Behavioral Responses of *Phormia regina* (Meigen) to Labellar Stimulation with Amino Acids

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ABSTRACT   Behavioral responses to labellar stimulation with 19 L-amino acids were predicted on the basis of electrophysiological responses of largest labellar hairs. With the exceptions alanine, aspartic and glutamic acids, and valine tests of these predictions confirmed that *Phormia* can discriminate amino acids, and that these acids may be grouped according to their effects. Electrophysiological investigation of the four exceptions was repeated and results were consistent with the behavioral data. In particular, these acids elicited previously unreported responses from the salt receptor. The discrepancies between this and earlier studies may be explainable, in part, on methodological grounds. There was evidence for response differences among hairs of different sizes and among the largest labellar hairs themselves. The significance of amino acid discrimination for the problem of protein recognition can only be speculated upon until more complete electrophysiological and nutritional information is available.

INTRODUCTION

The adult blowfly can exist on a diet of carbohydrate and water, but reproductive activity, in females at least, depends on ingestion of protein. Protein feeding by females varies cyclically in correlation with stages of oocyte development (Strangways-Dixon, 1961); males typically increase consumption during the first few days of life and maintain a rathar low level thereafter (Dethier, 1961). Discrimination between protein and cardohydrate is accomplished by chemoreceptors on the fly's tarsi and mouthparts.

The labellar chemoreceptors are contained in cuticular hairs 30–300 μm long and comprising six groups according to size and position (Wilczek, 1967) (Fig. 1). Each hair contains a mechanoreceptor that terminates at the hair base and three to four bipolar chemosensory neurons whose axons extend without synapsing to the central nervous system. Their dendrites penetrate a terminal papilla at the hair tip. This is the only sensitive region.

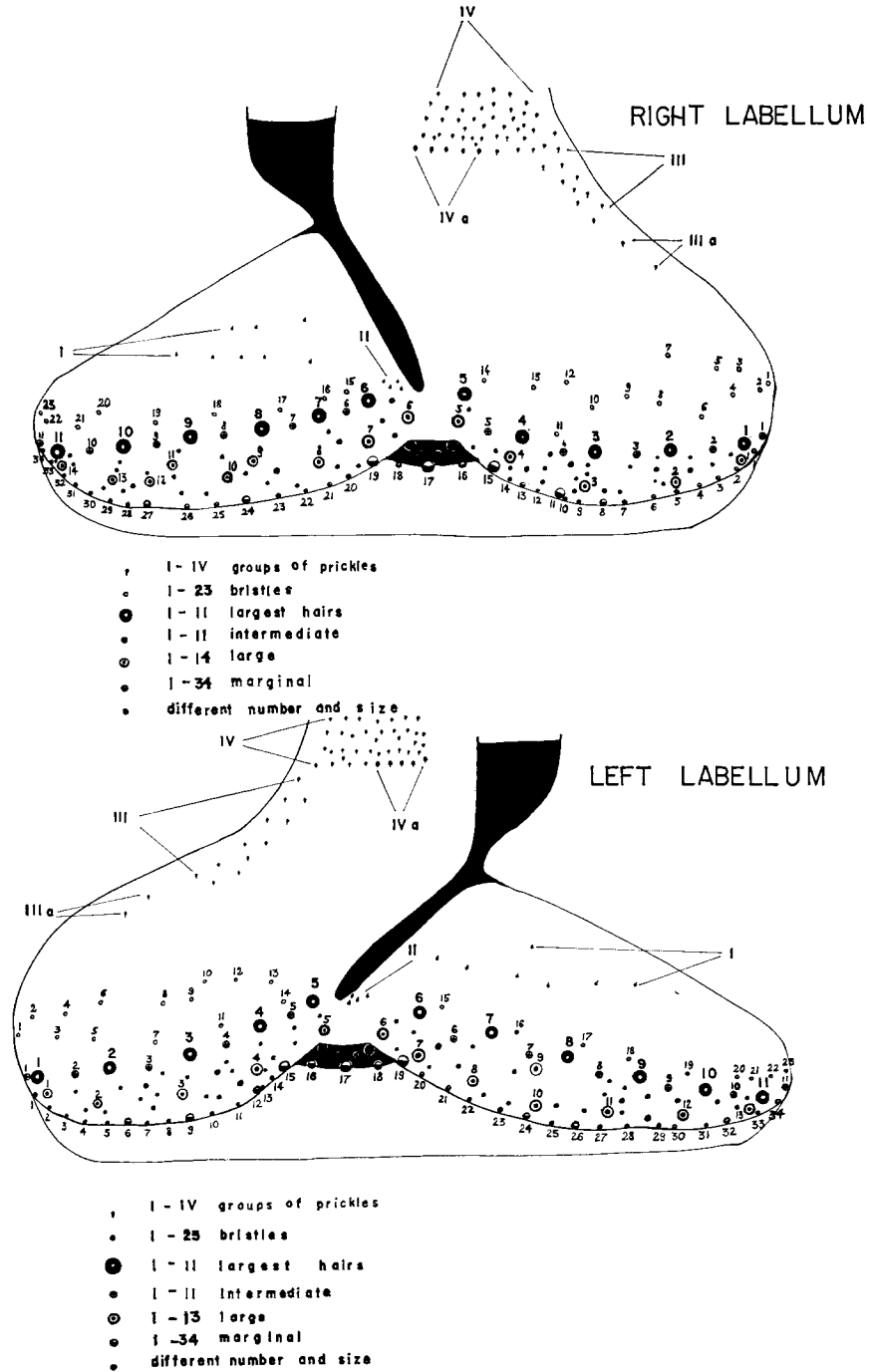One neuron has been designated a sugar receptor (S cell of Hodgson and

