10-1973

Sweetclover Weevil: Adenosine As a Feeding Stimulant

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Sweetclover Weevil:[1] Adenosine as a Feeding Stimulant[2]

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ABSTRACT

Adult *Sitona cylindricollis* Fåhraeus responded to adenosine in bioassays for feeding-stimulant activity. The molar concentration of adenosine which elicited maximum feeding in the bioassay was less than $\frac{1}{10}$ of the sucrose concentration required for maximum feeding.

Various purines, pyrimidines, nucleosides, nucleotides, and related compounds were compared with adenosine in bioassays for weevil-feeding stimulant activity. Of all compounds tested, only adenosine triphosphate and adenosine monophosphate appeared to act as feeding stimulants; at the concentrations used, their activities were substantially lower than that of adenosine.

Free adenosine was isolated from leaf extracts of weevil-susceptible *Melilotus officinalis* (L.) Lam. by ion-exchange and paper chromatography and was identified by spectrophotometric and paper chromatographic techniques. The quantity of adenosine present, ca. 12 μg/leaflet, was sufficient to indicate that this compound probably influences feeding of the weevil on *M. officinalis* plants.

Hsiao (1969) observed that adenine and related purine compounds act as potent feeding stimulants for larvae of the alfalfa weevil, *Hypera postica* (Gyllenhal). His report apparently provides the 1st recognized case of a phytophagous insect that responds to free purine- or pyrimidine-related compounds; although the adult yellow fever mosquito, *Aedes aegypti* L., and the house fly, *Musca domestica* L., and a blood-feeding hemipteran, *Rhodnius prolixus* Ståhl, responded to purine and pyrimidine nucleotides in buffer or salt solutions (Hosoi 1959; Galun et al. 1963; Friend 1965; Robbins et al. 1965; Friend and Smith 1971).

In previous work on feeding preference of the adult sweetclover weevil, *Sitona cylindricollis* Fåhraeus, sucrose, glucose, and fructose were identified as feeding stimulants in leaves of the host, *Melilotus officinalis* (L.) Lam. (Akeson et al. 1969a). However, the possible occurrence of other feeding stimulants was not ruled out. Reported here are the evaluation of various purines, pyrimidines, and related compounds as weevil-feeding stimulants, and the isolation of one test compound, adenosine, from extracts of *M. officinalis* leaves.

Methods and Materials.—The sweetclover root-disk bioassay described by Akeson et al. (1967) was used to evaluate the test compounds for stimulant activity. Adult weevils were collected in the vicinity of Lincoln, NE, and held in cold storage until used. Samples of 0.15 ml of each solution to be tested and water blanks of 0.15 ml were applied to separate sets of 5 bioassay disks as one replication, and the treated disks were randomly positioned in paraffin-layered petri dishes. Weevils (4 or 5/disk) were then introduced into each dish and allowed to feed for 4 or 6 h, then measurements were made of disk consumption. Each test was replicated 5 times. Duncan's multiple range test was used to evaluate the significance of differences between treatment means.

Leaves of 'Goldtop' sweetclover, a weevil-susceptible variety of *M. officinalis*, were investigated for the presence of adenosine by a modification of the procedure of Bickoff et al. (1968). Stems of greenhouse-grown plants were harvested, and the 2 youngest fully expanded leaves from each stem were combined and extracted with hot water (10 ml/g fresh tissue). Extracts were lyophilized to dryness. Lyophilized powder, representing 2.5 g of dry leaf tissue, was extracted for 6 h in a Soxhlet apparatus with 80% ethanol. The resulting ethanol extract was concentrated, applied to a Dowex®-50 (H+) ion-exchange column,[5] washed in with 0.05 N HCl, and eluted with 5 N NH_4OH. The effluent was concentrated, applied to a Dowex®-1 (Cl^-) ion-exchange column, washed into the column with water, and eluted with CO_2-saturated water. Fractions of the effluent from this column were concentrated, and a sample of each was examined by paper chromatography (Whatman no. 3MM paper) with adenosine as the control. The solvent was composed of butanol, glacial acetic acid, and water (65:15:35, vol/vol/vol). Developed chromatograms were viewed under 254-nm UV light and were then sprayed with the purine-detection reagent described by Dikstein et al. (1956). Effluent fractions containing a UV-absorbing, purine-positive spot corresponding in R_f to adenosine were combined, and an aliquot was applied in a band to each of several paper chromatograms. The chromatograms were developed in the aforementioned solvent, and the absorbing band corresponding to adenosine was cut out from each and eluted with water. Eluates were compared with known adenosine solutions in spectrophotometric, chromatographic, and feeding-stimulant tests.

