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EVIDENCE FOR THE PRESENCE OF GABAMIDE ON THE WEB OF ORB WEAVING SPIDERS

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Abstract—The principal water-soluble amine on the webs of some orb weaving spiders has been variously identified as GABamide and 2-pyrrolidone. We present evidence supporting the identification of this compound as GABamide.

INTRODUCTION

Although it has been known since the turn of the century that protein is a major component of the web of orb weaving spiders (Fischer, 1907), it was the discovery that more than half the web weight of *Araneus diadematus* is due to water soluble compounds (Fischer and Brander, 1960) that revealed the true complexity of the orb web. Fischer and Brander (1960) identified one of the principal water soluble web compounds as GABamide (4-aminobutyramide).

Fischer and Brander's evidence for the presence of GABamide on orb webs was based on paper chromatographic and electrophoretic data. In a later study, using infra-red and mass spectrographic data, Schildknecht *et al.* (1972) concluded that the compound in question was 2-pyrrolidone (2-pyrrolidinone). Our own studies (Anderson and Tillinghast, 1980), employing the methods of thin-layer chromatography and high voltage electrophoresis, lent support to the earlier conclusion of Fischer and Brander (1960). Because GABamide reportedly has inhibitory pharmacological properties (Farquharson and MacLean, 1961) and may be the major water soluble amine on the web of many orb weaving spiders (Fischer and Brander, 1960; Anderson and Tillinghast, 1980; Tillinghast and Christenson, 1984), we have sought more definitive evidence on the chemical identity of the compound in question.

MATERIALS AND METHODS

2-Pyrrolidone was obtained from Aldrich Chemicals, Milwaukee, Wisconsin. GABamide was synthesized at Degussa Facbereich Forschung Chemie, Hanau, West Germany. Other chemicals were obtained from Sigma Chemical Company, St. Louis, Missouri.

Mature female *Argiope aurantia* Lucas were collected locally (New Hampshire) and web was collected in the laboratory as previously described (Tillinghast, 1984). The extraction of water-soluble compounds from the web and fractionation of this extract were performed as described earlier (Tillinghast, 1984). Briefly, this procedure involved washing the web with distilled water, drying the aqueous extract in a Savant Speed Vac Concentrator, extracting it with 95% ethanol and drying the resulting water and ethanol-soluble extract. The water–ethanol soluble extract was reconstituted in 0.2 M pyridine–acetic acid buffer, pH 2.6, and fractionated on a Dowex 50W (100–200 mesh) column. Ninhydrin-reactive material was detected by the method of Moore and Stein (1948). The compound in question eluted in fractions 40–52 (labelled D in Tillinghast, 1984). Following further purification by high voltage electrophoresis (Anderson and Tillinghast, 1980) and elution with water, the compound was again brought to dryness.

The methods employed to characterize the compound were high-performance liquid chromatography (HPLC), proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) and two-dimensional thin layer chromatography (TLC). HPLC was performed on a Beckman Model 334 liquid chromatograph using a Beckman 5 μm Ultrasphere ODS reverse phase column. Substances were chromatographed as their *o*-phthalaldehyde derivatives using a two buffer system and detected by a Beckman Model 157 fluorometer. Buffer A consisted of 50 mM sodium acetate, pH 6.8, methanol and tetrahydrofuran (79:20:1 v/v). Buffer B consisted of 50 mM sodium acetate, pH 6.8, and methanol (20:80 v/v). Flow rate was 1 ml/min, using the following proportions of A and B: 10 min 80:20; 12 min 50:50; 5 min 0:100; 9.5 min 80:20. For ^{1}H-NMR, compounds were dissolved in methanol, transferred to Norell XR-55 NMR tubes and the methanol was vaporized under a flow of nitrogen. NMR spectra were recorded on a Bruker WM-250 spectrometer at a frequency of 250.13 MH_z using D_2O as the solvent. Two-dimensional TLC was performed on 20 × 20 cm Merck precoated cellulose TLC plates, 0.1 mm thickness, using the solvent systems given by Schmidt (1974); pyridine acetone–ammonium hydroxide–water (45:30:5:20, v/v) for the first dimension and isopropanol–formic acid–water (75:12.5:12.5, v/v) for the second dimension. The spider compound was chromatographed either alone or with synthetic GABamide.

The bioactivities of GABA (4-aminobutyric acid) and hydrolyzed and unhydrolyzed spider compound, synthetic GABamide and 2-pyrrolidone were measured using the horseshoe crab (*Limulus polyphemus*) heart (Pax and Sanborn, 1967). Horseshoe crabs were collected from the Great Bay Estuary, NH and maintained at 25°C in recirculating tanks filled with natural seawater. Hearts were removed and prepared for recording as described in Augustine *et al.* (1982). Tension was monitored with a Grass FT03C force transducer and displayed on a Grass Model 79D polygraph. Hearts prepared in this manner were sensitive to 10^{-8} M GABA.

