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Activation of the Parasympathomimetic Alkaloid Slaframine by Microsomal and Photochemical Oxidation

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SUMMARY

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Slaframine (1-acetoxy-6-aminooctahydroindolizine) is photochemically converted to two major products. On the basis of chemical studies done on the $NaBH_4$-reduced materials, structures have been assigned. The first compound arises from deamination and rearrangement to a cyclic imine. The second compound is apparently formed by direct electron transfer and hydrolysis to produce a ketoimine. The two compounds can be separated from each other and from slaframine by ion-exchange chromatography; the latter product is biologically active while the former is not. The same products are formed with rat or porcine liver microsomes in the presence and absence of pyridine nucleotides; the microsomal activation is also insensitive to carbon monoxide, 2-diethylaminoethyl 2,2-diphenylvalerate, cyanide, and prior treatment of the animals with phenobarbital or 3-methylcholanthrene. Purified porcine hepatic microsomal flavoprotein oxidase was shown to oxidize slaframine to the active metabolite; either reduced or oxidized pyridine nucleotides enhanced product formation by stabilizing the enzyme. This enzyme appears to oxidize slaframine directly and is postulated to be primarily responsible for slaframine activation *in vivo*.

INTRODUCTION

Slaframine (I, Fig. 1; 1*S*,6*S*,8*aS*-1-acetoxy-6-aminooctahydroindolizine) is an alkaloid produced by the fungus *Rhizoctonia leguminicola* (1–6). The compound does not appear to be biologically active per se; however, after bioactivation *in vivo*, a potent agent capable of stimulating the activity of exocrine glands is produced (7–10). The stimulation appears to result from direct binding to and stimulation of muscarinic acetylcholine receptors (8, 9).

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Bioactivation can also be achieved *in vitro*, either with hepatic microsomes or with flavins in the presence of visible light (9). Flavin reduction and slaframine bioactivation have been shown to be kinetically correlated. The microsomal activation, as assayed with the ileum bioassay, appeared to be NADPH-dependent and was insensitive to the removal of oxygen or addition of carbon monoxide. These results seemingly are in conflict, as the photochemical work suggests an oxidative pathway of activation for slaframine while the microsomal

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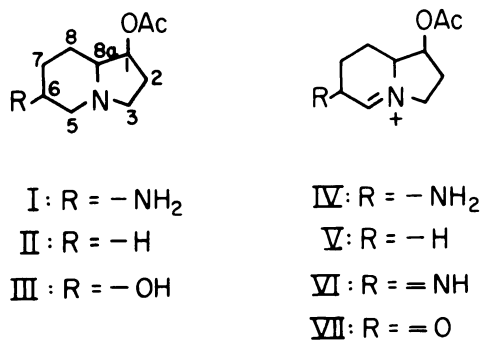

I: R = $-NH_2$
II: R = $-H$
III: R = $-OH$
IV: R = $-NH_2$
V: R = $-H$
VI: R = $=NH$
VII: R = $=O$

FIG. 1. *Structures of slaframine and related compounds*

data suggest a reductive pathway.

All previous attempts to isolate or demonstrate a chemically defined metabolite of slaframine, with the exception of the inactive deacetyl derivative, have been unsuccessful (8, 9). Moreover, earlier work did not identify the enzyme system responsible for activation. In this report evidence is presented that slaframine undergoes a direct, 4-electron oxidation to an active ketoimine and that this reaction is carried out by the hepatic microsomal flavoprotein oxidase characterized by Ziegler and his associates (11, 12).

MATERIALS AND METHODS

Materials. Slaframine was isolated from the fungus *Rhizoctonia leguminicola* as previously described (6), recrystallized from 95% ethanol as the dipicrate, and converted to the dicitrate salt before use. 1-Hydroxyoctahydroindolizine was synthesized as described (13, 14); reaction with pyridine–acetic anhydride gave 1-acetoxyoctahydroindolizine (II). 1-Hydroxy-8-aminooctahydroindolizine was a gift from Dr. K. L. Rinehart, Jr., University of Illinois, Urbana. NaB^2H_4 was obtained from Alfa Inorganics, and NaB^3H_4, from New England Nuclear. FMN (from Sigma) was used without further purification. Pyridine nucleotides were purchased from Sigma and dissolved immediately before use (older preparations of NADH inhibited microsomal slaframine activation). Glass-distilled water was used in all experiments.

Microsomal preparations. Microsomes were prepared from immature male Sprague-Dawley rats (80–100 g) essentially according to van der Hoeven and Coon (15). After the pyrophosphate wash, the microsomes were homogenized in 10 mM Tris-acetate buffer containing 1 mM EDTA and 20% glycerol, stored under nitrogen at −20°, and used within 3 days, unless otherwise noted.