Results and Discussion.—In initial tests, 0.01M solutions of adenosine, adenine, and sucrose, the sugar with the greatest feeding stimulant activity (Akeson et al. 1970), were compared in the root-disk bioassay. Sucrose and adenosine stimulated weevil feeding appreciably and to about the same extent (31.2 and 29.5% disk area consumed, respectively); the adenine treatment (5.2% consumed) did not differ significantly from the water control (6.0% consumed).

The percentages of disk area consumed by sweetclover weevils feeding on bioassay disks treated with 0.0001, 0.001, 0.01, and 0.1, and 0.0M concn of adenosine were 24.7, 40.8, 29.6, 7.4, and 6.8, respectively.

[1] Coleoptera: Curculionidae.

[2] A cooperative investigation of the Nebraska Agricultural Experiment Station, University of Nebraska-Lincoln, and the Agricultural Research Service, USDA. Supported in part by Cooperative Agreement no. 12-14-100-10052(34). The work was conducted under Nebraska Agricultural Experiment Station Projects 12-27 and 17-5. Contribution no. 366 of the Department of Entomology, University of Nebraska. Published as Paper no. 3526, Journal Series, Nebraska Agricultural Experiment Station. Received for publication Mar. 26, 1973.

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[4] The technical assistance of Mrs. Patricia Underwood is gratefully acknowledged.

[5] Mention of a proprietary product does not necessarily imply endorsement by the authors or their agencies.

Table 1.—Comparisons of adenosine, fructose, glucose, sucrose, and a mixture of the 3 sugars at 3 concn for sweetclover weevil feeding response.[a]

Compound	Disk area consumed[b] (%)		
	0.0001 M	0.001 M	0.01 M
Adenosine	24.9 a	34.0 a	27.4 a
Sugar mixture[c]	4.5 b	22.4 b	27.1 a
Sucrose	5.1 b	12.5 c	18.7 b
Fructose	7.3 b	7.4 cd	8.7 c
Glucose	4.3 b	13.2 c	7.0 c
Water (control)	5.1 b	5.5 d	6.7 c

[a] Separate bioassays were conducted at each of the concentrations. Weevil population, 4/disk; test duration, ca. 6 h.
[b] Within each concentration, means followed by the same letter do not differ at the 0.05 level of significance by Duncan's multiple range test.
[c] The sugar mixture contained equimolar amounts of sucrose, fructose, and glucose, with the total concentrations as shown.

Treating disks with the apparently optimum concentration (0.001M) resulted in the application of ca. 8 μg of adenosine/disk. It is noteworthy that the 0.0001M treatment (equivalent to 0.8 μg/disk) stimulated an appreciable degree of feeding, but the highest concentration tested (0.1M) was not more effective than water. Concurrent bioassays of 0.0001, 0.001, 0.01, 0.1, and 0.0M sucrose solutions resulted in 3.3, 11.8, 29.0, 38.4, and 5.1% disk area consumed. Sucrose was only slightly effective in stimulating weevil feeding at the optimum adenosine concentration (0.001M). Over the range 0.001–0.1M, increasing the concentration of sucrose resulted in increases in extent of weevil feeding, but increasing adenosine concentration over the same range resulted in decreases in feeding. Schoonhoven (1969) offered several suggestions to explain the observation that high concentrations of some feeding stimulants become deterrent when offered to phytophagous insects. However, current knowledge of sweetclover weevil chemoreceptors is not sufficient to indicate the mechanism responsible for the observed decreases in feeding at the 0.01 and 0.1M adenosine concn.