RESULTS

With the solvent systems used, GABamide and the spider compound were indistinguishable by two-dimensional TLC (Fig. 1) and the relative intensities

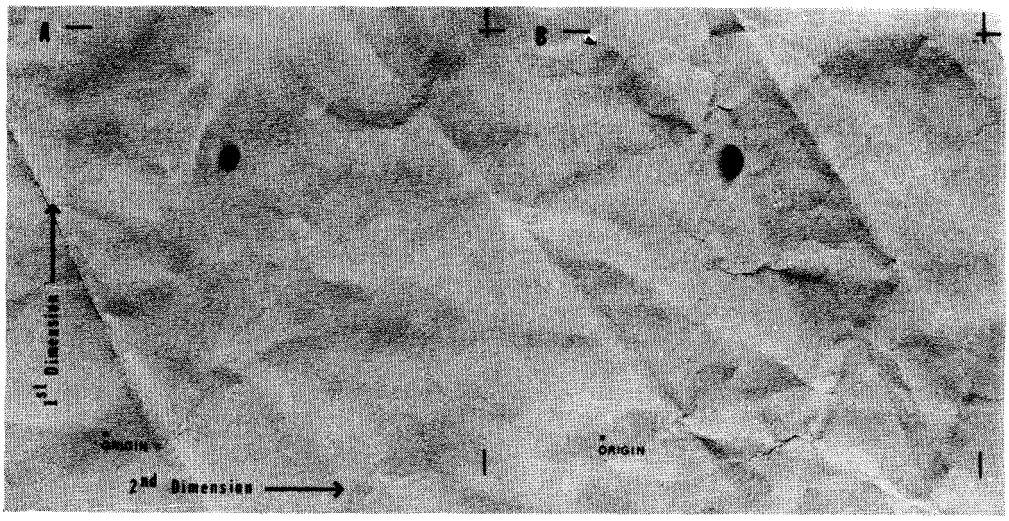

Fig. 1. Two-dimensional TLC of (A) 8 μg of the spider compound and (B) 8 μg of the spider compound with 8 μg of GABamide. Opposing parallel bars indicate solvent fronts. Development was 16 cm in the first dimension using pyridine–acetone–ammonium hydroxide–water (45:30:5:20, v/v) and 15 cm in the second dimension using isopropanol–formic acid–water (75:12.5:12.5, v/v).