Porcine liver microsomal flavoprotein oxidase preparations (11) were generously provided by Dr. D. M. Ziegler and his associates at the University of Texas, Austin.

Assay procedures. The guinea pig ileum bioassay for slaframine activation was used essentially as described (9); in all cases a standard curve was obtained using acetylcholine iodide. Preparations were tested before and after the addition of each test compound for acetylcholine response. Gas-liquid chromatography was performed on 6 ft. × 1/8 in. glass columns of 3% OV-17, 5% SE-30, and 15% Carbowax 20M on Gas-Chrom Q (100–120 mesh; all materials were purchased from Applied Science Laboratories); flow rates were 20–30 ml of N_2 per minute. Mass spectra were obtained using an LKB 9000 gas-liquid chromatograph–mass spectrometer with a 3% OV-1 column. Ammonia was trapped according to Conway and Byrne (16) and assayed with Nessler's reagent (17). Oxygen was removed from solutions (in Thunberg cuvettes) by repetitive cycles of vacuum and scrubbing with nitrogen which had been passed through two towers of Fieser's solution (18). Protein concentrations were determined according to Lowry *et al.* (19).

Activation of slaframine. Unless otherwise noted, photochemical activation of slaframine was accomplished by illuminating an aerobic mixture of 1 mM slaframine dicitrate and 2 mM FMN in 0.1 M potassium phosphate buffer (pH 7.0) for 10 min with a 150-W floodlamp at a distance of 15 cm, with the mixture maintained at 25° by means of a water bath. Approximately 5 mg of crystalline $NaBH_4$ were added, and the reduction was allowed to proceed for 20 min at room temperature. The pH was raised to 10 with 1 N NaOH, and the mixture was extracted three times with chloroform; the combined chloroform extracts were dried with anhydrous Na_2SO_4 and concentrated under vacuum

prior to analysis by gas-liquid or thin-layer chromatography.

In the microsomal activation of slaframine, a mixture of 1 mM slaframine dicitrate, rat liver microsomes (2–4 mg/ml) or purified flavoprotein oxidase (0.1–0.5 mg/ml), and any other added compounds was incubated in 0.1 M potassium phosphate buffer (pH 7.7; total volume, 1.0 ml) at 37° for 10 min with rotary shaking (150 rpm). The reaction was stopped by the addition of 0.1 ml of 10% $ZnSO_4 \cdot 7H_2O$, followed by chilling on ice and centrifugation (20). The supernatant was transferred to a second tube, and the pellet was suspended in 1.0 ml of H_2O and recentrifuged; the combined supernatants were washed twice with 5 ml of ether, with centrifugation each time. The pH of the aqueous layer was raised from 5.5 to 7.7 by the addition of 0.06 ml of 1 N NaOH, and then 0.5 μmole of NaB^3H_4 (119 mCi/mmole) in 1 μl of 40% NaOH was added; after mixing, reduction proceeded for 30 min at room temperature. The pH was lowered to 3.5 by the addition of 0.10 ml of 1 N HCl; the mixture was washed four times with 5 ml of chloroform (with centrifugation between washes), and the chloroform extracts were discarded. The contents of each tube were then adjusted to pH 10 with 1 N NaOH (monitoring with test papers) and extracted twice with 5 ml of chloroform, centrifuging between extractions. The combined extracts were dried with Na_2SO_4. An aliquot (2–3 ml) was transferred to a scintillation vial; solvent was thoroughly removed by leaving the vials under a hood fan overnight. To each vial were added 15 ml of a mixture consisting of 0.4%, 2,5-diphenyloxazole, 0.04% 1,4-bis[2-(5-phenyloxazolyl)]benzene, and 20% absolute ethanol in toluene for counting; tritium counting efficiency was determined with [^{3}H]toluene internal standards (New England Nuclear).

RESULTS

Photochemical oxidation of slaframine: characterization of products and reactions. Previous attempts to find metabolites of slaframine *in vivo* had been unsuccessful (8, 9). Moreover, when slaframine was activated with either liver microsomes or flavins in the presence of light, no metabolites were detected by thin-layer chromatography (8). Since FMN is reduced (under anaerobic conditions) during the photochemical activation of slaframine, it was suspected that slaframine might be oxidized in the process. However, no EPR signals were observed in the reaction mixture (8); thus neither an *N*-oxide of slaframine nor a radical arising from 1-electron oxidation of slaframine would appear to be involved in slaframine activation.

