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PHOTOINACTIVATION OF MONURON, 3-(*p*-CHLOROPHENYL)-1,1-DIMETHYLUREA, BY RIBOFLAVIN 5-PHOSPHATE

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SUMMARY

FMN has been found to cause a photochemical inactivation of monuron, 3-(*p*-chlorophenyl)-1,1-dimethylurea, and other substituted phenylureas. A high-molecular-weight compound, which was no longer antiphotosynthetic, was isolated from the monuron–FMN reaction mixtures. A close relationship was found between the ability of phenylurea compounds to react photochemically with FMN and their ability to inhibit photosynthesis. Molecular models of FMN and 3-(3,4-dichlorophenyl)-1-1-dimethylurea, diuron, show surprising similarity in structure. These findings suggest that the monuron inhibition of photosynthesis may result from an interaction of monuron with FMN or a flavoprotein in the photosynthesis pathway.

INTRODUCTION

The herbicide monuron, 3-(*p*-chlorophenyl)-1,1-dimethylurea[1], and other substituted ureas are among the most specific and sensitive inhibitors of photosynthesis known[2,3]. A molar ratio of monuron of only 1/50th to 1/200th that of chlorophyll will completely inhibit photosynthesis of algae. Light re-emission studies indicate that the site of monuron reaction is early in the energy-transfer process[4]. Studies with hydrogen-adapted *Scenedesmus* indicate that the urea herbicides are acting on the oxygen evolution of photosynthesis[5]. The exact nature of the monuron inhibition, or for that matter, the oxygen-liberating pathway, is not known.

FMN has been implicated as a cofactor for photosynthetic phosphorylation in isolated chloroplasts and as a component of the electron-transport system of photosynthesis[6,7]. The effect of monuron on cyclic photosynthetic phosphorylation with phenazine methosulfate or FMN as cofactors[8,9] suggest that the monuron block of photosynthesis may be close to the site of the FMN electron-transport system. It was therefore of interest to study the effect of FMN on the inhibition of photosynthesis by monuron. The results of this study are given in this paper.

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METHODS

Chlorella pyrenoidosa and *Euglena gracilis* were grown as previously described[4,10].

The rate of CO_2 fixation by the algal suspensions was determined by measuring the difference in CO_2 level of air, enriched with CO_2 to about 600 ppm, before and after passing through the algal solution. A Mine Safety Appliance Company Model-200 Infrared CO_2 Analyzer was used to measure the carbon dioxide concentrations.

The photochemical inactivation of monuron by FMN was carried out in flat-sided flasks which were illuminated by a 150-W G.E. reflector spot lamp. Heat from the light was filtered out with a water-cooled cell, made of "Plexiglas" acrylic resin.

The concentration of the monuron remaining in the solution after the photochemical reaction with FMN was estimated by adding an aliquot of the reaction mixture to a culture of *Chlorella* or *Euglena* which contained a standard excess of FMN. Under these conditions the time required to overcome the monuron inhibition of CO_2 fixation of the algae was proportional to the monuron concentration present in the added aliquot provided that the illumination intensity and FMN concentration of the culture were held constant.

Paper chromatography was used in studying the monuron–FMN photoreaction products. FMN, riboflavin, and the photoreaction products of FMN were located on the chromatograms by their fluorescence under ultraviolet light. Monuron and its reaction products were detected by following the radioactivity from ^{14}C-tagged monuron. When the level of ^{14}C activity was low, the chromatograms were cut into sections and counted in a scintillation counter by extracting each section with the scintillation medium. In some instances the radioactivity was sufficiently high to follow the activity on the chromatograms by scanning the paper with a flow counter.

