$ and DPIP reduction while at high light intensity these values were $6–9 \times 10^{-7}$ M for $Fe(CN)_6^{3-}$ and DPIP and $4–5 \times 10^{-6}$ M for $NADP^+$ reduction. These consistent observations suggest that the 'chloroplast reaction' is less sensitive to monuron at high light intensity than at low light intensity. Using a bioassay method for the measurement of monuron absorption Izawa and Good[8] reported that the amount of monuron absorbed by the isolated chloroplasts was closely related to the degree of inhibition, but varied under high and low light conditions. At 50% inhibition, the concentrations of monuron in the chloroplasts and at the active center were higher under high light conditions. This would imply that either the inhibitor-sensitive center in the chloroplasts is light-dependent or the differential response is due to the affinity of binding which may be affected under different light conditions. In this investigation we describe the mechanism of monuron association with the chloroplasts and its relation to the inhibition of Hill reaction under a variety of experimental conditions.

RESULTS

Relationship of Monuron Uptake and Inhibition

Our results indicate that at low light intensity the I_{50} was roughly equal to $1·6 \times 10^{-7}$ M for both $Fe(CN)_6^{3-}$ and DPIP reduction and $2·4 \times 10^{-7}$ M for $NADP^+$ reduction. At high light intensity these values were 7×10^{-7} M and 2×10^{-6} M respectively. The results agree with those reported by previous workers.[6,9–11] There was no difference in the inhibition curve obtained either with whole chloroplasts or with broken chloroplasts. However, the pH of the reaction mixture greatly affects inhibition by monuron (Fig. 1).

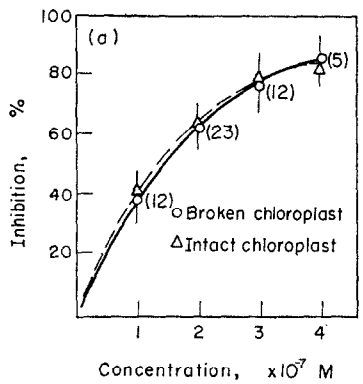

FIG. 1(a). EFFECT OF CHLOROPLAST INTEGRITY ON THE INHIBITION OF HILL REACTION BY MONURON. THE NUMBERS IN PARENTHESES ALONG THE CURVE INDICATE THE NUMBER OF EXPERIMENTS. pH OF THE REACTION MIXTURE WAS 7·8 AND DPIP WAS USED AS HILL OXIDANT.

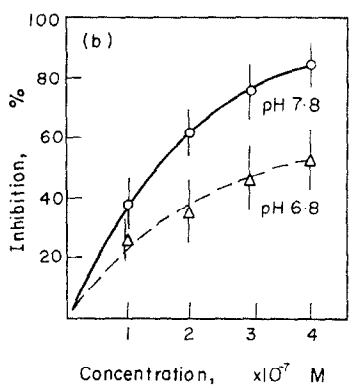

FIG. 1(b). EFFECT OF pH OF THE REACTION MIXTURE ON THE INHIBITION OF HILL REACTION BY MONURON. EXPERIMENTAL CONDITIONS WERE SIMILAR TO THOSE IN FIG. 1(a). THE VERTICAL BAR IS THE DEVIATION FROM THE MEAN VALUE.

It can be seen from Table 1 that the degree of inhibition of the Hill reactions by monuron was linearly proportional to the amount of monuron absorbed by the chloroplasts. Of the three oxidants used, the lower inhibition of $NADP^+$ reduction was parallelled by absorption

[10] E. S. BAMBERGER, C. C. BLACK, C. A. FEWSON and M. GIBBS, *Plant. Physiol.* **38,** 483 (1963).
[11] L. J. LABER and C. C. BLACK, *J. Biol. Chem.* **244,** 3463 (1969).

TABLE 1. RELATIONSHIP OF MONURON ASSOCIATION WITH CHLOROPLAST AND INHIBITION OF HILL REACTION

Monuron concn ($\times 10^{-7}$ M)	Ferricyanide reduction		DPIP reduction		$NADP^+$ reduction	
	Inhibition (%)	Radioactivity* (cpm)	Inhibition (%)	Radioactivity (cpm)	Inhibition (%)	Radioactivity (cpm)
1	24	80	33	95	26	70
2	56	157	57	160	42	128
3	75	225	70	190	50	160
4	90	255	82	235	—	—
5	100	340	100	280	—	—

* cpm/0·02 mg chlorophyll; 1 μmol of ^{14}C-monuron = 4150 cpm.