Attention was turned to the possibility that an imine might result from the photochemical oxidation of slaframine and be responsible for bioactivity. Preliminary experiments showed that $NaBH_4$ completely destroyed the bioactivity (measured by the ileum assay) of photochemically activated slaframine (after washing, the ileum preparation was still responsive to both acetylcholine and activated slaframine). After such $NaBH_4$ reduction, three compounds could be extracted into chloroform at basic pH and separated by gas-liquid chromatography: retention times on 3% OV-17 (170°) were 1.1, 2.6, and 3.5 min.

The first peak was identified as 1-acetoxyoctahydroindolizine (II) by co-chromatography on 3% OV-17 (150°), 5% SE-30 (125°), and 15% Carbowax 20M (185°) and by the identity of the mass spectrum with authentic material. The compound was found to be exclusively of the *cis* configuration [*cis* and *trans* isomers are separated on the Carbowax 20M column (21)]; thus the configuration of slaframine about carbons 1 and 8*a* is retained. The second peak was identified as slaframine (I) by co-chromatography on 3% OV-17 (170°) and 5% SE-30 (170°) with authentic material and by its mass spectrum.

The third peak has been assigned the structure 1-acetoxy-6-hydroxyoctahydroindolizine (III), based upon the following data. The mass spectrum, after computer subtraction of residual slaframine, exhibited a molecular ion at *m/e* 199; the spectrum resembled that of slaframine (M^+ at *m/e* 198) in its fragmentation pattern, except that several of the major peaks were 1 mass unit higher. The compound contained an acetoxy group, as judged by the presence of a base peak at *m/*

e 43 in the mass spectrum and since basic hydrolysis (0.5 N NaOH, 100°, 10 min) produced a compound with a similar mass spectrum, but with a parent ion at *m/e* 157. Reaction with hexamethyldisilazane-trimethylchlorosilane (in dry pyridine) gave a monosilylated derivative [*m/e* 271 (M^+), 270 ($M-1$), 256 ($M-CH_3$), 228 ($M-COCH_3$), 211 ($M-CO_2CH_3$)]. The underivatized material was trapped (from gas-liquid chromatography) and allowed to react with Dragendorff's reagent (22) to give an orange spot test typical of tertiary amines, but the material did not give a positive ninhydrin test, indicating that the primary amine of slaframine had been lost. The compound was identical, by gas-liquid chromatography on 3% OV-17 (170°) and mass spectrometry, with the major product of nitrous acid deamination of slaframine [another product was routinely obtained that probably resulted from an elimination reaction to give an olefin, *m/e* 181 (M^+)]. A larger amount of material was prepared and purified by preparative thin-layer chromatography in chloroform–methanol–concentrated NH_4OH (25:25:1, v/v/v) and sublimination. The 220-MHz NMR spectrum (K. L. Rinehart, Jr., University of Illinois) was consistent with the assigned structure; however, apparent trace impurities prevented spin decoupling of the sample.

When the reduction of photochemically activated slaframine was done using NaB^2H_4 (95% atomic excess), 1 deuterium was incorporated into compound II and 2 deuteriums were incorporated into compound III, as judged by mass spectrometry (all major ions were enriched in both cases) and NMR. The slaframine did not appear to contain any deuterium; however, the large excess of starting material would prevent observation of incorporation even if this occurred. When the reduction was done with NaB^3H_4, all three of the compounds (I–III) contained tritium (Table 1). These results, along with the finding that compounds II and III are not detected without $NaBH_4$ reduction, suggest strongly that compounds IV–VII are produced in the photochemical oxidation of slaframine. Moreover, compounds I–III contained tritium after NaB^3H_4 reduction following microsomal activation, implying that the same products are produced in both the microsomal and photochemical activations. (The positions of the double bonds are assigned arbitrarily to the piperidine rings; attempts to assign the positions of incorporated deuterium definitively, using NMR, were unsuccessful.)

TABLE 1

Radioactive products isolated after NaB^3H_4 reduction of activated slaframine

In each case 2.5 μmoles of slaframine dicitrate (in 2.5 ml) were activated either photochemically or with rat liver microsomes (1 mg/ml) and NaB^3H_4 was added (see MATERIALS AND METHODS). The chloroform extracts were chromatographed on silica gel G with the systems chloroform–methanol (3:1, v/v) and chloroform–methanol–concentrated NH_4OH (25:25:1, v/v/v); areas corresponding to the standard materials [compounds I, II, and III; located with I_2 vapors, ninhydrin, and Dragendorff's reagent (22)] were scraped and counted in 15 ml of toluene containing 33% Triton X-100, 0.3% 2,5-diphenyloxazole, and 0.04% 1,4-bis[(2-(4-methyl-5-phenyloxazolyl)]benzene) after the addition of 1.0 ml of H_2O per vial.