The monuron–FMN reaction product (I) was prepared as follows: 15 g of FMN was added to 6 l of distilled water in an 8-l bottle. To this was added 5 g of monuron which had been dissolved in 400 ml of methyl ethyl ketone. The bottle was placed between 6 20-W fluorescent lamps and air was bubbled continuously through the solution. After 3–4 days of light exposure, a considerable amount of precipitate had formed in the solution. This precipitate was dissolved by adding 600 ml of methyl ethyl ketone, and after 1–2 h a ball of gummy material formed on the surface of the solution. This was removed by washing with water and ether. An infrared spectrum indicated this material was identical to the material purified by paper chromatography. Further purification was carried out by precipitation from methyl ethyl ketone with *n*-butyl ether and from methanol with ether.

RESULTS AND DISCUSSION

The addition of monuron to a culture of *Chlorella* results in the immediate inhibition of photosynthesis and a return of the CO_2 concentration of the exit gas to the dark respiratory level. The addition of FMN to the culture returns the CO_2 fixation rate to the pre-inhibition level within a few minutes. The addition of a second aliquot of monuron results in only a temporary inhibition of CO_2 fixation of the culture (see Fig. 1).

Since FMN and riboflavin have been reported to cause the photodestruction of

a number of biologically active compounds, a check was made as to the light stability of a solution containing both monuron and FMN. One portion of a monuron–FMN solution was exposed to white light and a second portion was kept in the dark. After 5 h the solutions were tested for monuron by adding a suitable aliquot to a *Chlorella* culture. The solution exposed to the light no longer caused inhibition of CO_2 fixation, while the dark control gave full inhibition. From these results it was concluded that FMN photochemically inactivated monuron both in the presence and absence of light.

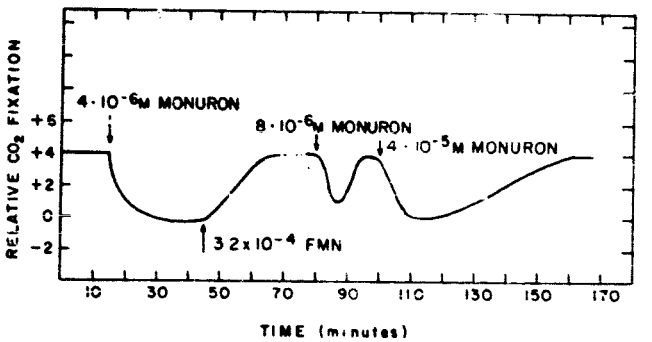


Fig. 1. Effect of FMN on the monuron inhibition of CO_2 fixation of a culture of *Chlorella pyrenoidosa*.

This conclusion was further substantiated by examining photosynthesis by *Euglena* in red light of sufficient intensity to saturate photosynthesis. FMN does not absorb in the red region of the spectrum, and under these conditions FMN did not prevent inhibition of photosynthesis until the red cellophane filter was removed from the light source.

The photochemical inactivation of monuron by FMN was found to take place in an aqueous buffer solution of pH 3.6–6.5. The citric acid, glycine and glutamic acid of the *Euglena* medium had no noticeable effect on the rate of inactivation of monuron. The photoinactivation reaction did not take place under anaerobic conditions, under acidic or basic conditions (0.2 N HCl and 4 N NH_4OH, respectively), or in methanol–water solutions (1:1). The photoinactivation experiments carried out in the presence of *Chlorella* or *Euglena* required FMN–monuron ratios between 1:2 to 1:1; however, when the photoreaction was carried out in the absence of the algae in an aqueous solution at pH 5.2 (0.05 N phosphate), the molar ratio of FMN to monuron was decreased to as much as 1:20.