Experimental conditions and the control rate of Hill reaction are described in Experimental. Hill reaction of the broken chloroplasts were carried out at pH 7·8 and low light intensity.

of monuron. When the Hill reaction was carried out with DPIP as oxidant under the high light or the low light condition, using either the whole or the broken chloroplast preparation, the degree of inhibition was always related to the absorption of monuron (Fig. 2). However,

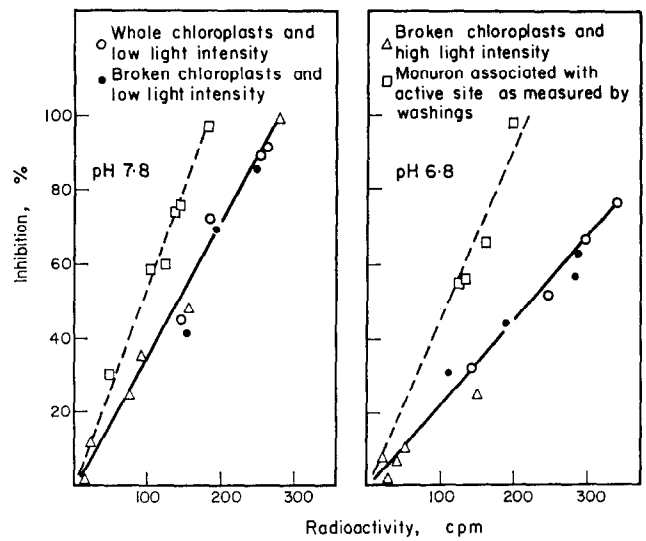

FIG. 2. RELATION BETWEEN THE AMOUNT OF MONURON ASSOCIATED WITH CHLOROPLASTS AND THE DEGREE OF INHIBITION UNDER DIFFERENT EXPERIMENTAL CONDITIONS.

a difference in slope was observed between those carried out at pH 7·8 and those at pH 6·8, at which pH approximately 60% more monuron absorption was required for the same degree of inhibition.

Compartmentation of Monuron in the Chloroplasts

Figure 3 indicates a biphasic removal of absorbed monuron by successive washes. The monuron in the fast removal compartment (compartment 1) exchanged very rapidly with the rinsing solution and presumably representing the adsorbed monuron on the outermost surface. Removal of the monuron from compartment 1 completely restored the Hill activity

of isolated chloroplasts. More than 90% of the absorbed monuron was in the compartment 1, all of which can be removed after two washings. Chloroplast integrity seemed to affect the rate of monuron removal from compartment 1. Figure 3 shows the comparison of the rate of removal and the relative amount of absorption of monuron in this compartment between the broken and intact chloroplasts of spinach. Greater surface area of broken chloroplasts

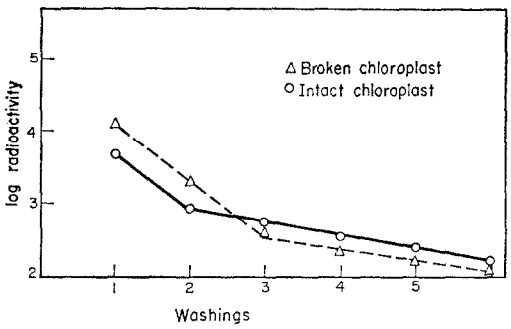

FIG. 3. COMPARISON OF THE RATE OF REMOVAL AND RELATIVE AMOUNT OF ABSORPTION OF MONURON IN COMPARTMENT I AND II BETWEEN BROKEN AND INTACT CHLOROPLASTS.

