Arylamine N-Oxidation by the Microsomal Fraction of Germinating Pea Seedlings (*Pisum sativum*)

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The microsomal fraction of germinated pea seeds was found to oxidize 4-chloroaniline primarily to 4-chloronitrosobenzene, although (4-chlorophenyl)hydroxylamine was also a major product at high substrate concentration. The enzyme-catalyzed oxidation of (4-chlorophenyl)hydroxylamine was faster than that for 4-chloroaniline. The oxidation of 4-chloroaniline was dependent on H_2O_2 and would not proceed when O_2 and NADPH were substituted for H_2O_2. Further slow oxidation of 4-chloronitrosobenzene to 4-chloronitrobenzene was found to be an enzymatic process that was also dependent on H_2O_2. The enzymatic oxidation of arylamines appears to be a fairly general process, since four other arylamine substrates were also found to be oxidized to the corresponding nitroso aromatic. Two arylamines not oxidized by this microsomal system are 4-nitroaniline and 2,6-dichloro-*p*-phenylenediamine. This peroxidatic activity is comparable to the action of chloroperoxidase but is quite unique in certain aspects. The significance of such enzymatic oxidations to the environmental fate of arylamines is discussed.

The environmental chemistry of arylamines is a major problem confronting agricultural science today. Many arylamines and their derivatives are employed as pesticides. In particular, acylanilides and phenylureas are used extensively as herbicides. The hydrolysis of such anilide and ureide pesticides accounts for the release of large quantities of arylamines into the environment. The ultimate fate of these arylamine residues is not well documented, although it is widely believed that mineralization is a very slow process (Zeyer and Kearney, 1982; You and Bartha, 1982). The complexity of arylamine chemistry in the environment was recently reviewed (Parris, 1980). We have been interested in identifying biochemical processes that cause oxidation of the nitrogen atom of arylamines. Previous reports on the microbial N-oxidation of arylamine pesticide residues suggest that such oxidative processes are of great importance to an understanding of the fate and effects of arylamines in the environment (Kaufman et al., 1973; Bordeleau et al., 1972).

Most research on the enzymology of N-oxidation has dealt with those N-oxidation processes effected by microsomal oxidases (Hlavica, 1982; Weisburger and Weisburger, 1973). The oxidation of a primary arylamine, **1**, to the corresponding arylhydroxylamine, **2** (Figure 1), appears to be a function of cytochrome P-450 dependent mixed-function oxidases (Smith and Gorrod, 1978). Further enzymatic or chemical oxidation of the arylhydroxylamine, **2**, to the nitroso oxidation state, **3**, may also occur (Kadlubar et al., 1973). Research on the ability of nonmicrosomal oxidative enzymes to catalyze N-oxidation of primary arylamines has been less extensive; however, we reported on the unique ability of the fungal enzyme, chloroperoxidase, to catalyze H_2O_2 oxidation of arylamines to the nitroso compounds (Corbett et al., 1978, 1979, 1980). Most notable in the case of chloroperoxidase was the efficiency of this conversion, since the oxidation proceeded rapidly to the nitroso oxidation state and was nearly quantitative for most arylamine substrates. On the other hand, the microsomal oxidation of arylamines is less specific in that ring hydroxylation products are major cometabolites.

Our interest in peroxidases and their ability to convert arylamines, **1**, to nitroso aromatics, **3**, led us to investigate such processes by the microsomal fraction of germinating pea seeds (*Pisum sativum*). In a study on the H_2O_2-catalyzed oxidation of indole by pea seed microsomes, the oxidation of aniline to phenylhydroxylamine was reported; however, no details were presented (Ishimaru and Yamazaki, 1977). We have investigated this possibility by employing techniques suitable for the analysis of arylhydroxylamines, **2**, and nitroso aromatics, **3**, and now report that N-oxidation by a component of pea seed microsomes is a major oxidative process.

Table I. Oxidation of Arylamine Substrates to Nitroso Aromatics[a]

substrate	HPLC solvent	retention time for nitroso product, min	rate of nitroso production, mmol L^{-1} min^{-1}
4-chloroaniline	70% CH_3OH	4.2	0.0022
3,4-dichloroaniline	70% CH_3OH	6.4	0.0015
4-bromoaniline	70% CH_3OH	4.5	0.0022
p-toluidine	60% CH_3OH	6.1	0.0029
aniline	60% CH_3OH	4.9	0.0031
4-nitroaniline	50% CH_3OH	6.1	0.0000

[a] Each substrate at 1.0 mM was allowed to react with 2.0 mM H_2O_2 in the presence of 0.14 mg/mL pea seed microsome preparation. Aliquots were analyzed after 1.0 min by HPLC employing the solvent indicated at a flow rate of 1.5 mL/min. The HPLC column was a μBondapak C_{18}. The retention times for each nitroso aromatic product were found to be identical with those of authentic nitroso aromatic standards. The rates of production for all nitroso aromatic products were computed from peak height measurements. The arylamine substrate 2,6-dichloro-*p*-phenylenediamine was not oxidized by the pea seed enzyme to any detectable degree. An authentic sample of the nitroso metabolite expected from 2,6-dichloro-*p*-phenylenediamine was not available.