Activation system	Total radioactivity recovered		
	I	II	III
	dpm	*dpm*	*dpm*
Photochemical, 3 min	2.8×10^6	3.6×10^6	8.8×10^6
Photochemical, 15 min	1.0×10^6	9.2×10^6	9.4×10^6
Microsomal	1.6×10^4	0.7×10^4	3.0×10^4

The anaerobic reduction of FMN by slaframine was found to have a pH optimum of about 7.3. The production of both V and VII (as judged by assay of II and III after reduction) was dependent upon light and flavin (Fig. 2); the two curves were quite different, however, consistent with two separate reactions. While the production of VII was dependent upon the FMN concentration over a wide range, the curve for production of V is consistent with the flavin playing only a catalytic role (as opposed to an electron acceptor role) in this reaction. Ammonia is a reaction product; the production of ammonia was nearly stoichiometric with the production of deaminated products (Fig. 3). Under aero-

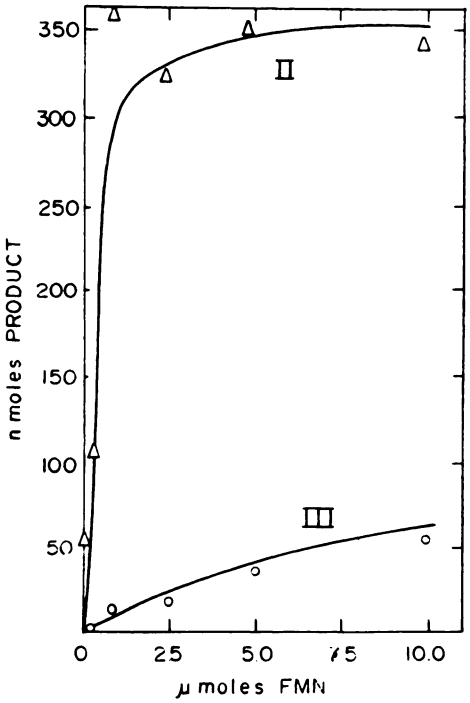

FIG. 2. *Production of slaframine metabolites as a function of flavin concentration*

Slaframine dicitrate (2.5 μmoles) was placed in the main compartment of a Thunberg cuvette along with 0.2 M potassium phosphate buffer (pH 7.0) to bring the total volume to 3.0 ml; varying amounts of FMN were placed in the neck vessel. After anaerobiosis was achieved, the contents were mixed and illuminated for 15 min. The cuvette was opened, and $NaBH_4$ was added in the dark; the products were extracted with chloroform and quantitated by gas-liquid chromatography. △, compound II; ○, compound III.

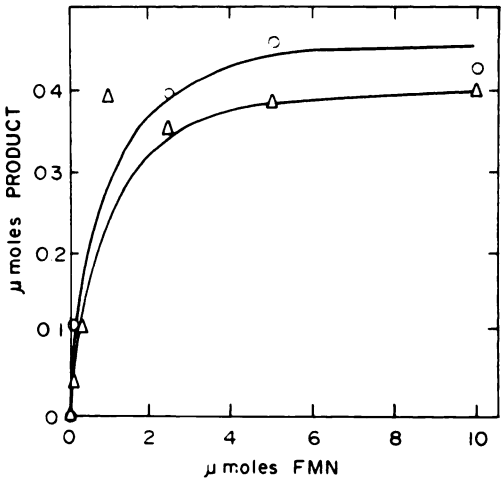

FIG. 3. *Production of ammonia during photochemical activation of slaframine*

Conditions were the same as in the experiment of Fig. 2; after illumination, ammonia (○) and the deaminated products II and III (△) were assayed as described under MATERIALS AND METHODS.

TABLE 2

Reduction of FMN by substituted octahydroindolizines

FMN (0.25 μmole) was placed in the neck vessel of a Thunberg cuvette; the main compartment contained 2.5 μmoles of the appropriate octahydroindolizine in 0.05 M potassium phosphate buffer (pH 7.0; total volume, 3.0 ml). The cuvette was closed and made anaerobic in the dark. After degassing, the components were mixed and illuminated (see MATERIALS AND METHODS); the reduction of FMN was measured at 450 nm. The calculated rate constants (all pseudo-first-order) have been corrected for FMN self-reduction.

Octahydroindolizine	k
	min^{-1}
Slaframine (I)	0.34
1-Hydroxy-8-aminooctahydroindolizine	0.07
1-Acetoxyoctahydroindolizine (II)	0.02

bic conditions, the observed yield of V was decreased while the yield of VII increased (see Fig. 4).