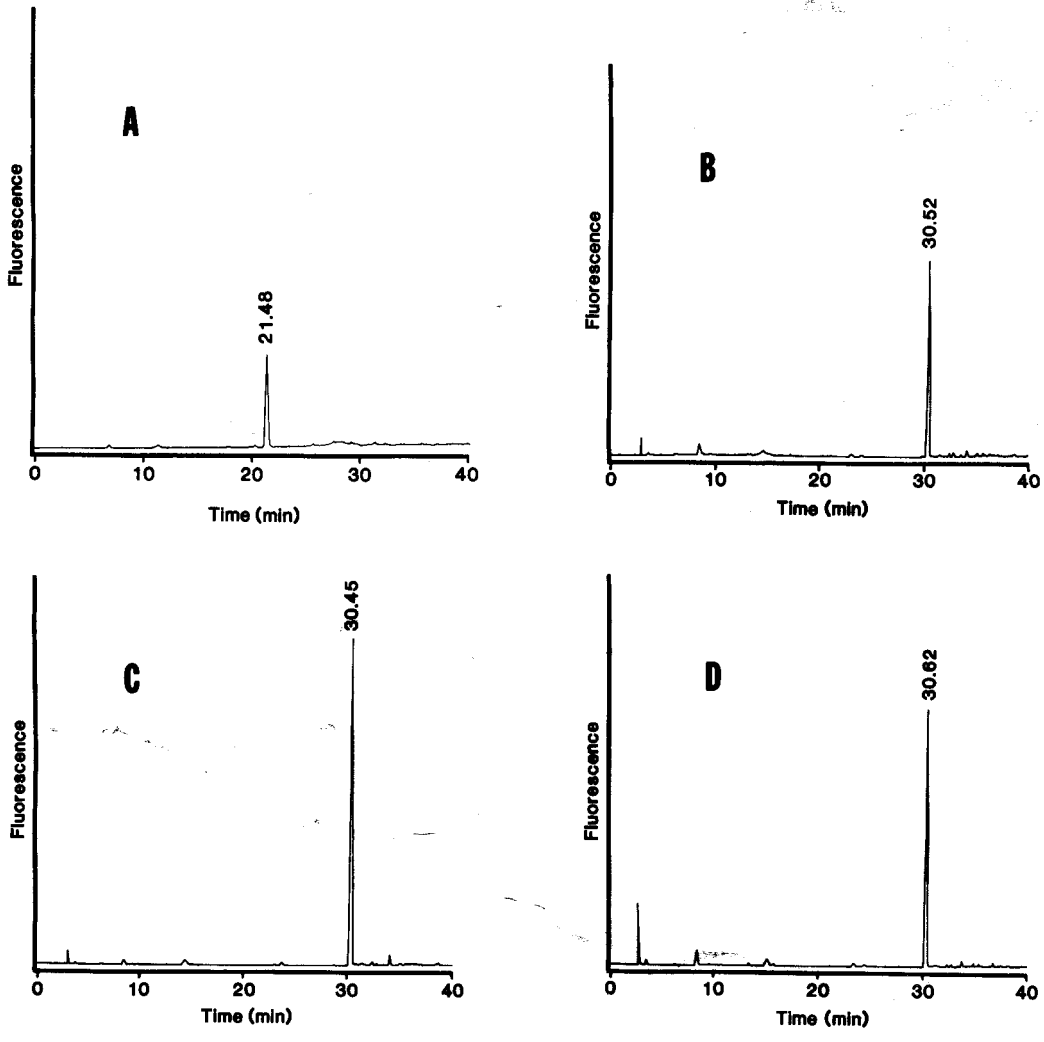

Fig. 2. HPLC analyses of the *o*-phthalaldehyde derivatives of (A) GABA; (B) GABamide; (C) spider compound and (D) GABamide and the spider compound.

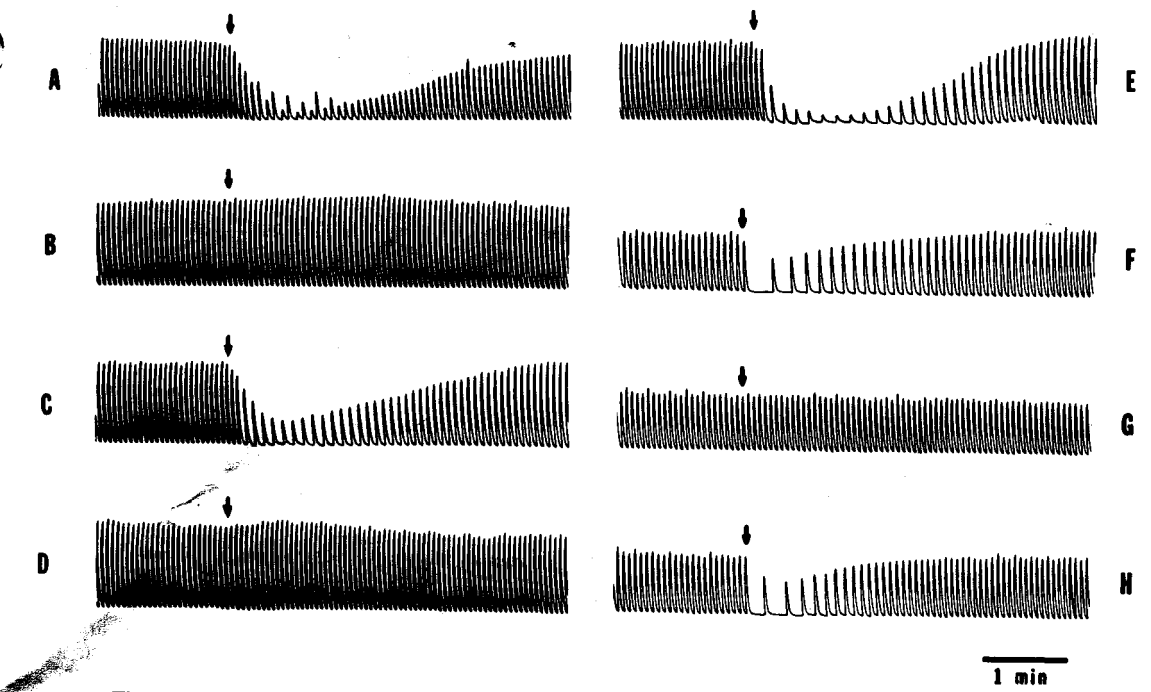

Fig. 3. Polygraph tracings of the contractions produced by a *Limulus* heart exposed to (A) GABA; (B) GABamide; (C) hydrolyzed GABamide; (D) 2-pyrrolidone; (E) hydrolyzed 2-pyrrolidone; (F) GABA; (G) spider compound and (H) hydrolyzed spider compound. Arrows indicate the time of compound addition. Recordings A through E and F through H were made from separate heart preparations. All compounds were present at a concentration of approx. 10^{-5} M.

of spots on separate plates, as visualized with a ninhydrin solution spray, were as one would expect for the amounts of spider compound or spider compound and GABamide applied.

