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ACTIVATION OF SLAFRAMINE BY LIVER MICROSOMES AND FLAVINS*

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Abstract—Liver homogenates, in the presence of NADPH, are capable of activating slaframine to a form capable of stimulation *in vitro* of the guinea pig ileum. Activation activity has been located in the microsomal fraction and required NADPH; however, the reaction was not inhibited by carbon monoxide. In the presence of light, flavins are also capable of activating slaframine, and slaframine will photochemically reduce flavins. Photoreduction and activation seem to be correlated. The product of the photochemical reaction seems to be the same as that produced by NADPH and liver microsomes. FMN activated slaframine has been tested *in vivo* and is a very potent stimulator of salivation.

The active metabolite seems to exert its pharmacological activity by binding to and directly stimulating cholinergic fibers. The active metabolite has a high affinity for the receptor. Its action cannot be easily reversed by washing nor can it be reversed by atropine; however, atropine will inhibit its action if given prior to the metabolite.

THE FUNGUS *Rhizoctonia legumincola* produces a potent cholinomimetic alkaloid capable of stimulating the activity of exocrine glands.[1,2] The fungus produces the alkaloid when growing on red clover and has been found responsible for the production of "slobber forages".[3] When cattle consume these "slobber forages" they soon salivate profusely and refuse further feed.[4,5] The causative agent has been isolated and identified as 1-acetoxy-6-aminooctahydroindolizine and given the name slaframine.[6,7] The pure compound is not active *in vitro*;[8] however, *in vivo* experiments showed that it was a potent stimulator of exocrine gland secretions.[9] After a brief delay the compound causes a dramatic increase in the production of pancreatic fluid and an increase in the activity of pancreatic enzymes.

Evidence that slaframine is activated by the liver mixed-function oxidases responsible for the metabolism of most xenobiotics has been presented.[10] Ligation of the portal vein of rats prevents all activity. The activity of slaframine was enhanced by pretreating animals with compounds such as phenobarbital,[11] which are known to induce the drug-metabolizing enzymes of the liver. Pretreating the animals with known inhibitors of this enzyme system, such as SKF-525A,[12] resulted in a decrease in the effectiveness of slaframine.

All attempts to isolate or demonstrate a chemically defined metabolite of slaframine have been unsuccessful. This report concerns the bioactivation of slaframine by liver microsomes, demonstrated by its effects on the guinea pig ileum and the production of what appears to be a similar specie by flavins and light.

Slaframine. Slaframine was isolated from *Rhizoctonia leguminicola.*[1] It was repeatedly recrystallized as the dipicrate before conversion to the dicitrate for use.

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Preparation of rat liver fractions. The rats (Sprague–Dawley strain, males, 250–350 g) were exsanquished and the liver perfused *in situ* with 10 ml of ice-cold 1·15% potassium chloride via the portal vein. The livers were removed and homogenized in 4 vol. of 1·15% potassium chloride in a Potter–Elvehjem homogenizer equipped with a motor-driven Teflon pestle. The homogenate was centrifuged at 1000 *g* for 30 min and the resulting supernatant used as the "crude homogenate". This homogenate was then further fractionated into mitochondrial, microsomal and supernatant fractions by the following method: The crude homogenate was centrifuged at 10,000 *g* for 20 min The pellet was resuspended in Tyrode's solution and recentrifuged to obtain a washed mitochondrial fraction. The 10,000 *g* supernatant was centrifuged at 105,000 *g* for 90 min to separate the pellet (microsomal fraction) from the supernatant fraction. The microsomal pellet was used immediately or resuspended in 0·05 M Tris-HCl (pH 7·5) containing 50% glycerol and frozen under nitrogen at −20°. No difference in activities could be observed because of storage. The protein concentration was assayed by the method of Lowry *et al.*[13] Incubations were carried out in a Dubnoff metabolic shaker under air at 37° using 0·4 mM NADPH, 0·2 mM slaframine and enzyme in 5 ml of Tyrode's solution.

Guinea pig ileum assay. About 350-g guinea pigs which had been starved about 24 hr were sacrificed and the ileum removed immediately, chilled and washed with ice-cold Tyrode's solution. Pieces of midileum about 3 cm long were suspended in a vertical 2 × 10 cm organ bath built into a constant temperature bath maintained at 37°. The organ bath was equipped with a two-way valve at the bottom so that the ileum could be rapidly washed by draining and refilling the bath with fresh Tyrode's solution pre-equilibrated at 37°. The sections of ileum were anchored at one end and fastened to a force-displacement transducer* at the other end. A small capillary tube bubbled air into the bath at all times. The bath was always filled to the same level, which was measured to contain 20 ml. Treatments were made by injecting the test substances directly into the bathing medium with a syringe. Contractions were recorded on a Sargent model SRL recorder.