EXPERIMENTAL SECTION

Chemicals. Arylamine substrates (Table I) were obtained from commercial sources and recrystallized to obtain pure substrates. Nitroso aromatic standards were prepared by standard procedures (Lutz and Lytton, 1938), except for 4-nitronitrosobenzene, which was prepared by the method of Kuhn and Weygand (1936). (4-Chlorophenyl)hydroxylamine (**7**) was prepared and purified by

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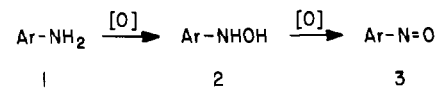

Figure 1. Conversion of an arylamine, 1, to the arylhydroxylamine, 2, and nitroso aromatic, 3, oxidation states. Ar = aromatic ring.

the method of Smissman and Corbett (1972). 2-Amino-5-chlorophenol was synthesized by a previously described method (Corbett and Corbett, 1981). Glucose 6-phosphate, glucose-6-phosphate dehydrogenase, NADP, and 30% aqueous H_2O_2 were obtained from Sigma Chemical Co. (St. Louis, MO). The stock solution of 30% aqueous H_2O_2 was assayed by the thiosulfate oxidation method (Pietrzyk and Frank, 1974); then portions were diluted with H_2O on a daily basis to 1.0 M or lower concentrations as needed.

Microsomal Preparation. The microsomal fraction of germinated pea seeds (*P. sativum*, "Texas Cream 40 Cowpeas") was prepared as previously described (Ishimaru and Yamazaki, 1977), except that the final washing was achieved by suspending the microsomal pellet equivalent to 80 g of germinated seeds in 100 mL of 0.1 M potassium phosphate buffer, pH 7.2, followed by centrifugation at 34000*g* for 2.5 h. The pellets were combined and resuspended in 200 mL of 0.1 M potassium phosphate buffer, pH 7.2. Protein concentration was determined by the biuret method with bovine serum albumin as a reference. The microsomal preparations contained either 1.4 or 2.2 mg of protein/mL.

Incubation Procedure. The enzymatic reactions were conducted at 22 °C in 10 cm × 2.5 cm (internal diameter) test tubes fitted with silicone stoppers. To each tube was added 8.9 mL of 0.1 M potassium phosphate buffer, generally pH 7.2, 50 μL of an ethanolic solution of the substrate, 100 μL of a diluted aqueous solution of H_2O_2, and then 1.0 mL of the microsomal preparation to initiate the reaction. Aliquots of the reaction were quenched at the desired time by dilution of a 1.0-mL volume with a solution of 1.0 mL of methanol containing 0.10 mL of 10% (w/v) aqueous trichloroacetic acid precooled to −20 °C. In one experiment H_2O_2 was replaced with NADP (2 μmol), glucose 6-phosphate (10 μmol), and glucose-6-phosphate dehydrogenase (20 units).

HPLC Quantitation of 4-Chloroaniline (6) Oxidation Products. HPLC was carried out with two identical systems composed of Waters Associates (Milford, MA) Model U6K septumless injector, Model 6000A solvent pump, and Model 440 (dual-wavelength option) spectrophotometric detector. The HPLC column was a μBondapak C_{18}, 30 cm × 3.9 mm internal diameter (Waters Associates). The quantitative determinations of all reaction products were achieved by analysis of each quenched aliquot on the μBondapak C_{18} column with three different solvent systems, each at a flow rate of 1.5 mL/min. Injection volumes of 10 μL each were analyzed within 2 h following the sampling of the enzymatic reactions. 4-Chloronitrosobenzene (4) and 4-chloronitrobenzene (8) concentrations were determined with the solvent 70% aqueous methanol employing 313- and 254-nm detectors. The concentrations of 4-chloroaniline (6) and (4-chlorophenyl)hydroxylamine (7) were determined with the solvent 40% aqueous methanol employing a 254-nm detector, and the concentration of 4,4′-dichloroazoxybenzene (9) was determined with the solvent system 2-propanol–methanol–water (2:6:2) employing a 313-nm detector. The analysis of reaction mixtures for the presence of 2-amino-5-chlorophenol was achieved with the solvent 40% aqueous acetonitrile. Authentic standards of known concentration were chromatographed under identical conditions, and absolute amounts of each product were calculated on the basis of peak heights.

Identification of 4-Chloronitrosobenzene (4). A 9.0-mL volume of a 2-h incubation of 0.50 mM 4-chloroaniline (6), 10 mM H_2O_2, and 0.22 mg/mL protein was treated with 82 mg (0.9 mmol) of glyoxylic acid monohydrate. Aliquots were analyzed by HPLC until the peak for 4-chloronitrosobenzene (4) had nearly disappeared, and then the reaction mixture was extracted twice with 10 mL of ethyl acetate. The combined organic layers were dried over Na_2SO_4 and evaporated to give a yellow residue, which was dissolved in 50 μL of methanol. Aliquots were chromatographed on TLC plates (5 × 20 cm EM silica gel 60-F254) with 5% CH_3OH–$CHCl_3$, along with authentic *N*-(4-chlorophenyl)formohydroxamic acid (5) (Corbett and Corbett, 1980). The hydroxamic acid spots were visualized by spraying with 1% $FeCl_3$ in methanol. Quantitative determination of the amount of hydroxamic acid produced was achieved by HPLC analysis employing 0.01% desferal mesylate in 40% aqueous methanol as a conditioned solvent on a μBondapak C_{18} column (Corbett and Chipko, 1979a,b).