HPLC analyses of GABA, GABamide and the spider compound are shown in Fig. 2. The retention times observed were: GABA 21.5 min, GABamide 30.5 min and spider compound 30.5 min. When GABamide and the spider compound were mixed and injected together, a single peak was obtained, eluting at 30.6 min. 2-Pyrrolidone, lacking a free amino group, yields weak fluorescence with OPA. A slight peak was obtained at a retention time of 22.3 min which is possibly ascribable to 2-pyrrolidone. A small peak was also seen at 21.3 min, presumably due to contaminating GABA (from hydrolysis of 2-pyrrolidone). NMR spectra of GABamide and spider compound were congruent. GABamide showed triplets centering at 2.258 and 2.887 ppm and a quintuplet at 1.798 ppm. Corresponding peaks were seen for the spider compound at 2.185, 2.809 and 1.729 ppm. 2-Pyrrolidone, on the other hand, under the same conditions showed triplets at 2.149 and 3.223 ppm and a quintuplet at 1.930 ppm.

Figure 3 demonstrates the inhibitory effects of GABA on the *Limulus* heart. Since GABamide and 2-pyrrolidone yield GABA upon acid hydrolysis, their hydrolysates also produced inhibition. Unhydrolyzed GABamide and 2-pyrrolidone had no apparent effect on the *Limulus* heart. In addition, hearts that were continuously perfused with GABamide responded normally to GABA, indicating that GABamide did not block GABA receptors. Thus, by itself, GABamide does not appear to act as an agonist or antagonist to GABA in this system. The spider compound, consistent with either a GABamide or 2-pyrrolidone identity, also inhibited *Limulus* heart contractions following, but not prior to, acid hydrolysis.

DISCUSSION

The evidence is now overwhelming in support of Fischer and Brander's (1960) GABamide identification for the molecule in question. These data include: (1) identical R_f values for synthetic GABamide and the spider compound with paper and thin-layer chromatography (Fischer and Brander, 1960; Anderson and Tillinghast, 1980; this communication, Fig. 1); (2) identical mobilities of both compounds with high voltage electrophoresis (Fischer and Brander, 1960; Anderson and Tillinghast, 1980); (3) identical retention times of both compounds with HPLC (this communication, Fig. 2); and (4) identical ^{1}H-NMR spectra for both compounds (this communication). Moreover, upon acid hydrolysis, a compound with properties identical to GABA is released. These data include: (1) the ready reactivity of the hydrolytic product in the "GABase" preparation (Anderson and Tillinghast, 1980); (2) identical chromatographic characteristics as commercial GABA (Anderson and Tillinghast, 1980); and (3) identical pharmacological properties of the hydrolytic product and commercial GABA on the heart of *Limulus* (this communication, Fig. 3). Finally, when the water soluble extract of freshly collected webs

is directly high voltage electrophoresed using the formic–acetic acid buffer, pH 2.1, of Schmidt (1974), without any additional extracting or fractionating, the compound of interest is easily detected with a ninhydrin solution spray. As this technique does not hydrolyze 2-pyrrolidone and as 2-pyrrolidone is ninhydrin negative, it does not seem possible for the ninhydrin positive character of the compound to be the result of 2-pyrrolidone hydrolysis during purification.

GABamide is confined to the adhesive spiral of the web of orb weavers (Anderson and Tillinghast, 1980) and is probably a product of the aggregate glands or their ducts. While it is the principal water soluble amine on the webs of *Araneus diadematus* (Fischer and Brander, 1960) and *Argiope aurantia* (Anderson and Tillinghast, 1980), it is second in quantity to an as yet unidentified compound on the web of *Argiope trifasciata* (Anderson and Tillinghast, 1980). In addition, GABamide is found on the web of the black widow, *Latrodectus mactans* (Tillinghast and Christenson, 1984). The black widow does not produce an orb web, but does produce adhesive strands and does possess aggregate glands.

The role of GABamide on the spider's web, if any, is as yet unknown. It does not appear to act either as an agonist or antagonist of GABA receptors in the one system we examined. However, Farquharson and MacLean (1961) have reported that it decreases the frequency or completely inhibits the generation of potentials in stretched slowly adapting stretch receptors of the crayfish, *Astacus fluviatilis*. Whether it exerts any effect upon the spider's prey is unestablished. Schildknecht *et al.* (1972) suggested that 2-pyrrolidone, being hygroscopic, may help to retain the moisture essential to the adhesive properties of the web. Since GABamide is also hygroscopic, their suggestion is still valid. Clearly, further studies are required before a definitive answer can be given.

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