The structure of slaframine appears to play a critical role in the photochemical activation. As shown in Table 2, the rate of anaerobic FMN reduction is considerably faster than expected on the basis of the data obtained with the analogues. Reoxidation of slaframine-reduced FMN is rapid and unaffected by the presence of light; no stable intermediates ($t_{1/2} \geqq 10$ sec) were detected (23). A number of slaframine analogues were tested for activity: while some of these reduced FMN slowly, no bioactivity was observed after attempted photochemical activation (8). The octahydroindolizine ring system, the 1-acetoxy moiety, and the free 6-amino group all appear to be necessary for bioactivity *in vitro* and *in vivo*. In contrast with previous work (8, 9), no deactivation of photochemically activated slaframine was observed in the dark or light, as judged both by the ileum bioassay and by tritium incorporation from NaB^3H_4 into the metabolites.

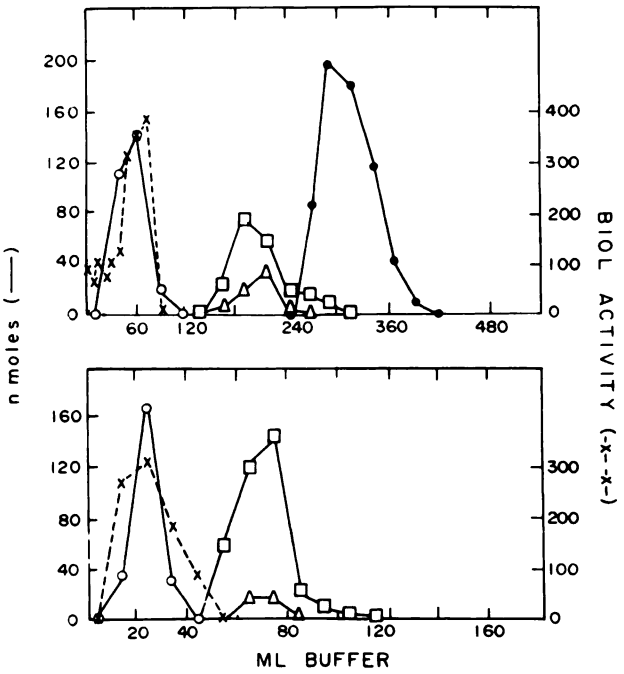

FIG. 4. *Ion-exchange chromatography of metabolites of slaframine and of biological activity*
Upper: Slaframine (5 μmoles) was activated with FMN (10 μmoles) in a total volume of 2.5 ml (with 100 μmoles of potassium phosphate, pH 7.0) aerobically for 15 min under a 150-W floodlamp at a distance of 15 cm. The mixture was applied to a 0.9 × 25 cm column of Dowex 50-X8 (H^+ form, 200–400 mesh). The column was eluted with 0.1 M ammonium acetate–1.0 M NaCl buffer (pH 6.8). Biological activity *in vitro* (×) is expressed as the percentage of the response of 0.1 μg of acetylcholine iodide obtained with 0.3 ml of each 5-ml fraction from the column. Each five 5-ml fractions was combined, reduced with $NaBH_4$, extracted into chloroform at pH 10, and assayed for slaframine and its metabolites by gas-liquid chromatography on 3% OV-17 at 165°. ○, compound III; □, compound II; △, deacetylated II; ●, slaframine (I). Lower: Activation, chromatography, and assays were performed essentially as in the upper frame, except that the column (0.9 × 25 cm) used was Bio-Rex 70 (Bio-Rad, 200–400 mesh, equilibrated with 0.25 N sodium citrate, pH 3.4) eluted with 0.38 N sodium citrate, pH 5.26.

Bioactivity of slaframine metabolites. It was found that the reaction products of the photochemical activation of slaframine could be separated by ion-exchange chromatography and quantitated after $NaBH_4$ reduction and extraction of the column fractions. The separations in two such systems are shown in Fig. 4. In both cases fractions were assayed for bioactivity *in vitro* with the ileum assay system, and this activity co-chromatographed with the fractions that contained compound III after $NaBH_4$ reduction. Thus it is inferred that the compound assigned structure VII is biologically active. When fractions from this peak were injected into adult male rats (via the femural vein under ether anesthesia), the animals salivated within 5 min; two out of four animals died of apparent "drowning" in their saliva.

Compound V is not active, consistent with the finding that production of this compound by prolonged illumination of II with FMN does not produce activity. Although no bioactivity was found to chromatograph with the fractions containing slaframine after $NaBH_4$ reduction, it should be recalled that in the photochemical activation most of compound IV produced is apparently converted to the ketone (VII) (Table 1).