FIGURE 1. Distribution of hairs on the labellum of *Phormia regina* (Meigen) (from Wilczek, 1967). (Reprinted from *J. Morphol.* **122(3)**:175 by permission of The Wistar Institute Press.)

Roeder, 1956; type 2 of Rees, 1968, 1970). An array of molecular structures stimulate this fiber, but an α-D-glucopyranoside linkage characterizes the most effective (Hodgson, 1965). Activity in the sugar fiber is accompanied by proboscis extension and feeding; inhibition or nonstimulation is not (i.e., the proboscis is extended only partially or not at all or, if previously extended, is retracted) (Steinhardt et al., 1966). In general, salts decrease both electrophysiological and behavioral responses to sugars, but the effect is complex. This receptor is excited by neutral salt solutions at high pH even with no sugar present (Morita and Hidaka, 1966; Shiraishi and Morita, 1969).

Activity in the water receptor neuron (W cell of Hodgson and Roeder, type 3 of Rees) also mediates a feeding response. Moderate (0.1–0.5 M) concentrations of electrolytes and osmotic pressures of nonelectrolytes inhibit firing and feeding (Evans and Mellon, 1962).

There are probably two neurons that respond to monovalent salts, one primarily to cations and the other to anions (Steinhardt, 1965; Gillary, 1966; Dethier and Hanson, 1968). Activity in the cation receptor (L cell of Hodgson and Roeder, type 1 of Rees) is associated with behavioral rejection (i.e. no feeding response). The anion receptor, or "fifth cell" (type 4 of Rees), is not well understood and may not be present in all hairs. Dethier and Hanson (1968) tested sodium salts of fatty acids and found that C_{6+} salts depressed the cation receptor and stimulated the fifth cell. They concluded that salt is not the most effective stimulus for this cell as it induced only a low rate of firing.

This classification of sugar, water, and salt receptors is an oversimplification since the various neurons respond to several classes of compounds, including proteins (Dethier, 1952; Dethier and Chadwick, 1947, 1948; McCutchan, 1969; Steinhardt et al., 1966; Wallis, 1961). Furthermore, the fly is capable of discriminating among proteins, for the female prefers homogenized liver to brain-heart infusion and the latter to hemoglobin although all three support egg development (Dethier, 1961).

Behavioral tests with houseflies showed that amino acids in low concentrations would elicit proboscis extension and feeding (Robbins et al., 1965); however, Wolbarsht and Hanson (1967) reported that there were neither behavioral nor electrophysiological effects in *Phormia regina*.