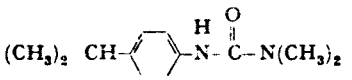
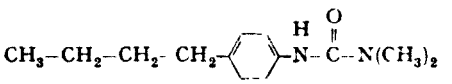
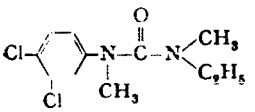
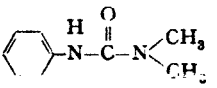
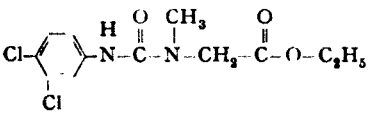
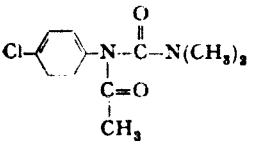
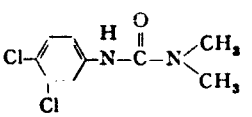
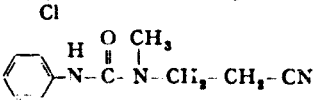
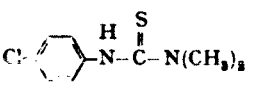
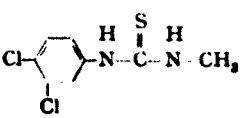
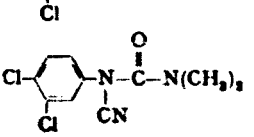
The inactivation of monuron by FMN has some similarity to the photooxidation of indoleacetic acid by FMN. However, other fluorescent dyes which have been reported to cause photoinactivation of indoleacetic acid and other compounds[11,12] did not cause inactivation of monuron. For example, fluorescein, eosin, erythrosin, β-carotene, and methylene blue did not cause photoinactivation of monuron when tested under conditions which were effective with FMN. Riboflavin was not active when added to the algal solution containing monuron but was active in the tests in which no algae were present during the light exposure.

A number of other substituted ureas have been tested for their ability to interfere with the photochemical inactivation of monuron by FMN (see Table I). The test used to indicate reactivity of a non-antiphotosynthetic substituted urea was an indirect

TABLE I

EFFECT OF UREA DERIVATIVES ON FMN INACTIVATION OF MONURON

The tests were made with 100 ml of an *Euglena* culture which was carrying out photosynthesis at a steady rate. 5 ml of a 200 ppm solution of the test compound was added to determine its effect on CO_2 fixation, after which was added 1 ml of 200 ppm monuron and 2 ml of 1000 ppm FMN. The normal inactivation time of the monuron under these conditions was 15–20 min.

Urea derivatives	*Effect on CO_2 fixation*	*Effect on inactivation time of monuron*
	Inhibition	Increased inactivation time
	Inhibition	Increased inactivation time
	No effect	No effect
	No effect	No effect
	No effect	No effect
	Inhibition	Increased inactivation time
	Inhibition	Increased inactivation time
	No effect	No effect
	No effect	Increased inactivation time
	No effect	Increased inactivation time
	Inhibition	Increased inactivation time

one since the photoreaction of these compounds could not be followed directly by CO_2-fixation measurements. Evidence for reactivity with FMN was taken as the ability of a compound to increase the time required for an excess of FMN to inactivate a known amount of monuron. Although this assumption may not be completely justified, it could be shown that the time required for FMN to overcome the inhibition of CO_2-fixation by monuron was directly proportional to the amount of monuron added to the solution. The addition of compounds which reacted with FMN in a similar fashion to that of monuron should increase the total time required for the inactivation of monuron. This was indeed the case with substituted urea compounds which were themselves antiphotosynthetic. Thus, the time to inactivate a compound + monuron was proportional to the time required to inactivate the compound and monuron separately.

There is surprisingly good correlation between the ability of the compounds to react photochemically with FMN and their antiphotosynthetic properties. Thus, substituted ureas which are antiphotosynthetic (inhibit CO_2 fixation of *Chlorella* cultures) are also inactivated by FMN; however, quite similar ureas which are poor inhibitors of photosynthesis do not appear to undergo a photochemical reaction with FMN. However, some thio derivatives of substituted ureas were exceptions. These were not antiphotosynthetic but did lengthen the time of the FMN inactivation of the monuron solutions. It is possible that the discrepancy is due to oxidation of the thio compounds to the normal urea derivatives which then undergo a FMN photoinactivation. However, no evidence for this reaction was found.