Enzymatic activation of slaframine. Previous studies have provided evidence that slaframine requires bioactivation, that the principal site of activation is the liver, and that this activity is localized in

the endoplasmic reticulum (7, 9). Experiments to date with bioactivation have been complicated by the lack of a suitable assay: assays *in vivo* (intravenous injections) are not very quantitative, and, like ileum assays *in vitro*, are inconvenient and subject to several artifacts. In particular, NADPH alone produces a rather large contraction in the ileum assay (9). A radioisotope derivative assay was devised, using the incorporation of tritium from NaB^3H_4 into the metabolites of slaframine. Since about 90% of the radioactivity that is extracted into chloroform (in the step preceding chromatography) migrated with slaframine and 1-acetoxy-6-hydroxyoctahydroindolizine (III), in both initial (Table 1) and subsequent microsomal experiments (even with various additions), the tritium in the organic phase was used as a measure of bioactivation via the electron transfer pathway.

Initial experiments showed that the microsomal activation of slaframine proceeded in the absence of added cofactors. The assay was linear with respect to the concentration of microsomal protein, to at least 4 mg/ml. Table 3 shows the results of a series of experiments in which attempts to modify the activation of slaframine were made (experiments done on different days are normalized with respect to the complete system containing no additions). The activation was almost totally abolished by boiling, indicating that the activation is indeed enzymatic and not due to free flavins; moreover, light and the absence of light did not affect the assays. Treatment of the rats with either phenobarbital or 3-methylcholanthrene did not significantly increase the level of activation. The reaction was not significantly affected by the presence of CO, SKF 525-A,[1] or cyanide. Moreover, the activation did not require pyridine nucleotides when fresh, carefully prepared microsomes were used (this point will be treated later).

The results of Table 3, along with other evidence using the ileum bioassay (9), strongly suggest that cytochromes P-450 are not responsible for slaframine activation. Moreover, mixed-function oxidation (i.e., utilizing reduced pyridine nucleotides) does not appear to be involved. The studies with the model photochemical system suggested that perhaps a microsomal

[1] The abbreviation used is: SKF 525-A, 2-diethylaminoethyl 2,2-diphenylvalerate.

TABLE 3

Microsomal metabolism of slaframine: effects of various additions and alterations of the system

The complete system (using 4 mg of microsomal protein per milliliter) is described under MATERIALS AND METHODS. All assays were performed in triplicate, and the results are expressed as normalized means ± standard deviations. The complete system metabolized slaframine at the rate of 21,000 dpm of tritium incorporated into products per minute per milligram of protein.

Additions to complete system	Relative activity
	%
None	100 ± 33
Boiled microsomes[a]	8 ± 2[b]
Phenobarbital-induced microsomes[c]	165 ± 37
3-Methylcholanthrene-induced microsomes[d]	125 ± 28
CO[e]	107 ± 37
SKF 525-A (0.5 mM)	123 ± 37
KCN (2 mM)	138 ± 35
NADH (0.5 mM)	94 ± 11
NADPH[f]	110 ± 4
NADPH[f] + cytosol[g]	42 ± 15[b]
N,N-Dimethylaniline (5mM), NADPH[f]	113 ± 14
n-Octylamine (3 mM), NADPH[f]	105 ± 23

[a] Boiled for 10 min (in the absence of light).

[b] Significantly different ($p < 0.05$) from the control experiment. None of the other values is significantly different ($p < 0.10$).

[c] The rats were allowed to drink a 0.1% phenobarbital solution for 5 days before death.

[d] The rats were given 3-methylcholanthrene intraperitoneally (25 mg/kg) in corn oil 36 and 24 hr prior to death.

[e] Bubbled through the solution for 3 min at the rate of 5 ml/min.

[f] A generating system containing 0.5 mM $NADP^+$, 10 mM glucose 6-phosphate, and 0.5 unit of glucose 6-phosphate dehydrogenase per milliliter was used, and was fully active as judged by NADPH production (assayed at 340 nm) and support of the microsomal-catalyzed oxygenation of benzphetamine, benzpyrene, and other compounds in other assays. Similar results were obtained with 0.5 mM NADPH or with 1.0 mM $NADP^+$, 5 mM DL-isocitrate, and isocitrate dehydrogenase (2 units/ml).

[g] Added at 4 mg/ml (100,000 × *g* supernatant; protein concentration was equivalent to that of the microsomal fraction).

flavoprotein was accepting electrons directly from slaframine. Neither NADH-cytochrome b_5 nor NADPH-cytochrome P-450 reductase appears to be involved, since slaframine could not support microsome-catalyzed reduction of cytochrome *c* (the limit of detection of cytochrome *c* reduction was 10% of the amount needed to account for slaframine oxidation with the same preparation).