Incorporation of 4-Chloro[U-^{14}C]aniline into Enzyme Protein. To each of four 15-mL centrifuge tubes was added 5.0 mL of 0.1 M potassium phosphate buffer, pH 7.2, containing 0.20 mM 4-chloro[U-^{14}C]aniline (specific activity = 0.28 mCi/mmol). Two of the solutions were treated with 100 μL of 1.0 M H_2O_2, and then each of the four solutions was treated with 5.0 mL of pea seed microsome preparation containing 2 mg of protein/mL. After reaction at ambient temperature for 30 or 60 min, one complete reaction (+H_2O_2) and one control (−H_2O_2) were each treated with 1.0 mL of 10% trichloroacetic acid, followed by centrifugation to deposit the protein. The protein pellets were washed with 10 mL of 1% trichloroacetic acid and then twice with 10 mL of ethanol. The protein pellets were dried under high vacuum, and then approximately 5 mg of each was weighed and placed into a liquid scintillation vial with 200 μL of Protosol (New England Nuclear) and gently heated until the protein had dissolved. To each vial was added 0.5 mL of methanol and 14 mL of Aquasol 2 (New England Nuclear). After the mixture was allowed to stand in the dark for 24 h liquid scintillation counting was performed, employing a Searle Analytic 92 liquid scintillation counter. Each sample was recounted after the addition of a [^{14}C]toluene standard (45 000 dpm).

RESULTS

Seven arylamine substrates (Table I) were incubated at 1.0 mM concentrations with 2.0 mM H_2O_2 in the presence of 0.14 mg/mL pea seed microsome preparation. The analysis of these reaction mixtures at various times by HPLC was employed to determine the production of the nitroso metabolite. Five of the substrates gave an HPLC peak identical in retention time with that of the authentic nitroso aromatic product (Table I). In each case, the product peak height was found to be enhanced as arithmetically predicted when the sample was spiked with a known amount of authentic standard. This HPLC peak matching technique was suggestive of the oxidation of each of the five arylamine substrates to its nitroso metabolite. Further identification of these nitroso aromatic metabolites was achieved by comparison of relative peak height intensities at three wavelengths (254, 280, and 313 nm) as determined by multiple HPLC analyses employing a dual-wavelength detector. In all cases these ratios were identical with those measured for authentic nitroso aromatic standards. Positive identification of metabolites was

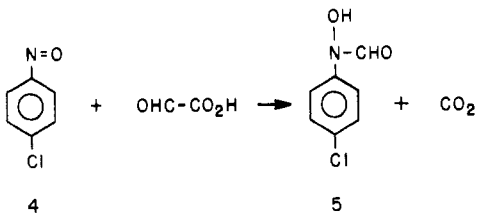

Figure 2. Conversion of 4-chloronitrosobenzene, **4**, to *N*-(4-chlorophenyl)formohydroxamic acid, **5**.

carried out in the case of 4-chloroaniline (**6**). The relative rates of oxidation of each of the arylamines to the nitroso metabolite were similar under identical conditions (Table I). All further kinetic studies on this enzymatic oxidation were conducted with 4-chloroaniline (**6**) as the arylamine substrate. Neither 4-nitroaniline nor 2,6-dichloro-*p*-phenylenediamine were oxidized to any product by this enzyme system (Table I).

4-Chloronitrosobenzene (**4**) was positively identified in the enzymatic reaction mixtures employing 4-chloroaniline (**6**) as the substrate by trapping the nitroso functional group with glyoxylic acid as previously described (Corbett and Corbett, 1980). This recently discovered and specific organic reaction converts nitroso aromatics to the corresponding formohydroxamic acids (Figure 2), which are then detected by HPLC and TLC. A specific TLC spray reagent for hydroxamic acids is an acidified solution of $FeCl_3$ in methanol, which gives violet complexes with this functional group. The R_f for *N*-(4-chlorophenyl)formohydroxamic acid (**5**) was 0.35 on silica gel plates developed with 5% CH_3OH–$CHCl_3$. The HPLC retention time for the hydroxamic acid product was 8.0 min by employing 40% aqueous methanol containing 0.01% desferal mesylate as the solvent (Corbett and Chipko, 1979a). The product obtained from the glyoxylic acid trapping reaction on the enzymatic reaction mixture behaved in a manner identical with authentic *N*-(4-chlorophenyl)formohydroxamic acid (**5**). The total amount of 4-chloronitrosobenzene (**4**) in the original enzymatic reaction mixture was calculated to be 80 μg (0.51 μmol) by HPLC analysis, and 60% (0.33 μmol) of this enzymatic product was chemically converted to the hydroxamic acid, **5**, and recovered by solvent extraction as determined by HPLC analysis.

The oxidative conversion of 4-chloroaniline (**6**) to 4-chloronitrosobenzene (**4**) displayed a broad pH dependency in the range 6.0–8.5, with pH 7.2 being optimal. The rate of oxidation was closely dependent on H_2O_2 concentration. No oxidation occurred in the absence of H_2O_2, and an NADPH-generating system consisting of glucose-6-phosphate dehydrogenase, glucose 6-phosphate, and NADP would not support the enzymatic oxidation of 4-chloroaniline (**6**). The conversion of 4-chloroaniline (**6**) to 4-chloronitrosobenzene (**4**) increased in a nearly linear relationship with H_2O_2 concentration up to about 6 mM, and at 10 mM H_2O_2 the conversion was nearly saturated with respect to H_2O_2. The presence of 20 mM KBr had no effect on the enzymatic reaction and did not result in the production of brominated products as was the case with the fungal enzyme chloroperoxidase (Corbett et al., 1980).

The rate of oxidation of 4-chloroaniline (**6**) to 4-chloronitrosobenzene (**4**) was linearly dependent on the concentration of enzyme in the range of protein concentration of 0.03–0.29 mg/mL. Heat denaturation required treatment at 90 °C for 30 min to totally eliminate the oxidative ability of the microsomal preparation. The microsome preparation maintained full enzymatic activity for at least 60 days when stored at −20 °C. Preincubation of microsomal preparations with H_2O_2 resulted in a time-dependent inactivation of the ability to convert 4-chloroaniline (**6**) to 4-chloronitrosobenzene (**4**). At 10 mM H_2O_2 in the absence of 4-chloroaniline (**6**), complete inactivation of enzyme resulted within 20 min. Under similar conditions but in the presence of at least 0.05 mM 4-chloroaniline (**6**), enzyme oxidative capacity was prolonged to nearly 40 min. These observations of H_2O_2 sensitivity and substrate protection are similar to those observed with chloroperoxidase (Corbett et al., 1980), although the latter enzyme is much more susceptible to H_2O_2 inactivation than is the enzymatic activity of interest in the pea seed microsome preparation.