Animal assays. Assays *in vivo* for the active metabolite could be conducted by assuming that the active metabolite would have an immediate effect, whereas any slaframine in the test solution would not stimulate salivation until activation by the liver, which required several minutes.[10] Tests were therefore performed by injecting the test solution into the tail vein of rats or mice and observing for immediate salivation. Immediate salivation would have to be because of slaframine which was already activated without passage through the liver.

RESULTS

Metabolism of slaframine by rat liver homogenates. Portions of an incubation mixture (5-min incubation) containing a crude liver homogenate, slaframine, and NADPH in Tyrode's solution were added to an organ bath containing sections of guinea pig ileum. The untreated, complete incubation mixture caused a marked contraction of the ileum (Fig. 1, A) which persisted much longer (about 5 min) than contractions initiated by acetylcholine and could not be readily reversed by washing. An absolute requirement for NADPH was shown (Fig. 1, D). After a brief delay, the control incubation mixture

* Grass Instruments, Quincy, Mass.

(minus slaframine) caused a slight contraction (Fig. 1, B); however, this could be attributed to NADPH (Fig. 1, C).

Localization of the enzyme responsible for the metabolism of slaframine. The livers (28·7 g) from two rats (400 g each) were homogenized and fractionated as described in the Methods. Protein concentration and metabolizing ability were assayed on each resulting fraction. Activity was recorded as response obtained from injecting 0·2 ml of the incubation mixture into the ileum bath, compared to the response obtained from a previous injection of 0·1 μg of acetylcholine (Table 1).

Pretreatment of animals with phenobarbital gave a microsomal preparation roughly three times as active as those from control animals. Subsequently, all incubations

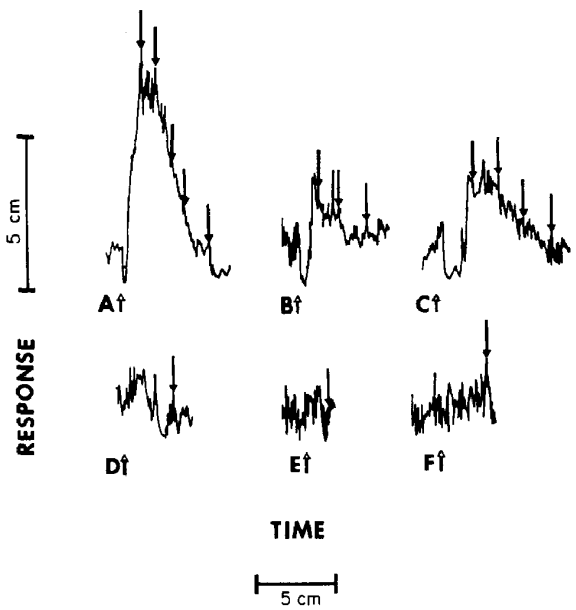

FIG. 1. Metabolism of slaframine by rat liver homogenates. Incubations were tested for their ability to cause contraction of the guinea pig ileum. A, Complete incubation mixture; B, minus slaframine; C, minus slaframine and liver homogenate; D, minus NADPH; E, slaframine alone; F, Tyrode's solution only. All incubations were for 3 min at 37°. Upper arrows indicate washings and lower arrows additions.

TABLE 1. ACTIVATION OF SLAFRAMINE BY RAT LIVER FRACTIONS

Fraction	Activity*
Crude homogenate (1000 *g* supernatant fraction)	1·32
10,000 *g* supernatant fraction	0·25
10,000 *g* pellet (mitochondrial fraction)	0·34
105,000 *g* supernatant fraction	0
105,000 *g* pellet (microsomal fraction)	0·98

* Activity equals ratio of response because of metabolite to response because of 0·1 μg acetylcholine per 10 mg protein in incubation mixture.

were with microsomes from animals pretreated with phenobarbital by including 1% phenobarbital in their drinking water.

An absolute requirement for NADPH could be shown and could only partially be replaced by NADH. The reaction was not inhibited by carrying out the incubations under either nitrogen or carbon monoxide atmosphere.