In order to define further the nature of the monuron–FMN reaction, several known characteristics of FMN were examined to see if they were involved in the inactivation of monuron. Riboflavin and FMN have been reported to form charge transfer type complexes with a number of organic compounds[13-15]. A comparison of the visible and ultraviolet spectra of FMN and monuron with the spectrum of monuron plus FMN solution gave no indication of complex formation. Solutions of FMN and monuron showed no color changes on freezing. Furthermore, monuron did not prevent the color change that occurs when tryptophan–FMN solutions[12] freeze. No change in the phosphorescence intensity or spectrum of FMN solutions was observed when monuron was added. There was also no indication that the photoinactivation of monuron was mediated *via* the semiquinone, or dihydroflavin form of FMN. From these observations it would appear that the FMN–monuron photoreaction is not mediated *via* a charge transfer type complex or by reduced forms of riboflavin.

Experiments with monuron tagged with ^{14}C in the 1-phenyl, methyl, and carbonyl positions gave no indication of breakdown of the monuron molecule during the photoreaction. Paper chromatograms of the [^{14}C]monuron–FMN reaction products, with a 3% $Na_2HPO_4 \cdot 7H_2O$ solvent, showed a well-defined spot at R_F zero although there were also other widely scattered zones of radioactivity. From these experiments it appears that there is at least one major reaction product and, possibly, a number of other compounds which result from side reactions or are precursors to the main product in the photochemical inactivation of monuron by FMN. The isolated reaction product (I) had a m.p. of around 300° (with decomposition), mol. wt. 1250–1300 (boiling point elevation with ethylene chloride and with acetone), and the following analysis: C, 57.27; H, 4.84; N_E, 13.58; N_D, 13.2; Cl, 7.39; 7.16. NMR proton resonance spectrum in deuterated acetone showed only phenyl and CH_3 protons with

no indication of hydroxy protons. The ratio of phenyl protons to methyl protons was 200:318. The ultraviolet spectra of II resembled that of reduced riboflavin but no evidence could be obtained for any portion of the riboflavin molecule in the purified product. Polarographic studies showed no reduction wave for I. Analysis of the reaction product (I) for *p*-chloroaniline[17,18] indicated a content of only 3–5 %, while a monuron control gave the theoretical 64 %.

On the basis of the above analytical data, it was not possible to assign a structure to the monuron–FMN reaction product. However, it appears that several monuron molecules have condensed to form a major portion of this molecule. It is possible that a portion of the riboflavin molecule is also incorporated in this molecule, although the only evidence for this is from the ultraviolet spectrum which resembles that of reduced FMN, and the fact that the chlorine content of I is low. Experiments with the monuron molecule labelled with ^{14}C in the phenyl, carbonyl, and methyl positions rule out complete loss of any of these groups but were not accurate enough to eliminate the possibility that some of the monuron molecules broke apart during the photochemical reaction.

The discovery of the photochemical inactivation of monuron by FMN points to the possibility that monuron may act at or close to the site of the FMN or flavoprotein in the photosynthetic pathway. Thus, it is possible that monuron may inhibit photosynthesis by acting as an energy trap in the electron-transport system near an FMN molecule. *In vitro*, this results in the photodestruction of monuron by FMN due to the transfer of energy from FMN to monuron. *In vivo*, this action is undoubtedly different as there is no evidence of photochemical destruction of monuron. Thus, in systems *in vivo* monuron may be able to pass on the acquired excitation energy received from FMN and thus prevent internal destruction of the monuron molecule. One of the most convincing arguments for the possible relationship of monuron inhibition in photosynthesis to that of the FMN photoinactivation is the close relationship between the antiphotosynthetic properties of the substituted urea molecules and their tendency to react photochemically with FMN. Thus, a simple change in location of the chlorine atom of monuron from the *para* to the *ortho* position caused almost complete loss of antiphotosynthetic activity and of ability to react photochemically with FMN. This effect does not seem to be a simple stereo effect around the N–H group, because the 2,4,6-trichloro derivative is an active antiphotosynthetic agent and undergoes photochemical reaction with FMN. Substitution on the N–H group of urea derivatives results in the loss of antiphotosynthetic activity and also of ability to interfere with the monuron–FMN reaction. Likewise, substitution of large groups in place of the *N*-methyl groups of monuron usually results in the loss of antiphotosynthetic activity and photochemical inactivation with FMN.