Table II. Binding of 4-Chloroaniline (6) to Protein[a]

incubation	incubation time, min	dpm/mg of protein	% of total dpm in protein
$-H_2O_2$	30	4 370	3.5
$+H_2O_2$	30	16 200	13.2
$-H_2O_2$	60	5 240	4.2
$+H_2O_2$	60	18 000	14.6

[a] Incubations of 4-chloro[U-^{14}C]aniline and pea seed microsomes with or without H_2O_2 were conducted as described under Experimental Section. The total level of radioactivity in each incubation reaction was 1.23×10^6 dpm.

In general, aromatic nitroso compounds are highly reactive chemicals with respect to biochemical systems. They are particularly reactive with sulfhydryl-containing chemicals (Eyer, 1979; Dolle et al., 1980), which is a probable explanation for their ability to bind with proteins. This possibility, and its potential to interfere with an accurate quantitation of 4-chloronitrosobenzene (**4**) generated in situ by pea seed microsome oxidation of 4-chloroaniline (**6**), was investigated. In the absence of H_2O_2, 4-chloronitrosobenzene (**4**, 0.01 mM) in the presence of 0.14 mg/mL microsomal protein was found to decrease according to a first-order rate constant of 0.016 min^{-1}. The products to which the substrate was converted could not be detected at this level by HPLC. The limit of detection of 4-chloronitrosobenzene (**4**) in enzymatic incubations by our HPLC method was 1.0 μM with a precision of ±0.2 μM at this low concentration. On the other hand, (4-chlorophenyl)hydroxylamine (**7**) had a lower limit of detection of 8 ± 2 μM. By use of radiotracer methodology it was concluded that a significant amount of 4-chloronitrosobenzene (**4**) produced by enzymatic oxidation was covalently bound to protein (Table II). Nevertheless, the first-order rate constant for the total loss of the nitroso product through secondary chemical reactions allowed us to conclude that such losses would have a negligible effect on the computation of initial rates (1 min) of formation of this product. An investigation of the stability of the nitroso product, **4**, in timed aliquots quenched by combination with an equal volume of methanol precooled to −20 °C indicated that this quenching procedure alone was inadequate. Oxidation of substrate **6** continued in such aliquots, although at a much slower rate. Through the addition of 0.1 volume of 10% trichloroacetic acid to the methanol we found that all enzymatic activity was inhibited and that such quenched aliquots were stable in composition for several hours.

Since the conversion of 4-chloroaniline (**6**) to 4-chloronitrosobenzene (**4**) is a four-electron oxidation, the possibility that the intermediary oxidation product, (4-chlorophenyl)hydroxylamine (**7**), might be a discrete intermediate was investigated. Previous studies with chloroperoxidase demonstrated that the arylhydroxylamine, **7**, was a fleeting intermediate during the conversion of

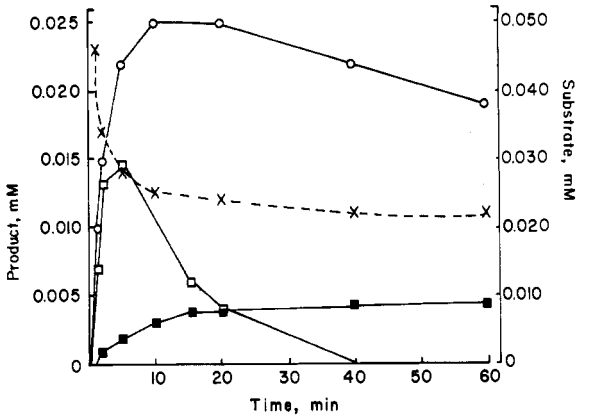

Figure 3. Effect of pea seed microsomes and H_2O_2 on 4-chloroaniline (6, 0.0625 mM) as a function of time. The reaction was carried out at 22 °C by the addition of 2.2 mg of enzyme preparation in 1.0 mL of 0.1 M potassium phosphate, pH 7.2, to 9.0 mL of 0.1 M potassium phosphate, pH 7.2, containing 100 μmol of H_2O_2 and 0.625 μmol of 4-chloroaniline (6). Aliquots were quenched at the indicated times and analyzed as described under Experimental Section. Left ordinate: (○) 4-chloronitrosobenzene (4); (□) (4-chlorophenyl)hydroxylamine (7); (■), 4-chloronitrobenzene (8). Right ordinate: (×) 4-chloroaniline (6).