Because of variations in ileum responses, all assays were corrected by comparing the response obtained with the slaframine metabolite to the response obtained with acetylcholine. In addition to the variation in ileum responses, a decrease in response to acetylcholine was seen after the ileum had been exposed to activated slaframine. There was also a gradual rise in the resting level base line. For these reasons each ileum section could only be used for about five assays.

Activation by flavins. A number of cofactors were tried in attempt to increase the production of the metabolite. Most cofactors tried were without affect; however, a marked increase in activity was observed with all flavins. FMN gave the largest increase and FAD the least. The fact that all flavins tried gave enhanced activity suggested the possibility that the enhancement was not because of a cofactor requirement but rather a reaction of the flavin itself. Further support of this hypothesis was the fact that the activity was not dependent upon the addition of microsomes or NADPH and was prevented by keeping the reaction mixture in the dark. The activity with FMN (1 mg/5 ml) and slaframine (0·2 mg/5 ml) gave roughly 100-fold greater activity than the microsomal incubation mixture.

Since the photochemical reduction of flavins by amines has been reported[14–17] it was postulated that slaframine was photochemically reducing flavins and the oxidized form of slaframine was the pharmacologically active species. This hypothesis was in-

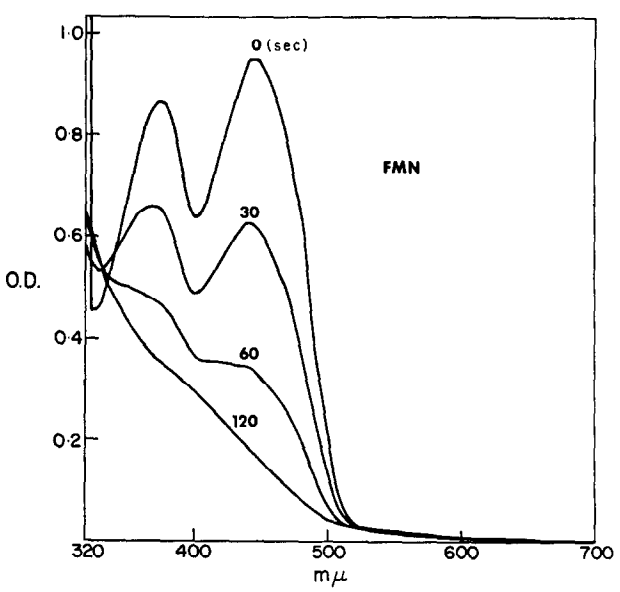

FIG. 2. Anaerobic FMN spectra in the presence of slaframine. Final concentrations of FMN and slaframine were 1×10^{-4} and 1×10^{-3} M (pH 7·0). The reaction mixture was exposed to sunlight for 0, 30, 60 and 120 sec. A spectrum identical to the zero time spectrum was obtained upon the admission of air after illumination was complete.

vestigated by first showing that slaframine was capable of anaerobic photoreduction of flavins. The absorption spectra of FMN at various times after the onset of photoreduction by slaframine were shown in Fig. 2. In daylight, the photoreduction was complete in 2 min. The reduced flavin was completely oxidized by the admission of oxygen to the system.

Additional evidence for oxidized slaframine being the pharmacologically active species came from the fact that the rate of reduction of flavins by slaframine was the same as the rate of formation of the active species. The activation process was observed to be dependent upon light intensity. By using a light of low intensity it was possible to slow the reduction and activation processes sufficiently to follow the rate of both activities and to test their correlation. The photoreduction process was assayed in a Thunberg cuvette by the decrease in absorption at 450 mμ, while the activation of slaframine was assayed by testing aliquots of a reaction mixture for its ability to contract the guinea pig ileum. Both reactions were carried out under the same light; the activation reaction was aerobic while the photoreduction reaction was anaerobic.

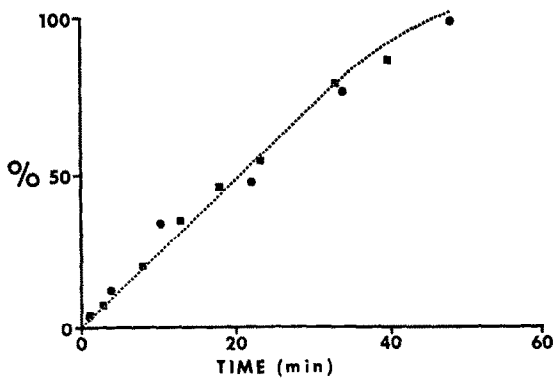

FIG. 3. Rate of FMN photoreduction and activation by slaframine. Slaframine (1×10^{-3} M) was mixed with FMN (1×10^{-4} M) under reduced light and assayed for photoreduction of FMN (anaerobically at 450 mμ) and activation of slaframine (aerobically with the guinea pig ileum). Data are expressed as per cent of final. Squares are FMN reduction and circles are slaframine activity.

