Microsomal N-Demethylation, by a Cotton Leaf Oxidase System, of 3-(4′-Chlorophenyl)-1,1-Dimethylurea (Monuron)

Abstract. *A cotton leaf microsomal oxidase system that* N-*demethylates 3-(4′-chlorophenyl)-1,1-dimethylurea (monuron) to 3-(4′-chlorophenyl)-1-methylurea has been partially characterized. The enzyme system is associated with a microsomal fraction separated by differential centrifugation and requires molecular oxygen as well as either the reduced form of nicotinamide adenine dinucleotide phosphate or the reduced form of nicotinamide adenine dinucleotide as cofactors.*

Several *N*-methylphenylurea herbicides have recently been shown to undergo *N*-demethylation and subsequent hydrolysis to anilines in plants or plant tissue sections (*1–3*). This report describes the partial characterization of a microsomal oxidase system from cotton leaves which catalyzes the *N*-demethylation of 3-(4′-chlorophenyl)-1,1-dimethylurea (monuron) to 3-(4′-chlorophenyl)-1-methylurea.

Leaf tissue (50 g) was excised from 1-month-old Stoneville 213 cotton (*Gossypium hirsutum*, L.) plants grown in the greenhouse in one-half strength Hoagland's nutrient solution. Active microsomal preparations were obtained by the differential centrifugation of crude homogenates prepared with a modified Loomis and Battaile extraction medium (*4*). The excised leaf tissue was washed with distilled water and ground to a fine powder in a mortar and pestle with liquid nitrogen and 10 g of sand. Because of the presence of various phenols and phenol-oxidizing enzymes in cotton leaf homogenates, the frozen leaf powder was slurried with 200 ml of 0.3*M* potassium phosphate buffer, *p*H 7.5, containing 0.25*M* sodium isoascorbate, 0.001*M* NaCN, and 25 g of Polyclar AT (insoluble polyvinylpyrrolidone). After standing for 15 minutes with occasional stirring, the slurry was squeezed through cheesecloth and differentially centrifuged. All enzyme extraction and centrifugation procedures were carried out at 0° to 4°C.

Enzyme activity was determined by following the rate of 3-(4′-chlorophenyl)-1-methylurea formation. The standard reaction mixture contained approximately 7.5 mμmole of ^{14}C-ring-labeled 3-(4′-chlorophenyl)-1,1-dimethylurea (specific activity 5.81 μc/μmole), 0.05 μmole of NaCN, 50 μmole of potassium phosphate, *p*H 7.5, 1 μmole of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), and 1 to 3 mg of microsomal protein in a final volume of 1.0 ml. The reaction was initiated by the addition of NADPH and incubated for 30 minutes at 25°C. Controls were run without NADPH and the reaction was terminated by rapid freezing in a dry ice–acetone bath followed by lyophilization. The lyophilized reaction mixture was extracted with 500 μl of methanol, and the reaction product, 3-(4′-chlorophenyl)-1-methylurea, was separated from the substrate by thin-layer chromatography (*3*). The radioactivity of the separated reaction product was quantitatively determined by the method of Snyder and Stephen (*5*). Under the assay conditions used, only one radioactive reaction product was observed after autoradiography. This product cochromatographed with known 3-(4′-chlorophenyl)-1-methylurea. Recoveries of radioactivity from the lyophilized reaction mixtures were better than 95 percent, while recoveries from thin-layer chromatograms were 90 percent or better.

Protein concentration was determined by the method of Lowry *et al.* (*6*), with crystalline bovine serum albumin as the standard.

Under standard assay conditions with unwashed microsomal preparations, enzyme activity was linear up to 40 minutes and was proportional to enzyme concentration up to 3 mg of protein. The *p*H optimum with phosphate and *N,N*-bis(2-hydroxyethyl) glycine (bicine) buffer systems was between 7.4 and 7.8.