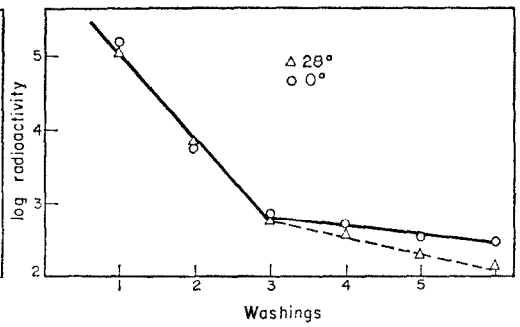

FIG. 4. COMPARISON OF THE RATE OF REMOVAL OF MONURON IN COMPARTMENT I AND II AT TWO DIFFERENT TEMPERATURES.

and faster rate of diffusion may be responsible for this difference. The monuron of the slow removal compartment (compartment 2) was not responsible for the suppression of Hill reaction activity. In addition to these two compartments, a small amount of monuron was bound very tightly to the chloroplasts membrane and could not be removed by washing (irreversible compartment).[8] This binding is presumably the consequences of biochemical reactions between the monuron molecule and chloroplast constitutents; and the amount of monuron bound to this compartment largely depends on both the temperature and the time of incubation.

In an attempt to ascertain the nature of association between the monuron molecule and chloroplasts, chloroplast preparations, which had been incubated in 1×10^{-5} M ^{14}C-labeled monuron for 1 hr, were washed several times at 0 and 28° separately (Fig. 4). The activation energy calculated according to the Arrhenius equation was approximately 0 and 4 kcal per mol for the association of monuron in compartments 1 and 2, respectively. This observation indicates that interaction of monuron with chloroplast constituents (compartment 2) involves rather weak bonds, such as electrostatic interaction, van der Waals forces and perhaps hydrogen bonding, and that this interaction is not responsible for the inhibition of Hill reaction.

DISCUSSION

Irrespective of which Hill oxidants are used, the amount of monuron absorption is linearly related to the inhibition (Table 1). This implies that the unidentified reactive sites between monuron and the chloroplasts are common for all three reactions. With the same levels of monuron, the inhibition of $NADP^+$ reduction was less in comparison to the inhibition of DPIP or $Fe(CN)_6^{3-}$ reduction. This variation could be due to components of the reaction mixture, some of which might compete with chloroplasts for the binding of monuron.

High light intensity greatly alters the inhibition curve of monuron on Hill reaction; it also affects the concentration of monuron in compartment 1 of chloroplasts, but it does not change the relationship of inhibition of Hill reaction to the concentration of monuron in the chloroplast. The pH also similarly affects the inhibition of Hill reaction by monuron. In both cases, the I_{50} values measured under low light conditions were increased from $1{\cdot}6 \times 10^{-7}$ M at pH 7·8 to $4{\cdot}0 \times 10^{-7}$ M at pH 6·8, and those measured at pH 7·8 were increased from $1{\cdot}6 \times 10^{-7}$ M at low light intensity to 7×10^{-7} M for high light intensity. Only pH affects the slope of a plot between the inhibition and the monuron adsorption by chloroplasts. More monuron association was required to give the same inhibition of the reaction when the pH was changed from 7·8 to 6·8 (Fig. 2). A similar observation has been reported with simazine and propanil.[12] It is assumed that high light intensity may alter the size and permeability of isolated chloroplasts, thereby affecting the extent of monuron uptake. Changing pH may affect the internal organization of isolated chloroplasts by opening up additional inactive binding sites, thereby requiring more monuron association to achieve the same degree of inhibition. Our results on the percent inhibition vs. monuron association with chloroplasts, as shown in Fig. 2, gives abscissa intercepts near 0 at pH 7·8 and 6·8. However, if the curves were plotted with the data obtained only from low light experiments we would also obtain a plot similar to that reported by Izawa and Good.[8]