Under these conditions the rate of photoreduction of FMN and the rate of activation of slaframine were essentially the same (Fig. 3). The photochemical reduction of FAD by slaframine was much slower than the reduction of FMN. The activation of slaframine by FAD was also observed to be much slower than the activation by FMN (assayed by the guinea pig ileum).

Evidence that the pharmacological activity observed was because of slaframine and not some other product of the photochemical reaction (such as hydrogen peroxide arising from air oxidation of reduced flavin) was obtained by using an analogue of slaframine to reduce the flavin. Deaminoslaframine (synthesized by Robert A. Gardiner*) would photochemically reduce FMN at essentially the same rate as would slaframine; however, it was without activity when tested on the guinea pig ileum. These results also point out the necessity of the primary amino group of slaframine for pharmacological activity.

* Robert A. Gardiner, Ph.D. Thesis, University of Illinois, Urbana, Ill.

Several lines of evidence suggest that the metabolite produced by microsomes and NADPH was the same as that produced by FMN and light. The active metabolite was very labile and the same conditions resulted in the loss of activity independent of the method of activation. Heating, raising the pH to 10 or lowering it to 2 consistently resulted in immediate loss of activity. All attempts to observe a metabolite of slaframine or a degradation product of the active metabolite were unsuccessful. In all attempts only slaframine was observed and within experimental error all of the starting material was recovered. Thin-layer chromatography of the metabolite produced from ^{3}H-(acetate)-slaframine (350 mc/mM)* by either microsomes or FMN failed to show any radioactive spot other than for slaframine. However, in each case the presence of the active metabolite could be shown: *in vitro* in the case of the microsomal reaction and *in vivo* in the case of the photochemical reaction.

The concentration of the active metabolite produced by microsomes of NADPH was too low to allow testing by injecting the incubation mixture into the tail vein of mice. Also the mice would react to the microsomal protein in the incubation mixture. However, the concentration of metabolite produced by FMN and light was sufficient to permit testing *in vivo*. The product of this reaction elicited an immediate salivation response which appeared identical to the response obtained by slaframine alone without the delay required for activation. An equivalent response would normally require more than 10 times the quantity of slaframine and would have required at least 15 min for activation by the liver before any response could be observed.

Stability of the active metabolite. Experiments preliminary to the isolation of the active metabolite slaframine (changes in pH, boiling, etc.) consistently resulted in loss of activity. The loss of activity with time was observed by activating slaframine with

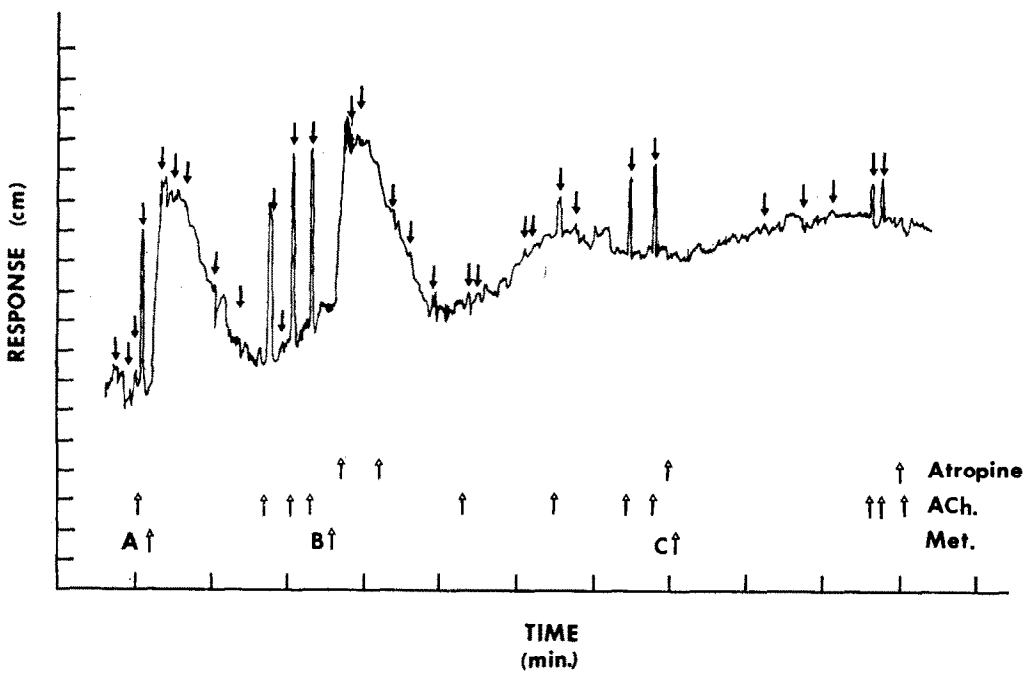

FIG. 4.