The results of a representative differential centrifugation experiment are shown in Table 1. The distribution of enzyme activity was typical of a microsomal particle. The lack of any appreciable enzyme activity in the cell-free and the 17,500*g* supernatant fractions is apparently due to presence of a soluble endogenous inhibitor. Electron-microscopy studies and the chemical characterization of washed microsomal preparations have subsequently confirmed the presence of endoplasmic reticulum membranes with associated ribosomes.

The results of a typical experiment that demonstrates the requirement for molecular oxygen are shown in Table 2. The partial pressure of oxygen in air was apparently sufficient to saturate the system under the assay condi-

Table 1. Distribution of 3-(4′-chlorophenyl)-1,1-dimethylurea *N*-demethylase in cotton leaf extracts. Cell-free extract (supernatant after centrifugation at 300*g* for 5 minutes) was centrifuged at 1500*g* for 10 minutes, 17,500*g* for 20 minutes, and 78,000*g* for 80 minutes. The 1500*g*, 17,500*g* and 78,000*g* unwashed pellets were resuspended in 0.1*M* potassium phosphate buffer, *p*H 7.5, containing 1×10^{-3}*M* NaCN. The reaction mixture contained 1.8 to 2.8 mg of protein, 99,900 disintegrations per minute (dpm) of ^{14}C-ring-labeled 3-(4′-chlorophenyl)-1,1-dimethylurea (specific activity, 5.81 μc/μmole), 50 μmole of potassium phosphate, *p*H 7.5, 0.5 μmole of NaCN and 1 μmole of NADPH. The reaction was run for 30 minutes at 25°C. Controls were run without NADPH.

Fraction	Specific activity [dpm 3-(4′-chlorophenyl)-1-methylurea/mg of protein, per 30 minutes]
Cell-free extract	0
1500*g* pellet	0
17,500*g* pellet	21
17,500*g* supernatant	83
78,000*g* pellet	596
78,000*g* supernatant	49

Table 2. Oxygen requirement for 3-(4′-chlorophenyl)-1,1-dimethylurea *N*-demethylase activity in cotton leaf microsomal fraction. The reaction mixture contained 3.2 mg of protein, 99,900 dpm of ^{14}C-ring-labeled 3-(4′-chlorophenyl)-1,1-dimethylurea (specific activity, 5.81 μc/μmole), 50 μmole of potassium phosphate, *p*H 7.5, 0.5 μmole of NaCN, and 1 μmole of NADPH. The reaction was run for 30 minutes at 25°C. Controls were run without NADPH.

Treatment	3-(4′-chlorophenyl)-1-methylurea formed (dpm)
Air	2850
Oxygen	2933
Nitrogen	333

Table 3. Effect of NADPH and NADH on 3-(4′-chlorophenyl)-1,1-dimethylurea *N*-demethylase activity. The reaction mixture contained 2.6 mg of protein, 99,880 dpm of ^{14}C-ring-labeled 3-(4′-chlorophenyl)-1,1-dimethylurea (specific activity, 5.81 μc/μmole), 50 μmole of potassium phosphate, *p*H 7.5, 0.5 μmole of NaCN, and a total of 1 μmole of NADPH or NADH or both. The reaction was run for 30 minutes at 25°C. Controls were run without NADPH or NADH.

Cofactor	3-(4′-Chlorophenyl)-1-methylurea formed (dpm)
NADPH	2852
NADH	1253
NADH + NADPH	2454

tions used, since no increase in activity was observed in the presence of oxygen alone. A representative experiment which shows the reduced pyridine nucleotide specificity of cotton leaf microsomal *N*-demethylase is shown in Table 3. Both NADPH and NADH (the reduced form of nicotinamide adenine dinucleotide) served as cofactors for the *N*-demethylation of 3-(4′-chlorophenyl)-1,1-dimethylurea, but NADPH was approximately twice as effective as NADH. The combination of NADPH and NADH was only as effective as NADPH alone.

The observed requirements for molecular oxygen and reduced pyridine nucleotides, together with the localization of enzyme activity in the microsomal fraction, indicate that the *N*-demethylation of 3-(4′-chlorophenyl)-1,1-dimethylurea to 3-(4′-chlorophenyl)-1-methylurea is catalyzed by a mixed-function oxidase system in cotton leaves. Further studies are needed to purify and characterize this and other in vitro plant microsomal systems as possible sites for the metabolism of pesticides in plants.