Direct comparisons were made also at 3 concn of the feeding stimulant activities of adenosine, fructose, glucose, sucrose, and a mixture of the 3 sugars (Table 1). All these sugars are present in a fraction of *M. officinalis* leaves that stimulates weevil feeding (referred to as Stimulant A by Akeson et al. 1969a). At the lowest concentration (0.0001M), adenosine was the only compound of those tested that stimulated a significant amount of feeding. At the 0.001M level, adenosine had the greatest activity, followed in order by the sugar mixture and the individual sugars. The sugar mixture was as active as adenosine at the 0.01M concn, and sucrose was the only individual sugar which differed significantly from the water control. Lack of appreciable feeding stimulant activity of glucose and fructose in these comparisons should not be taken as contradictory to the conclusion of Akeson et al. (1970) regarding these monosaccharides. The present tests included the sugar mixture and adenosine, both of which stimulated more extensive feeding than any single sugar. The sugar comparisons of Akeson et al. (1970), however, included only individual compounds and extended to considerably higher than 0.01M concn.

In further tests, adenosine was compared with several purines, pyrimidines, and related compounds in feeding-stimulant bioassays. As shown in Table 2, separate comparisons were made with groups of similar compounds at each of 3 concn. At the 0.01M concn, adenosine was the only compound that stimulated a significant amount of feeding. Weevil-feeding levels on disks treated with 0.001 or 0.0001M adenosine triphosphate or 0.0001M adenosine monophosphate were intermediate between levels on the water and adenosine controls. Other compounds tested at the 0.001 and 0.0001M concn failed to stimulate feeding significantly more than the water control. Thus, of the tested com-

Table 2.—Comparisons of adenosine with various purines, pyrimidines, and related compounds for sweetclover weevil feeding response.[a]

Compound tested	Disk area consumed[b] (%)		
	0.0001 M	0.001 M	0.01 M
Comparison 1			
Adenosine	28.1 a	36.5 a	35.6 a
Cytosine	11.2 b	7.3 b	13.7 b
Uracil	7.6 b	5.4 b	4.9 c
Thymine	7.1 b	5.2 b	3.7 c
Water (control)	9.2 b	5.6 b	11.2 b
Comparison 2			
Adenosine	26.2 a	33.8 a	43.1 a
Cytidine	8.6 b	9.5 b	13.0 b
Uridine	5.9 b	7.7 b	0.9 c
Thymidine	5.3 b	4.0 b	0.7 c
Water (control)	6.0 b	4.4 b	10.8 b
Comparison 3			
Adenosine	17.0 a	29.8 a	41.0 a
Adenine	8.0 b	8.8 b	1.7 c
Guanine	1.7 c	2.4 c	—
Hypoxanthine	3.1 c	3.9 c	5.5 c
Xanthine	2.6 c	1.8 c	1.3 c
Purine	2.2 c	2.1 c	0.6 c
Water (control)	8.9 b	5.1 bc	14.3 b
Comparison 4			
Adenosine	32.1 a	42.0 a	36.9 a
Guanosine	10.8 bc	8.9 b	4.3 b
Inosine	4.8 c	2.3 c	3.7 b
Xanthosine	12.6 b	7.4 bc	1.6 b
Water (control)	6.8 bc	6.4 bc	6.6 b
Comparison 5			
Adenosine	19.2 a	37.9 a	29.3 a
Adenine	5.7 b	7.8 b	2.3 c
Adenine HCl	4.1 b	4.6 bc	0.7 c
Benzoyl adenine	1.7 b	0.7 c	2.3 c
Water (control)	3.8 b	5.7 b	9.1 b
Comparison 6			
Adenosine	22.7 a	31.0 a	21.3 a
Adenosine-5′-triphosphate	16.5 ab	11.7 b	1.2 c
Adenosine-3′-monophosphate	11.3 b	2.4 c	0.2 c
Guanosine-3′-monophosphate	4.1 c	3.2 c	5.1 bc
Water (control)	3.9 c	2.7 c	7.1 b

[a] Separate bioassays were conducted at each of the concentrations for each comparison. Weevil population 4/disk; test duration ca. 6 h.
[b] Within each comparison and concentration, means followed by the same letter do not differ at the 0.05 level of significance by Duncan's multiple range test.