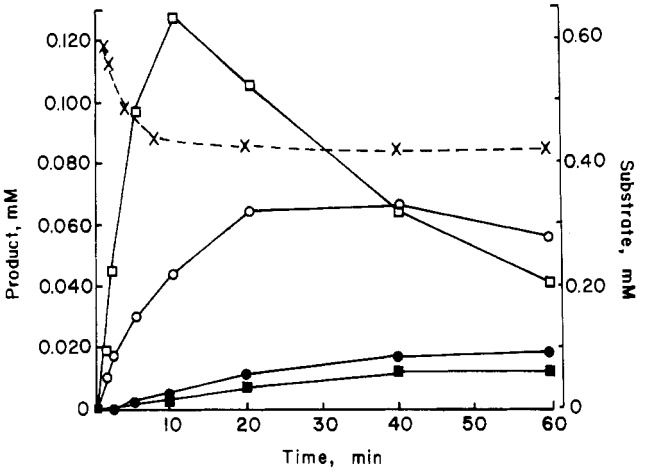

Figure 4. Effect of pea seed microsomes and H_2O_2 on 4-chloroaniline (6, 0.625 mM) as a function of time. The reaction was carried out by the procedure described in the legend to Figure 3 except that 6.25 μmol of 4-chloroaniline (6) was used. Left ordinate: (○) 4-chloronitrosobenzene (4); (□) (4-chlorophenyl)-hydroxylamine (7); (■) 4-chloronitrobenzene (8); (●) 4,4'-dichloroazoxybenzene (9). Right ordinate: (×) 4-chloroaniline (6).

4-chloroaniline (6) to 4-chloronitrosobenzene (4) by that peroxidase (Corbett et al., 1980). The report by Ishimaru and Yamazaki (1977) was suggestive since they reported that phenylhydroxylamine was the product of aniline oxidation by the pea seed microsome preparation; however, their methodology did not allow for the direct observation of phenylhydroxylamine. We found that the production of a discrete hydroxylamine intermediate, 7, during the microsomal enzyme oxidation of 4-chloroaniline (6) was dependent on the concentration of the substrate arylamine 6 (Figures 3 and 4). At substrate concentrations below 0.05 mM, no (4-chlorophenyl)hydroxylamine (7) could be detected by HPLC analysis. As the substrate concentration increased, this intermediate rapidly increased relative to the amount of the nitroso metabolite, 4, that was produced. At substrate concentrations in excess of 0.5 mM, the hydroxylamine, 7, became the major initial product of this enzymatic oxidation, even when the H_2O_2 concentration was in large excess so that its depletion was not a factor.

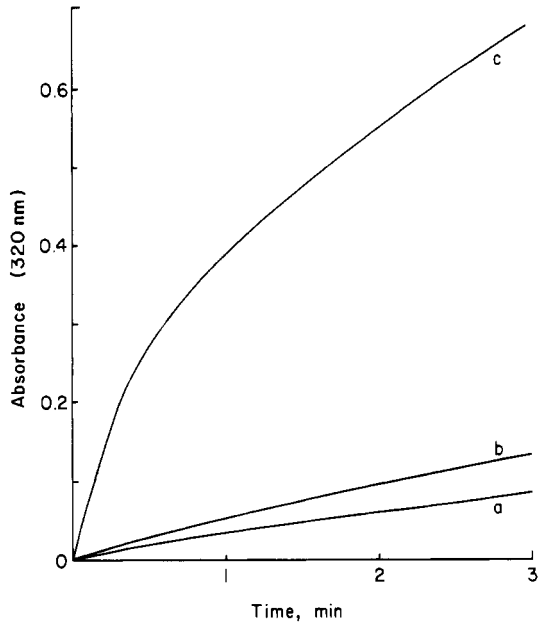

Figure 5. Spectrophotometric determination of the chemical and enzymatic oxidation of (4-chlorophenyl)hydroxylamine (7). The reactions were carried out in quartz UV cells placed in a Beckman Model 35 spectrophotometer at 24 °C by adding 1.7 mL of 0.1 M potassium phosphate, pH 7.2, and 21.5 nmol of (4-chlorophenyl)hydroxylamine (7) as a solution in 10 μL of 95% ethanol. (Note: 7 must be recrystallized on a daily basis and ethanolic solutions prepared just prior to use.) H_2O or H_2O_2 (0.2 M aqueous solution) was added as 100 μL just prior to the addition of substrate. For the enzymatic reaction, 200 μL of the microsome suspension containing 0.44 mg of protein was added immediately after the addition of substrate 7 to initiate the reaction. Curve a: 7 in buffer. Curve b: 7 in buffer containing 10 mM H_2O_2. Curve c: 7 in buffer containing 10 mM H_2O_2 and 0.22 mg/mL pea seed microsomes.

The observation that the hydroxylamine, 7, was an intermediate product during the conversion of 6 to the nitroso product 4 necessitated additional studies. The spontaneous oxidation of arylhydroxylamines in aqueous solution is a known reaction (Kalhorn et al., 1981). The possibility was eliminated that the nitroso compound, 4, was produced simply by chemical oxidation of the hydroxylamine, 7, and that the hydroxylamine, 7, was the only product resulting from enzymatic oxidation. Figure 5 illustrates the rapid enzymatic oxidation of 7 relative to the much slower chemical oxidation of this hydroxylamine. Except for the substrate employed, the reaction conditions for the process illustrated in Figure 5 were the same as those employed in the kinetic study of 4-chloroaniline (6) oxidation (Figure 6). From the data in Figure 5, pseudo-first-order rate constants were calculated to be 1.9 min^{-1} for the initial rate of enzymatic oxidation of 7, 0.046 min^{-1} for the H_2O_2-facilitated chemical oxidation of 7, and 0.037 min^{-1} for oxidation of 7 in the aqueous buffer. Under identical conditions, (4-chlorophenyl)hydroxylamine (7) was oxidized to 4 by the pea seed microsome system at an initial rate that was about 4 times faster than was the combined oxidation of 4-chloroaniline (6) to 4 and 7 (Table III). This study was conducted with HPLC in order to detect 7 and 4 simultaneously during the enzymatic oxidation of 4-chloroaniline (6). Thus, the rate-determining step in the conversion of 6 to 4 by the pea seed microsome system is the initial oxidation to give the hydroxylamine, 7.