More recent electrophysiological experiments have been confined to L-amino acids and largest labellar hairs (according to Wilczek, 1967) and have provided intriguing results. Shiraishi and Kuwabara (1970) divided 19 amino acids into four groups on the basis of the firing patterns they produce in the salt, sugar, and water receptors of 6-day old male *Phormia regina* and *Boettcherisca peregrina*. Class 1 acids (alanine, cystine, glycine, serine, threonine, tyrosine) neither stimulate nor inhibit any receptor. Class 2 acids (arginine-

HCl, aspartic acid, glutamic acid, histidine-HCl, lysine-HCl) inhibit all three receptors at high concentrations. Class 3 acids (hydroxyproline, proline) stimulate the salt receptor and inhibit the water receptor. Class 4 acids (isoleucine, leucine, methionine, phenylalanine, tryptophan, valine) stimulate the sugar receptor.

If these electrophysiological differences among amino acids are significant for the fly, as they are for other compounds, they should be reflected in differences in behavioral responses to the four classes of amino acids. This study attempted to investigate these responses.

MATERIALS AND METHODS

The flies used in this study were provided from a stock culture maintained by Dr. V. G. Dethier at Princeton University. Larvae were raised on an artificial diet (Hill, Bell, and Chadwick, 1947; Orr, 1964) and adults were kept at room temperature in constant light. All experiments were performed on 3-day old animals.

Amino acids were all L configuration (Nutritional Biochemicals, Cleveland, Ohio). Solutions were prepared in distilled water and were of pH 3–7. Within this range the chemosensory cells are not affected by pH (Gillary, 1966; Shiraishi and Morita, 1969; Shiraishi and Kuwabara, 1970). Stimulus concentrations were those that had given maximum electrophysiological responses as reported by Shiraishi and Kuwabara (1970).

Behavioral Tests

All flies were starved from emergence. Those tested thirsty received no water at all; those water-adapted were water-deprived for 24 h before testing and allowed to drink until the proboscis was retracted just before application of the stimulus. Flies were anesthetized with CO_2, fastened to wax blocks on applicator sticks, and allowed at least 90 min for recovery. Sexes were tested separately until it became apparent there were no differences in responses. With the aid of a dissecting microscope, stimulus solutions were applied on filter paper simultaneously to the tips of all the largest labellar hairs. Each stimulus was applied for up to 3 s and not more than twice in succession. A minimum of 20 min elapsed between tests of different stimuli on the same fly. The response criterion was the initiation of proboscis extension since once the proboscis protrudes labellar receptors in shorter hairs come in contact with the stimulus and affect subsequent response. Z-scores were computed to assess staistical significance of response differences.

Using the extensive literature on chemoreceptor activity and its relation to feeding responses in *Phormia* outlined above, it was possible to design stimulus situations such that when amino acid was not included in the solutions the electrophysiological and behavioral responses could be predicted accurately. Incorporating the electrophysiological data on the amino acids provided by Shiraishi and Kuwabara (1970) it was then possible to predict how the addition of a given amino acid in a particular stimulus situation should affect the receptors and the feeding response. For example, thirsty flies offered water will drink because their water receptors are activated. If

the class 1 acids are nonstimulatory and noninhibitory as reported, adding a class 1 acid to the water should not interfere with acceptance. If the class 2 acids indeed inhibit all receptors, adding one of these acids should cause the flies to reject the water. Other tests would be necessary to demonstrate inhibition of the salt and sugar receptors by class 2 acids, for instance, checking whether the presence of one of these acids causes flies to reject a normally acceptable concentration of sucrose and accept a normally rejected concentration of salt. The expected responses to all four classes of amino acids in the four stimulus situations are summarized in Table I.