The microsomal flavoprotein oxidase described by Ziegler and his associates (11) was considered, although attempts to modify the activation of slaframine in microsomes with the addition of *N,N*-dimethylaniline (a substrate for mixed-function oxidation) or *n*-octylamine (a nonsubstrate activator) were unsuccessful. A homogeneous preparation of porcine hepatic microsomal flavoprotein oxidase was found to oxidize slaframine; however, in repeated experiments, NADPH was found to stimulate activity (Table 4). The activity could be further stimulated with the addition of *n*-octylamine. More than 90% of the incorporated radioactivity (in experiments both with and without NADPH) migrated with slaframine and compound III and traces of their deacetylated derivatives (arising from hydrolysis during experimental treatment) upon subsequent thin-layer chromatography using chloroform–methanol (3:1, v/v). Similar radiochromatographic profiles were obtained with the thin-layer chromatographic system chloroform–methanol–concentrated NH_4OH (25:25:2, v/v/v) and with gas-liquid chromatography using 3% OV-17 at 170°.

The NADPH stimulation suggested that mixed-function oxidation was occurring. However, Ziegler has observed that the oxidase is unstable in the absence of pyridine nucleotides at temperatures above 10°; the enzyme is almost totally inactivated in 3 min at 38°, but is stable for at least 10 min in the presence of 0.2 mM NADPH.[2] $NADP^+$, NADH, and NAD^+ were all found to prevent the inactivation of the oxidase and to thus increase the total amount of slaframine oxidized in the fixed-time assay (Table 4). [The rate of slaframine oxidation (approximately 1 nmole of product per nanomole of enzyme per minute) is too slow in relation to the endogenous oxidase activity (reduction of O_2 to H_2O_2) to rule out slaframine-dependent NADPH oxidation directly.]

TABLE 4

Metabolism of slaframine by hepatic microsomal flavoprotein oxidase

All incubations included 0.25 mg of flavoprotein oxidase, 0.1 M potassium phosphate (pH 7.7), and slaframine dicitrate (1.0 mM); where noted, NADPH (0.3 mM), *n*-octylamine (3 mM), $NADP^+$ (0.5 mM), NADH (0.5 mM), or NAD^+ (0.5 mM) was added. Incubation was performed for 10 min at 37° (with enzyme as the last addition after a 3-min preliminary incubation). Results are expressed (in net disintegrations per minute of product per milligram of enzyme) as means of triplicate experiments ± standard deviations.

Additions to system	Activity
	dpm × 10^6/*mg*
1. None	0.11 ± 0.03
2. NADPH	0.93 ± 0.16[a]
3. NADPH + *n*-octylamine	1.51 ± 0.11[a, b]
4. $NADP^+$	2.84 ± 0.14[a]
5. NADH	2.48 ± 0.27[a]
6. NAD^+	3.05 ± 0.70[a]

[a] Significantly different ($p < 0.025$) from value of line 1.

[b] Significantly different ($p < 0.025$) from value of line 2.

The flavoprotein appears to be more stable in microsomes, as no pyridine nucleotide stimulation was usually observed in recently prepared rat liver microsomes. However, older preparations (stored for 1 week or longer) showed stimulation with both NADPH and $NADP^+$. Porcine liver microsomes had about one-half the specific activity of rat liver microsomes toward both methimazole [a substrate for mixed-function oxidation (12)] and slaframine; NADPH stimulated slaframine oxidation about 2-fold.[3]

[2] D. M. Ziegler, personal communication. These observations have been confirmed in this laboratory.

[3] The low activity of the porcine liver microsomes is attributed to technical difficulties: we were unable to select the animals used (with respect to age and sex) and were also unable to excise the livers rapidly because of slaughterhouse restrictions. The importance of these factors has been discussed Ziegler and Mitchell (11).

DISCUSSION

Slaframine appears to be a unique compound, both in its mechanism of action and in its pathway of activation. After partial characterization of the active metabolite of slaframine, a sensitive assay was devised with which several questions about the enzymatic activation could be answered. Figure 5 summarizes the present view of slaframine activation, based upon the data presented here. Evidence is provided that the photochemical and enzymatic reactions produce the same products, although considerably less of compound V is formed in the assays with microsomes and with the purified flavoprotein than is formed in the photochemical system [previous evidence for the identity of the active metabolites from the two systems has been provided with bioassays (8, 9)].