Unless the affinity between the monuron molecule and the inactive site is much greater than that for the active site it would be inconceivable to think that a preferential binding of inactive site would take place prior to the association of the active site. Furthermore, the study of monuron removal from chloroplasts by repeated washing always shows the presence of both compartments 1 and 2, regardless of whether the chloroplasts were incubated at a concentration which gave about 20% or 100% inhibition. This indicates that the equilibration of monuron takes place between the active and inactive sites in addition to the equilibration of bound monuron and free monuron within the chloroplast. The estimation of inactive site as indicated may not include the total inactive site present. Since the association of monuron in compartment 1 is responsible for the inhibition of Hill activity and the amount of monuron in this compartment can be determined by the washing of pretreated chloroplasts, we measured the amount of monuron in compartment 1 at both pH 7·8 and 6·8 and at various degrees of inhibition. These results (Table 2) reveal that the inactive site as measured increased as the concentration of monuron increased. Also, the amount of monuron in compartment 2 increased as the pH of the reaction mixture changed from 7·8 to 6·8. This evidence supports the concept that pH affects the internal organization of chloroplasts thereby increasing the inactive binding sites (Fig. 2). Monaco[12] studied the partition and distribution of simazine and propanil in the spinach chloroplast and reported that the pH of the reaction mixture affected the partition of the inhibitors between the medium and the chloroplast, Ip:Im ratio; and also the distribution of inhibitors between the inactive and active sites within the chloroplast. With simazine, the change of distribution was a result of increasing the inactive site; with propanil, the change was a combination of an increase of inactive site and a slight decrease of active site.

A comparison of the partitioning behavior of monuron for two different pH reveals that more monuron is taken up by the chloroplast at pH 6·8 than at 7·8. Furthermore, at pH 7·8 less monuron is needed to give the same levels of inhibition. These results provide a full explanation for the difference in inhibition curves observed between these two pH values.

[12] T. J. MONACO, Ph.D. Thesis, North Carolina State University at Raleigh N.C. (1968).

Table 2 also shows the ratio of monuron to chlorophyll in the chloroplast at three levels of inhibition. At I_{50} level, the average of 535 we obtained for the ratio of chlorophyll to monuron at pH 6·8 agrees quite well with the values of 430 and 620 reported by Izawa and Good[8] who measured the reaction at pH 7·4. At 100% inhibition the ratio is one molecule of monuron for every 240 molecules of chlorophyll when measured at pH 6·8; and

TABLE 2. THE PARTITIONING AND DISTRIBUTION OF MONURON IN SPINACH CHLOROPLASTS AT DIFFERENT INHIBITION LEVELS

Inhibition level	pH	Monuron concn (M) Medium (I)M ($\times 10^{-7}$)	Chloroplasts (I)p Total* ($\times 10^{-5}$)	Inactive ($\times 10^{-5}$)	Active ($\times 10^{-5}$)	Partition ratio (I)p:(I)M	Chlorophyll:monuron ratio† Total	Active
I_{25}	7·8	0·6	0·97	0·23	0·74	162	1850	2780
I_{50}	7·8	1·3	2·0	0·50	1·50	154	900	1260
I_{75}	7·8	2·7	3·2	0·9	2·28	119	556	795
I_{25}	6·8	1·0	1·7	0·8	0·89	170	1070	2030
I_{50}	6·8	3·1	3·4	1·6	1·77	110	535	1020
I_{75}	6·8	5·9	5·2	2·6	2·60	88	344	693

* 1 mμmole of ^{14}C-monuron = 4150 cpm assumes the volume of chloroplasts containing 1·0 mg chlorophyll is 62 μl and 25 μg chlorophyll is 1·55 μl.[8]

† Photosynthetic particles containing 25 μg (28 mμmoles) chlorophyll were used.

The experimental conditions were described in the text DPIP photoreduction was carried out under low light intensity.

one for every 350 molecules at pH 7·8. These values are in agreement with the report of Bishop[13] that approximately one molecule of monuron is needed for every 200 molecules of chlorophyll to cause 100% inhibition of Hill reaction.

EXPERIMENTAL

Broken chloroplasts were prepared from fresh spinach leaves obtained from a local market according to the method described by Laber and Black[11] and were suspended in buffer diluted ten-fold. Intact chloroplasts were isolated according to the method of Walker[14] and were suspended in a medium containing 0·33 M sucrose, 0·1% $MgSO_4$ and 0·1% NaCl at pH 7·0.