Table 3.—Ascending chromatography of the isolated compound, adenosine, and adenine in 7 solvent systems.

Solvent composition (% by volume)		$R_f \times 100$		
		Isolated compound	Adenosine	Adenine
n-Butanol	60	56	55	61
Acetic acid	15			
Water	25			
n-Butanol	33	68	67	66
Pyridine	33			
Water	33			
Ethyl ether	76	11	12	49
Acetic acid	18			
Water	6			
Methanol	80	48	45	57
Formic acid (88%)	15			
Water	5			
n-Butanol	77	20	18	30
Formic acid (88%)	10			
Water	13			
Ethanol	67	48	46	58
Pyridine	20			
Water	13			
Water	100	55	58	37

pounds, only those containing adenosine were active as feeding stimulants. Some of the compounds, especially certain purine bases, appeared to deter feeding, but the bioassay was not designed to provide a reliable measure of feeding deterrent activity. Adenine hydrochloride, reported by Hsiao (1969) to be most effective as a feeding stimulant for alfalfa weevil larvae, was ineffective as a feeding stimulant for sweetclover weevils.

Spectrophotometric comparison of known adenosine and putative adenosine isolated from *M. officinalis* leaves gave a strong absorption peak at 259 nm and a valley at 228 nm for both compounds. Furthermore, the 228:259, 249:259, and 269:259 nm optical density ratios for the isolated compound (0.26, 0.79, and 0.81, respectively) agreed well with the respective ratios of 0.25, 0.80, and 0.84 for known adenosine. In paper-chromatographic tests employing 7 different solvent systems, the migration of the isolated compound was similar to that of known adenosine and markedly different, in several of the solvents, from that of adenine (Table 3). Bioassays for feeding-stimulant activity indicated no significant difference in weevil feeding on disks treated with approximately equal amounts of known adenosine (19.9% disk area consumed) and the isolated adenosine (16.3% disk area consumed).

From the optical density of a solution of chromatographically isolated adenosine (and assuming no loss during the extraction and isolation procedures), the adenosine content of young *M. officinalis* leaves was ca. 0.3% of the dry weight of the leaves. Losses during the various procedures are inevitable; therefore, the true content of adenosine probably was somewhat greater than 0.3%. Young fully-expanded *M. officinalis* leaflets have a dry weight of ca. 4 mg. Using the values of 0.3% and 4 mg, it is apparent that the adenosine content of young *M. officinalis* leaves was ca. 12 μg/leaflet. This quantity of adenosine applied to a root disk in the feeding stimulant bioassay would be sufficient to elicit significant weevil feeding. Root disks and leaflets are greatly different in some respects, but they are not vastly different in size. Therefore, it is reasonable to conclude that adenosine is present in the leaflets in sufficient quantity to play a significant role in weevil feeding.

The results presented here provide ample support for the addition of adenosine to the list of *M. officinalis* constituents that influence feeding by the adult sweetclover weevil. Levels of adenosine, as well as the previously reported feeding stimulants, sucrose, glucose, and fructose (Akeson et al. 1969a), the feeding deterrent, nitrate (Akeson et al. 1969b), and probably other as yet unidentified substances, are involved in the response of the sweetclover weevil to *M. officinalis* leaves. As pointed out by Schoonhoven (1969) and Dethier (1970), numerous factors are involved in the chemical interactions between phytophagous insects and their host plants. Results of our study and that of Akeson et al. (1969a, b) indicate that the feeding response of the sweetclover weevil to its host is similarly complex.

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