The effect of substrate concentration on the nature of metabolites produced is most evident in Figure 6; however, this effect also causes the production of additional me-

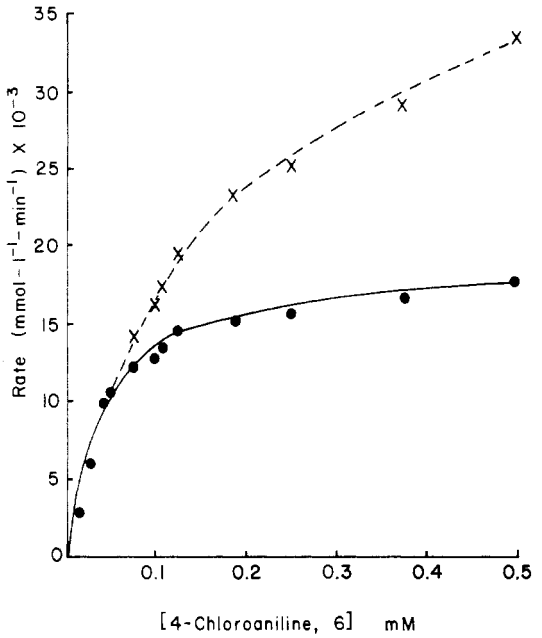

Figure 6. Effect of substrate concentration on the initial rate of reaction. The reactions were carried out at 22 °C by the addition of 2.2 mg of enzyme preparation in 1.0 mL of 0.1 M potassium phosphate, pH 7.2, to 9.0 mL of 0.1 M potassium phosphate, pH 7.2, containing 100 μmol of H_2O_2 and 4-chloroaniline (6) at the indicated concentration. Aliquots were quenched at 1 min and analyzed as described under Experimental Section. (●) Measured concentrations of 4-chloronitrosobenzene (4); (×) calculated sums of oxidation products expressed as equivalents of 4-chloronitrosobenzene (4).

Table III. Relative Rates of Enzymatic Oxidation of 4-Chloroaniline (6) and (4-Chlorophenyl)hydroxylamine (7)[a]

substrate	concentration, mM, of substrate or metabolite in enzymatic reaction mixture at 30 s (±0.002 SD) 6	7	4
4-chloroaniline (6)	0.106	0.006	0.007
(4-chlorophenyl)hydroxylamine (7)		0.074	0.040

[a] The enzymatic oxidation of both 6 and 7 were conducted in triplicate by employing a substrate concentration of 0.116 mM, H_2O_2 concentration of 10 mM, and pea seed microsome preparation at a concentration of 0.22 mg/mL. Aliquots were quenched after 30 s and then immediately analyzed by HPLC employing 40% aqueous methanol with a 254-nm detector and 70% aqueous methanol with a 313-nm detector.

tabolites in incubations conducted for extended periods. The time–course studies illustrated in Figures 3 and 4 were conducted at 10-fold differences in substrate concentration. Figure 3 illustrates that for 0.0625 mM 4-chloroaniline (6), the nitroso metabolite, 4, was produced most rapidly at all times during the enzymatic reaction. A 10-fold increase in 4-chloroaniline (6) concentration caused a reversal in the nature of the major metabolite (Figure 4), and the hydroxylamine, 7, was produced over twice as rapidly as was the nitroso metabolite, 4, for the first 10 min of the reaction. A complicating factor of this substrate concentration effect is the observation that 4,4′-dichloroazoxybenzene (9) is produced during the course of the reaction at high substrate concentration (Figure 4). This product was identified by HPLC retention time and peak height ratios (254, 280, and 313 nm) relative to authentic 9. The production of the azoxy compound, 9, is probably due to the chemical condensation of the hydroxylamine metabolite, 7, with the nitroso metabolite, 4. The chemical production of azoxy artifacts is a major problem in any system containing hydroxylamines and nitroso compounds and therefore necessitates a judicious choice of analytical methodology (Corbett et al., 1979).

Figures 3 and 4 also reveal the production of a fourth compound, which was identified as 4-chloronitrobenzene (8) by HPLC methods and on the basis of identical UV spectra of the product and authentic 4-chloronitrobenzene (8). The mass spectrum of this product following isolation by HPLC was identical with that of authentic 8. The production of 4-chloronitrobenzene (8) was found to be dependent upon the presence of an active enzyme in the microsomal preparation and is thus a metabolite. Although the oxidation of 4-chloronitrosobenzene (4) to the nitro oxidation state by H_2O_2 is a thermodynamically favorable process, the nitroso functional group is kinetically inert to simple chemical oxidation by H_2O_2 or O_2. The slow relative rate of enzymatic oxidation of the nitroso functional group is probably the result of the much lower nucleophilicity of the nitrogen atom of the nitroso group relative to that of the amino and highly nucleophilic hydroxylamino functional groups (Corbett and Corbett, 1980).

It is significant that the pea seed microsome system did not cause the conversion of 4-chloroaniline (6) to 2-amino-5-chlorophenol. Such a conversion would be suggestive of a C-hydroxylation process (Fletcher and Kaufman, 1979) or arylhydroxylamine rearrangement (Corbett and Corbett, 1981). The absence (<4 μM) of this potential C-hydroxylated metabolite in enzymatic reaction mixtures was established by HPLC analysis as previously described (Corbett and Corbett, 1981).