There is also a surprising similarity between the spacial structure of FMN and the urea herbicides, particularly diuron, 3-(3,4-dichlorophenyl)-1,1-dimethyl-urea. This similarity in structure is not immediately apparent from the structural formulas but is revealed upon inspection of their molecular models (see Fig. 2). From this figure it can be seen that the overall size of the molecules is very similar. The two chlorine atoms of diuron overlap the CH_3 groups of FMN. The N–H and C=O groups of the two molecules are in similar positions. The side chains of the riboflavin molecule in some instances can be made to approach the shapes of the two methyl groups of the diuron molecule. The similarity in size and location of the functional groups

between monuron and FMN could facilitate the transfer of excitation energy from FMN to monuron, and could also favor the adsorption of monuron by areas occupied by FMN or flavo-enzymes in the chloroplasts.

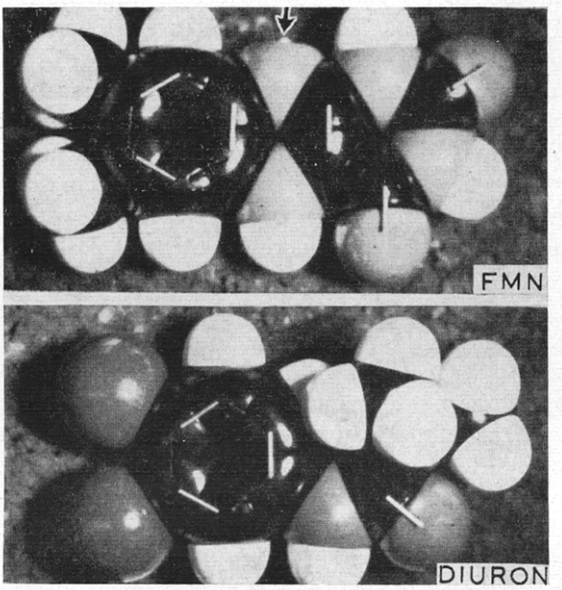


Fig. 2. Molecular models of riboflavin and diuron, 3-(3,4-dichlorophenyl)-1,1-dimethylurea. The ribityl group has been omitted from the molecular model of riboflavin in order to simplify the comparison of size and functional groups of the basic molecule.

In the above discussion it has been suggested that there may be a relationship between the mechanism of the monuron inhibition of photosynthesis and the photoinactivation of monuron by FMN; however, one should use a certain amount of reservation in this conclusion, as FMN is known to induce a number of photochemical reactions[11,12,19–24]. There does appear to be some difference between the FMN–monuron system and other photoreactions of FMN. In the case of auxins and 2,4-dichlorophenoxyacetic acid there are a number of other fluorescent dyes which also induce photodestruction of these molecules. However, in the case of monuron other dyes do not cause photoinactivation under conditions in which FMN is active. Furthermore, the product from the FMN reaction did not appear to result from simple oxidation but rather to be a complex high-molecular-weight material. A high degree of specificity of FMN for monuron is demonstrated by the ability of an FMN solution to prevent the herbicidal action of monuron on Pinto bean plants. Thus, by spraying the leaves of a bean plant with a 1% FMN solution for 4 days after the application of 0.5 mg of monuron to the roots, it was possible to prevent the herbicidal action of the monuron. The FMN spray had no effect on untreated bean plants.