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Estimating the Number of Animals: A Rapid Method for Unidentified Individuals

Abstract. *A proposed model yields the density of a mobile population from quick, cursory surveys in which the observer identifies none of the animals. When the spaces on which animals were seen are successively removed, the decline in the counts permits estimation of the average probability of seeing a given animal. The method showed promise in initial trials.*

Estimations of the density of populations (*1*), depend either on total counts on sample plots, on marking and later reobservation, or on physical removal of some animals (*2*), or on a "flushing count" (*3*). The model I now propose requires none of these methods and may lessen some of the difficulties in one of the central tasks of animal ecology.

A study area subdivided into equal sample spaces is inspected during a series of n cursory, randomized surveys, but the observer limits each survey to just the L_i spaces on which he has not previously seen animals in that given series of counts ($i = 1, 2, \ldots$ unless otherwise noted). During each i^{th} count the investigator notes the total number of observed animals, x_i, and the total number of spaces on which they occurred, l_i; following the survey he eliminates the l_i plots from further study in that given experiment (series of counts or surveys). The worker continues the surveys under uniform conditions, preferably until all plots have been removed or the remaining ones have no observed animals. The present "plot-removal method" is distantly related to the earlier removal procedures which Zippin and Tanaka (*4*) reviewed.

I consider the average probability, P, of seeing any one given animal, during any one survey that included its plot, under two cases: (i) the unusual one where the average value of P remains constant from one l_i group of plots to another l_i group (although not necessarily from plot to plot within each group); and (ii) the common case where it does not. The observer must make the surveys near each other in time, to prevent change in the size of the total population K but far enough apart to permit successive observations on any specific plot to be independent. Movements of animals on and off each l_i group of plots do not occur or the movements must tend to cancel out.

In the special case where P is constant and the investigator makes only two of the rapid, cursory counts on one study area (divided into numerous spaces), the following postulates and definitions can be made. If the assumptions are fulfilled, the number of animals seen on the first survey is given by

$$x_1 = PK \qquad (1)$$

and the number seen on the second survey is

$$x_2 = P\,(K - x_1 - y_1) \qquad (2)$$

where y_1 is the number of unobserved animals that occurred on the same l_1 spaces as the x_1 seen animals. The total number of unobserved animals on the entire area studied in the first survey, including those living on the l_1 plots as well as elsewhere, Y_1, is further defined by

$$Y_1 = K - x_1 \qquad (3)$$

The spatial distribution of the Y_1 animals is now a major concern, for one must form some idea about what fraction of them constituted y_1. Although the situation undoubtedly differs somewhat in different populations, the strongest assumption that one can make in the present circumstance probably is that the number of unobserved animals, per plot, is proportional to the number of observed ones. If this is true, one can write that

$$y_1 = Y_1\,(x_1/K) \qquad (4)$$

The preceding four equations contain the four unknowns y_1, Y_1, P, and K, of which P and K are the population parameters. After the equations are solved simultaneously, and after writing $\hat{K}$ for K, one obtains an estimate of abundance on one study area based on a constant P, $n = 2$, and the factor of proportionality x_1/K, as follows:

$$\hat{K} = x_1/[1 - (x_2/x_1)^{1/2}] \qquad (5)$$

In this equation it is obvious that

$$\hat{q} = (x_2/x_1)^{1/2} \qquad (6)$$

where q is defined as $1 - P$, and where the circumflex ($\wedge$) means estimated.

A different assumption about the distribution of the Y_1 unseen animals can be made, namely that their density throughout the study area is essentially uniform. If this is so, $(y_1/Y_1) = (l_1/L_1)$, and therefore

$$\hat{y}_1 = Y_1(l_1/L_1) \qquad (7)$$

When one uses Eq. 7, instead of Eq. 4, as the factor of proportionality in developing the previous type of deriva-