The effect of 4-chloroaniline (6) concentration on the initial rate of the enzymatic reaction was determined over the concentration range of 0.0125–0.50 mM with an H_2O_2 concentration of 10 mM, which was near the saturating concentration of H_2O_2 for the enzymatic oxidation reaction. The amount of 4-chloronitrosobenzene (4) produced vs. substrate concentration is given in Figure 6. A double-reciprocal plot of these data was meaningless; however, the simultaneous measurement of (4-chlorophenyl)hydroxylamine (7) produced in the incubations allowed us to calculate total oxidation products of the enzymatic reaction. The dashed line of Figure 6 represents this calculated amount of oxidation products, which was computed as the sum of the concentration of 4-chloronitrosobenzene (4) plus half the concentration of (4-chlorophenyl)hydroxylamine (7) in the same 1.0-min aliquot. The use of half the hydroxylamine concentration was based on the fact that this product results from a two-electron oxidation and the nitroso product results from a four-electron oxidation. A double-reciprocal plot of the data represented by the dashed line (Figure 6) plus the data points for substrate concentrations at and below 0.05 mM gave a very good indication of Michaelis–Menton-type kinetics for this enzymatic oxidation of 6. The K_m for 4-chloroaniline (6) was determined by graphical methods to be 0.12 mM and the V_{max} for the production of 4-chloronitrosobenzene (4) was found to be 0.037 mmol L^{-1} min^{-1} under the given incubation conditions.

DISCUSSION

The microsomal fraction of germinated pea seeds was found to oxidize 4-chloroaniline (6) to the hydroxylamine, 7, nitroso, 4, and nitro, 8, oxidation states (Figure 7).

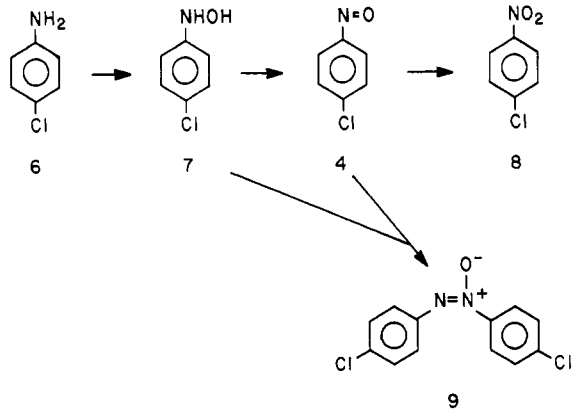

Figure 7. Metabolic and chemical fate of 4-chloroaniline (**6**) in the presence of pea seed microsomes and H_2O_2. (4-Chlorophenyl)hydroxylamine (**7**); 4-chloronitrosobenzene (**4**); 4-chloronitrobenzene (**8**); 4,4′-dichloroazoxybenzene (**9**).

Under all conditions investigated the nitroso metabolite, **4**, was the major product, except at high substrate concentrations in which case the hydroxylamine metabolite, **7**, was the major product. These results differ from the report that aniline is oxidized solely to phenylhydroxylamine (Ishimaru and Yamazaki, 1977); however, in that study the only aniline concentration employed was 1.0 mM. Studies with six additional arylamine substrates indicate that this oxidative ability is a rather general property for the microsomal fraction from pea seeds (Table I). The two arylamine substrates that were not oxidized by the microsomal system are extremes with respect to the electronic properties of arylamines. The amine group of 4-nitroaniline is a much weaker base than most arylamines (Rao et al., 1979) and has a substantially reduced electron density on the amine nitrogen relative to that of the other arylamines in Table I (Loew et al., 1979). These properties of 4-nitroaniline could explain the failure of the microsomal enzyme to effect N-oxidation. An explanation for the inability of this enzyme system to oxidize the electron-rich substrate 2,6-dichloro-*p*-phenylenediamine is not obvious. Those arylamines in Table I that were readily oxidized by the microsomal system comprise a good representation of major arylamine pesticide residues.

The N-oxidation of 4-chloroaniline (**6**) was dependent on H_2O_2. The microsome preparation would not effect N-oxidation or C-oxidation when H_2O_2 was replaced with an NADPH-generating system under aerobic conditions. This same observation was made in the case of indole oxidation by pea seed microsomes (Ishimaru and Yamazaki, 1977). In that study it was found that the responsible peroxidase in the microsomal fraction was unusual in that an oxygen atom of the peroxide substrate was directly incorporated into indole to give indoxyl. The term "peroxygenase" was proposed for such enzymatic action (Ishimaru and Yamazaki, 1977). No attempt was made in our study to determine whether the incorporation of a peroxide oxygen into the N-oxidation products of 4-chloroaniline (**6**) was occurring.

The similarity between the N-oxidation enzyme present in pea seed microsomes and the fungal enzyme chloroperoxidase (Corbett et al., 1980) is strong, although the latter peroxidase has no detectable ability to oxidize 4-chloronitrosobenzene (**4**) further to the nitro metabolite, **8**. Also, we have found that chloroperoxidase oxidizes 4-nitroaniline but the pea seed microsome preparation does not. The pea seed preparation did not cause ring halogenation of 4-chloroaniline (**6**), which is a major reaction for chloroperoxidase in the presence of Br^- or Cl^-. Another notable difference between these two enzymes is the product of indole oxidation. The pea seed microsome system converts indole to indoxyl (Ishimaru and Yamazaki, 1977), while chloroperoxidase selectively oxidizes indole to oxindole (Corbett and Chipko, 1979b). However, it is not yet known whether arylamine and indole oxidations are carried out by the same enzyme in pea seed microsomes.

The pea seed microsome system, like chloroperoxidase, is an unusual peroxidative enyzme with respect to its action on arylamines. Most peroxidases, including horseradish peroxidase, oxidize arylamines by one-electron processes to give complex products (Saunders et al., 1964), while the pea seed microsomal enzyme and chloroperoxidase give products expected from two-electron oxidations on the arylamine nitrogen. The relative contributions of such differing classes of peroxidases on the overall metabolic fate of arylamines would thus be expected to depend on the plant species under investigation. In a similar manner, the nature of residual peroxidase activity in soil (Bordeleau and Bartha, 1972) may depend on the nature of the microflora and on the types of plants growing in a particular soil.