Hill reactions were carried out in cuvettes having an optical path of 10 mm which were illuminated with two 300-W movie flood lamps through a layer of water (high light intensity, 32 000 lx) or with a band of four 20 W fluorescent lights (low light intensity, 5400 lx). Photoreduction of Hill oxidants was measured spectrophotometrically at the following wavelength: 420 nm for ferricyanide; 600 nm for 2,6-dichlorophenol-indolephenol (DPIP) and 340 nm for $NADP^+$. All experiments were carried out at room temperature (about 25°). The control rates of Hill reaction under different experimental conditions as expressed in μmol reduced/mg chlorophyll/hr were as follows: 50–70 for DPIP, 150 for $Fe(CN)_6^{3-}$ and 45 for $NADP^+$ reduction at pH 7·8 and low light intensity with broken chloroplasts; 120–150 for DPIP, 360–450 for $Fe(CN)_6^{3-}$ and 140–150 for $NADP^+$ reduction at pH 7·8 and high light intensity with broken chloroplasts; 30 for DPIP reduction at pH 7·8 and low light intensity with whole chloroplasts; 80 for DPIP reduction at pH 6·8 and low light intensity with broken chloroplasts; and 55 for DPIP reduction at pH 6·8 and low light intensity with whole chloroplasts.

The reaction mixture in a final volume of 3 ml contained chloroplasts equivalent to about 10–20 μg of chlorophyll, 1·2 μmol of ferricyanide, 45 μmol of methylamine-HCl and 150 μmol of Tricine-NaOH buffer, pH 7·8 for the reduction of ferricyanide; a mixture contained chloroplast (10–20 μg chlorophyll), 0·08 μmol of DPIP dye, 10 μmol of potassium chloride and 40 μmol phosphate buffer (pH 7·8, otherwise specified) for

[13] N. I. BISHOP, in *Plant Physiology* (edited by F. C. STEWARD), Vol. IB, p. 223, Academic Press, New York (1960).

[14] D. A. WALKER, *Biochem. J.* **92**, 22C (1964).

the reduction of DPIP; and a mixture of chloroplasts (20–30 μg chlorophyll), 0·6 μmol of $NADP^+$, 6 μmol of $MgCl_2$, 75 μmol of Tricine-NaOH (pH 7·8) and a saturated amount of spinach ferridoxin was employed for $NADP^+$ reduction.

Study of inhibition on Hill reaction by 3(*p*-chlorophenyl)-1,1-dimethylurea (monuron) was carried out with a sample of carbonyl-^{14}C-labeled monuron with a specific activity of 6760 dpm/mμmol. Various amounts of ^{14}C-labeled monuron were added to a series of tubes containing 3 ml of reaction mixture to give a concentration ranging 0·5–10 $\times$ 10^{-7} M of monuron. After illumination and measurement of photoreduction, the cuvettes were centrifuged immediately at 1000 *g* for 10 min to sediment the chloroplasts. An aliquot of the supernatant which contained the unabsorbed ^{14}C-monuron was carefully taken. The radioactivity of these aqueous solutions was determined by liquid scintillation technique, utilizing 10 ml scintillation medium of Prockop and Ebert.[15] The chloroplast pellet was extracted with 1·0 ml of ethanol and 0·1 ml of the extract was removed for counting. ^{14}C-Benzoic acid was used as an internal standard for correction of quenching. The radioactivity of the alcohol-insoluble residue was counted with a gas-flow G–M detector.

To study the relationship of monuron release from the chloroplasts and its restoration of Hill reaction activity, chloroplasts were incubated in a 5 ml reaction mixture containing 25 μmol of Tricine-NaOH buffer, 75 μmol of methylamine HCl with or without 1 mμmol of ^{14}C-monuron at 0° for 1 hr. The mixtures were then transferred to tubes and centrifuged. After removal of the supernatant solution the pellets were washed 6 times successively with the same reaction medium without ^{14}C-monuron. All washings were freeze-dried separately and the radioactivity in the washings was counted. Duplicate tubes were tested for Hill reaction activity after one and two washes. To check whether or not a loss of ^{14}C-monuron may occur during freeze-drying, a known amount of ^{14}C-monuron in 3 ml of reaction medium was subjected to the same procedure. In general, the loss of radioactivity was small and insignificant if the sample were removed soon after drying. Chlorophyll concentration was determined according to the method of Arnon.[16]

[15] D. J. Prockop and P. S. Ebert, *Anal. Biochem.* **6**, 263 (1963).
[16] D. I. Arnon, *Plant Physiol.* **24**, 1 (1949).