In Fig. 5, slaframine (I) is converted to the active ketoimine (VII) by electron transfer and hydrolysis. The oxidation of primary and tertiary amines to produce aldehydes in such a manner is well established (24, 25); piperidine rings (IV, VI) tend to maintain their cyclic imine structures in solution, as judged by the products isolated after reduction with either $NaBH_4$ or NADPH (26). The conversion of slaframine to V is a rather novel reaction: the nature of the products (V, NH_3) dictates a mechanism that does not involve net oxidation or reduction, in contrast with the upper scheme. At least one intermediate probably exists in the transformation. One possibility is 5,6-dehydroslaframine, which could arise from slaframine in a manner analogous to the reactions catalyzed by histidase (27) and aspartase (28). Such a reaction has not been demonstrated to date with a flavin; conceivably, nucleophilic attack of the flavin by slaframine (via the primary amine), followed by stepwise elimination of the olefin and then ammonia, could take place. A somewhat similar mechanism (i.e., group transfer) has been proposed for other flavin reactions (23, 29–31). The enamine 5,6-dehydroslaframine could undergo acid-catalyzed rearrangement to V. Alternatively, such a rearrangement might be flavin-catalyzed: evidence has been presented that olefins are raised to excited triplet states in the presence of photosensitizers (including flavins); *cis-trans* isomerization and destruction of olefins have been observed (32, 33). Other mechanisms are conceivable, however, for the conversion of slaframine (I) to V, and the pathway discussed here should be considered speculative at this time.

The activity observed with the purified oxidase appears to be sufficient to account for most of the microsomal activity toward slaframine: a rat liver microsomal preparation metabolized methimazole at the rate of 2.5 nmoles/min/mg and slaframine at the rate of 13 pmoles/min/mg, while the purified enzyme metabolized methimazole at 770 nmoles/min/mg and slaframine at 2300 pmoles/min/mg (assuming the incorporation of 2 tritium atoms per molecule of product). As mentioned above, the relative

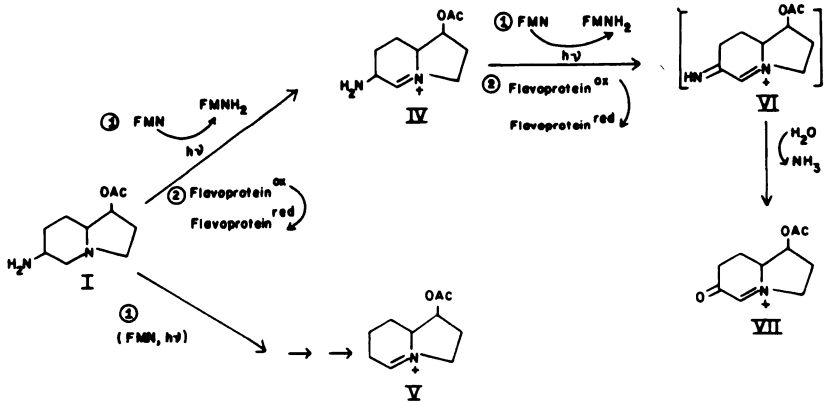

FIG. 5. *Postulated photochemical (1) and enzymatic (2) metabolism of slaframine*

rates in porcine liver microsomes are similar. Surprisingly, the substrate *N,N*-dimethylaniline (added at a concentration 10 times K_m) does not inhibit slaframine oxidation in rat or porcine liver microsomes, and slaframine (at 2 mM) has little effect on the activity of the purified enzyme toward *N,N*-dimethylaniline, even at dimethylaniline concentrations as low as one-half the K_m of 60 μM; thus it would appear that slaframine is bound to a site on the oxidase other than that used for *N,N*-dimethylaniline and other substrates.

The previous apparent requirement for NADPH in enzymatic activation can be explained by protection of flavoprotein oxidase and/or the ileum response to NADPH (9). However, the results and conclusions presented here cannot explain the induction of activity by treatment of mice with phenobarbital and inhibition of activity by treatment *in vivo* with SKF 525-A, 2,4-dichloro-6-phenylphenoxyethylamine, and other compounds (activation was judged by the comparative time lags *in vivo* before the onset of salivation) (7), because the flavoprotein oxidase is unaffected by phenobarbital and SKF 525-A treatment (12). (Some of the discrepancies may be due to the fact that label in both compounds I and III was measured in this work, although the ratios of two compounds were relatively constant in all the enzyme experiments that were checked chromatographically.) The contribution of other enzyme systems to the activation cannot be completely ruled out at this point. Although microsomal activation of slaframine was not stimulated by *n*-octylamine, Ziegler and his associates have also reported that such stimulation of the flavoprotein oxidase toward other substrates is negligible in rat liver microsomes (12).

The resemblance of compound VII to acetylcholine should be noted; i.e., a quaternary nitrogen separated from an acetoxy moiety by 2 carbon atoms. It can be postulated that this central portion of VII is involved in binding to the receptor, the 6-keto group is necessary for somehow stabilizing the nitrogen atom during binding, and the remainder of the molecule contributes to the observed specificity for the exocrine glands *in vivo*. Previous work with atropine suggests that activated slaframine is a muscarinic ligand; we have been unable to demonstrate significant inhibition of [^{125}I]α-bungarotoxin binding to a purified nicotinic acetylcholine receptor (34) with photochemically activated slaframine.[4]

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