Similar investigations with the microsomal fractions from corn (*Zea mays*) and wheat (*Triticum aestivum*) indicated a low level of *N*-oxidase activity in germinating corn seeds but none in wheat. From these limited studies, we conclude that the presence of a peroxidase with pronounced ability to effect N-oxidation of arylamines will be a property of individual plant species. An investigation of the metabolism of 4-chloroaniline (**6**) in germinating pea seeds is in progress to determine if the pea seed microsome peroxidase activity contributes significantly to the overall metabolic fate of this arylamine.

Registry No. **4**, 932-98-9; **6**, 106-47-8; **7**, 823-86-9; **8**, 100-00-5; 3,4-dichloroaniline, 95-76-1; 4-bromoaniline, 106-40-1; *p*-toluidine, 106-49-0; aniline, 62-53-3.

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Received for review Januray 26, 1983. Revised manuscript received May 19, 1983. Accepted August 29, 1983. This work was supported by Grant CA 32395 awarded by the National Cancer Institute and by Research Career Development Award ES 00120 to M.D.C. from the National Institute of Environmental Health Sciences, Department of Health, Education, and Welfare. This is journal series no. 4198 from the University of Florida Agricultural Experiment Station.

Relative Biological Activity of Nonphosphorylated Vitamin B-6 Compounds in the Rat

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The relative biological activity for rats of pyridoxine (PN), pyridoxal (PL), and pyridoxamine (PM) was examined in research concerning the quantitation of rat bioassays for vitamin B-6. With typical basal diets containing 19.8% casein, PM and PL elicited slightly lower growth and feed efficiency responses and essentially equivalent plasma pyridoxal 5′-phosphate (PLP) concentration, relative to PN. In contrast, PL and PM were markedly less active on the basis of erythrocyte aspartate aminotransferase activity and in vitro stimulation by exogenous PLP. Similar results were observed when high-protein (50% casein) basal diets were used for growth, feed efficiency, and plasma PLP indexes. The relative differences in biological activity were much less pronounced with low-protein (5% casein) diets. The specific activity of intestinal aromatic aminotransferase, which has been reported to be increased in severe vitamin B-6 deficiency, was not suitable for bioassay quantitation. The results of this study indicate the need to be cognizant of differences in the apparent biological activity of the various B-6 vitamers in rat bioassays employing PN dose–response curves. These results also support the validity of plasma PLP as an index of vitamin B-6 status.

Vitamin B-6 is the generic name for a family of compounds having similar biological activity. Although pyridoxal 5′-phosphate (PLP) and pyridoxamine 5′-phosphate (PMP) are the only two B-6 vitamers presently known to be active coenzyme forms of the vitamin, various B-6 vitamers can be interconverted both enzymatically (Snell and Haskell, 1971) and nonenzymatically in the presence of catalysts [e.g., Metzler et al. (1954), Matsuo (1957), and Lui et al. (1981)]. Numerous studies have been conducted with pyridoxine (PN) hydrochloride as a standard in the quantitation of biologically available vitamin B-6 in food [e.g., Yen et al. (1976), Gregory and Kirk (1978), Gregory (1980a), and Tarr et al. (1981)]. The vitamin B-6 of animal-derived foods is comprised largely of PLP and PMP, with small amounts of PL, PM, and PN (Vanderslice et al., 1980; Gregory et al., 1981), while the majority of the vitamin in plant tissues appears to occur as PN (Polansky et al., 1964; Polansky, 1969). The use of PN as a standard in animal bioassays is based on the greater stability of this vitamer and, to some extent, the assumed equivalent biological response to PN and the other vitamin B-6 compounds.

Although a great deal of information has been reported on differences in responses of a number of microorganisms to various B-6 vitamers (Toepfer and Lehmann, 1961; Haskell and Snell, 1970; Guilarte et al., 1980% Gregory, 1982), data concerning the relative response of animal species to different forms of vitamin B-6, especially as influenced by diet, are rather limited. An influence of the intestinal microflora on the apparent relative activity of B-6 vitamers has been suggested by several studies. Sarma et al. (1946) reported equivalent growth-supporting activity in rats for PN, PM, and PL when administered in solution per os or injected intraperitoneally; however, PL and PM elicited a lower growth-promoting response when fed in the diet. Similar results were obtained for dietary and intraperitoneally injected PLP. These findings were confirmed in subsequent experiments with rats (Linkswiler et al., 1951) and chicks (Waibel et al., 1952). In these early studies growth was the only criterion used for the evaluation of the relative potency of the B-6 vitamers. Lower apparent activity of dietary PL and PM for rats was suggested in further research in which B-6 vitamer concentrations and the activity of aspartate aminotransferase and alanine aminotransferase in several tissues were determined (Brin and Thiele, 1967; Thiele and Brin, 1968). The degree of enzyme stimulation by in vitro addition of PLP and plasma PLP concentration were not determined, however.

The purpose of the present study was to evaluate the relative activity of the nonphosphorylated B-6 vitamers when fed in semipurified diets under conditions of typical rat bioassays. Of primary interest was a comparison of the relative activity of the vitamers as determined by several routinely used biochemical indicators of vitamin B-6 nutriture. A second objective as to evaluate the relative activity of the B-6 vitamers as a function of dietary protein content.

MATERIALS AND METHODS

Animals and Diets. Male weanling Crl:CD(SD)BR Sprague-Dawley rats (Charles River Breeding Laborator-

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0021-8561/83/1431-1282$01.50/0