FMN and light and then storing in the dark for various periods of time prior to assaying with the guinea pig ileum. A logarithmic plot of the rate of decay yields a straight line, indicating a first-order rate constant for the decay process with an apparent half-life of about 8 min. Similar results were obtained when slaframine was activated by microsomes and then placed in the dark on ice.

Pharmacological effects of activated slaframine. Some unusual pharmacological actions of this metabolite would suggest that this compound is unlike any other cholinergic compound. The metabolite causes a rather slow but prolonged contraction of the ileum (Fig. 4, A). The slow contraction was intermediate between the action of acetylcholine and an anticholinesterase such as eserine. Further evidence that the comparative rat on anticholinesterase was obtained by the use of atropine. The addition of atropine to the organ bath at levels (8·6 μg) double that required for the reversal of eserine effects failed to reverse the effect of the metabolite (Fig. 4, B). These results also suggest a very strong binding of the metabolite to the receptor.

Further experiments with atropine did confirm the fact that the action of the metabolite was cholinergic. Addition of atropine before the addition of the metabolite to the organ bath (Fig. 4, C) completely blocked its action, which is consistent with data obtained in animals when observing salivation in response to slaframine.

DISCUSSION

Liver microsomes in the presence of NADPH have been found to be capable of producing a metabolite of slaframine as judged by its ability to cause contraction of the guinea pig ileum. A small amount of activity was localized in the mitochondrial fraction while the 100,000 *g* supernatant was devoid of activity.

The activation process was also found to be catalyzed by flavins and light. The most effective flavin was FMN, while FAD was the least effective. The photoreduction of flavins by tertiary amines has been reported,[18] and slaframine was found to photoreduce flavins and the rate of reduction of FMN correlated to the rate of activation of slaframine. The flavin-activated species could also be tested in animals and was a potent stimulator of salivation without the delay required for slaframine. McCormick[19] has reported that photoreduction of FMN is approximately four times faster than the photoreduction of FAD. Similarly, the activation of slaframine by FMN was very rapid; however, when FAD was used as the flavin, a much slower rate of activation was observed. The photochemical or microsomal-produced oxidized slaframine perhaps could be the active metabolite. This would give the tertiary nitrogen a partial positive charge perhaps necessary for cholinergic action. The nature of the active metabolite is currently under investigation.

The microsomal reaction could not be inhibited by carbon monoxide, thus the involvement of cytochrome P_{-450} is probably ruled out. The activity was increased, however, by pretreating the animals with phenobarbital, suggesting that perhaps part of the microsomal oxidase system must be involved. These results seem to confirm the results *in vivo* previously obtained.[10]

All previous attempts to isolate a metabolite of slaframine from the blood or urine of animals injected with slaframine have been unsuccessful. Attempts described in the communication to demonstrate a product of either the microsomal or flavin reactions were unsuccessful. When high specific radioactivity slaframine was used (350 mc/mM), under no circumstances were any radioactive products observed. This would indicate

that the active metabolite would have to be transitory in nature and be readily converted back to slaframine. Since the half-life of the active metabolite is about 8 min, any attempt to observe a metabolite must be performed in roughly this time period.

The mechanism by which this active metabolite stimulates the guinea pig ileum was investigated by employing the anticholinergic atropine. Since atropine will block the effects of cholinergic compounds and since it did block the action of the metabolite if given previous to the metabolite, its action must be through the acetylcholine receptor. Since atropine would not reverse the action of the metabolite, the active species cannot be acting by inhibition of acetylcholinesterase. Also, the binding of the metabolite must be greater than the binding of atropine since high levels of atropine would not reverse the effect of activated slaframine. The prolonged contraction of the guinea pig ileum even after repeated washing also suggests that the active compound has a very high affinity for the receptor. Indeed, the binding may be essentially irreversible. After the addition of the active metabolite and repeated washings, the ileum rarely completely relaxes and the response to acetylcholine diminishes.

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