It has recently been suggested[24] that in some FMN photochemical reactions the active agent is a complex between riboflavin, water and an activator. This complex is dissociated upon the absorption of radiant energy and leads to the formation of reduced riboflavin and an oxidized form of the activator. It is possible that a similar reaction takes place during the photoinactivation of monuron. That is, the monuron–

FMN reaction could be mediated *via* an FMN–H_2O–monuron complex. Moreover, the same property of monuron which results in an FMN–H_2O–monuron complex could account for the interference of monuron in the oxygen pathway of photosynthesis since here again an enzyme–H_2O complex may be involved. However, as pointed out above, no spectral evidence has been found for such an FMN–H_2O–monuron complex.

Although it has not been possible to describe the mechanism of the photoinactivation of monuron, it is apparent that in working with photosynthetic phosphorylation reactions in which both monuron and FMN are present, one must use caution in carrying out the experiments and in interpreting the results, otherwise photoinactivation of monuron by FMN in light may take place and thus result in erroneous conclusions.

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REFERENCES

1 H. C. BUCHA AND C. W. TODD, *Science*, 114 (1951) 493.
2 J. S. C. WESSELS AND R. VAN DER VEEN, *Biochim. Biophys. Acta*, 19 (1956) 548.
3 A. R. COOKE, *Weeds*, 4 (1956) 397.
4 P. B. SWEETSER, C. W. TODD AND R. T. HERSH, *Biochim. Biophys. Acta*, 51 (1961) 509.
5 N. I. BISHOP, *Biochim. Biophys. Acta*, 27 (1958) 205.
6 F. R. WHATLEY, M. B. ALLEN AND D. I. ARNON, *Biochim. Biophys. Acta*, 32 (1959) 32.
7 D. I. ARNON, F. R. WHATLEY AND M. B. ALLEN, *Science*, 127 (1958) 1026.
8 A. T. JAGENDORF AND M. MARGULIES, *Arch. Biochem. Biophys.*, 90 (1960) 184.
9 A. T. JAGENDORF AND M. AVRON, *Arch. Biochem. Biophys.*, 80 (1959) 246.
10 P. B. SWEETSER AND C. W. TODD, *Biochim. Biophys. Acta*, 51 (1961) 504.
11 A. W. GALSTON, *Proc. Natl. Acad. Sci. U.S.*, 35 (1949) 10.
12 M. G. FERRI, *Arch. Biochem. Biophys.*, 31 (1951) 127.
13 I. ISENBERG AND A. SZENT-GYÖRGII, *Proc. Natl. Acad. Sci. U.S.*, 44 (1958) 857.
14 H. HARBURY AND K. FOLEY, *Proc. Natl. Acad. Sci. U.S.*, 44 (1958) 662.
15 B. PULLMAN AND A. PULLMAN, *Proc. Natl. Acad. Sci. U.S.*, 44 (1958) 1197.
16 B. COMMONER AND B. B. LIPPINCOTT, *Proc. Natl. Acad. Sci. U.S.*, 44 (1958) 1110.
17 W. K. LOWEN AND H. M. BAKER, *Anal. Chem.*, 24 (1952) 1475.
18 W. E. BLEIDNER, H. M. BAKER, M. LEVITSKY AND W. K. LOWEN, *Agric. and Food Chem.*, 2 (1954) 476.
19 W. R. FRESELL, C. W. CHUNG AND C. G. MACKENZIE, *J. Biol. Chem.*, 234 (1959) 1297.
20 E. ZONDAG, J. POSTHUMA AND W. BERENDS, *Biochim. Biophys. Acta*, 39 (1960) 178.
21 CARROL, *Am. J. Botany*, 36 (1949) 281.
22 J. K. HANSEN AND K. P. BUCHOLTZ, *Weeds*, 1 (1952) 237.
23 L. P. VERNON, *Biochim. Biophys Acta*, 36 (1959) 177.
24 W. J. NICKERSON AND G. STRAUSS, *J. Am. Chem. Soc.*, 82 (1960) 5007.