Appropriate tests for each group of acids were chosen on the basis of the table, with one exception. The class 3 acids reportedly stimulate the cation receptor, their maximum effect comparable to 0.0625 M NaCl. This concentration of salt is below the rejection threshold of our 3-day old flies so tests A and C could not be used. However, since this amount of NaCl does interfere with acceptance of certain concentrations of

TABLE 1

EXPECTED BEHAVIORAL RESPONSES TO STIMULI CONTAINING AMINO ACIDS PREDICTED ON THE BASIS OF ELECTROPHYSIOLOGICAL RESPONSES (SUPRATHRESHOLD CONCENTRATIONS)

	Amino acids to be added and receptor activity they produce alone*			
Stimuli without amino acid and the responses they evoke	Class 1 acid Water cell 0 Salt cell 0 Sugar cell 0	Class 2 acid Water cell — Salt cell — Sugar cell —	Class 3 acid Water cell — Salt cell + Sugar cell 0	Class 4 acid Water cell 0 Salt cell 0 Sugar cell +
	Expected behavioral response to stimulus + acid			
A. H₂O offered to thirsty flies Water cell + Salt cell 0 Sugar cell 0	Acceptance	Acceptance	Rejection	Rejection	Acceptance
B. H₂O offered to water-adapted flies Water cell A Salt cell 0 Sugar cell 0	Rejection	Rejection	Rejection	Rejection	Acceptance
C. NaCl + H₂O offered to thirsty flies Water cell + Salt cell + Sugar cell 0	Rejection	Rejection	?-Depends on degree of inhibition of water cell relative to salt cell	Rejection	?-Depends on degree of activity of sugar cell relative to salt cell
D. Sucrose + H₂O offered to water-adapted flies Water cell A Salt cell 0 Sugar cell +	Acceptance	Acceptance	Rejection	?-Depends on degree of salt cell activity	Acceptance

Note: The table has 5 data columns (Stimuli + 4 acid classes). Row C and D have 5 cells each.

* From Shiraishi and Kuwabara (1970).
+, stimulation; —, inhibition; 0, no effect; A, adaptation.

sucrose, a direct comparison between NaCl and class 3 acids on responses to sucrose was made.

Electrophysiology

In four cases behavioral results did not fit predictions and the electrophysiology of the largest labellar hairs was reexamined. The sidewall technique of Morita (1959) as modified by Evans and Mellon (1962) was used on starved and water-adapted flies, according to the stimulus situations outlined in Table I. Carbohydrate-fed flies were tested also since the data of Shiraishi and Kuwabara (1970) were probably obtained on such animals. The indifferent electrode contained Calliphora Ringer solution and the recording electrode 0.33 M LiCl. The average preparation kept at 22–24°C. was viable for over 2 hr. Stimulus solutions were contained in glass capillaries and, to prevent concentration changes, solution was drawn out the tip before each trial. Solutions were replaced two to three times during the course of a session. Stimuli were applied to the tip of the hair for a maximum of 3 s with an interstimulus interval of 1–10 min depending on the stimulus. Concentrations were those that had previously given the maximum response (Shiraishi and Kuwabara, 1970). Reference stimuli were distilled water, 0.5 M sucrose, and 0.5 M NaCl. Response magnitude was taken to be the number of spikes generated in the first 0.5 s of stimulation, and the criterion for inhibition or enhancement was a 50 % decrease or increase as compared to controls.

RESULTS

Class 1 Amino Acids

Behavioral tests generally confirmed the finding that these acids are non-stimulatory and noninhibitory for largest labellar hairs. Thirsty flies accepted acid solutions precluding inhibition of the water fiber. Responses of water-adapted flies to saturated solutions were at chance level (38%) indicating nonstimulation of the sugar fiber, with the exception of alanine (Fig. 2). The wide range of response levels obtained was not due merely to concentration differences among the saturated solutions, since saturated glycine (1.33 M) and saturated tyrosine (0.003 M) were about equally stimulating (30% responded). Thirsty flies presented with NaCl 0.5 M in acid (0.1 and 1.0 M where possible) responded at the same level (about 70%) as controls offered NaCl in water; the salt fiber is therefore unaffected.

Responses of water-satiated flies (test B) implied that alanine might stimulate the sugar fiber. Electrophysiologically the response to aqueous solutions of alanine (0.5 M, 1.0 M, saturated) relative to response to pure water was low-level sugar and salt fiber activity. However, a mixture 0.5 M with respect to sucrose and 0.5, 1.0 M, or saturated with respect to alanine produced the same pattern of firing as 0.5 M sucrose alone. The same was true of salt cell responses to mixtures of alanine and NaCl. Fig. 3 was chosen from among 64 records from 14 flies. This is yet another indication of the complexity of the
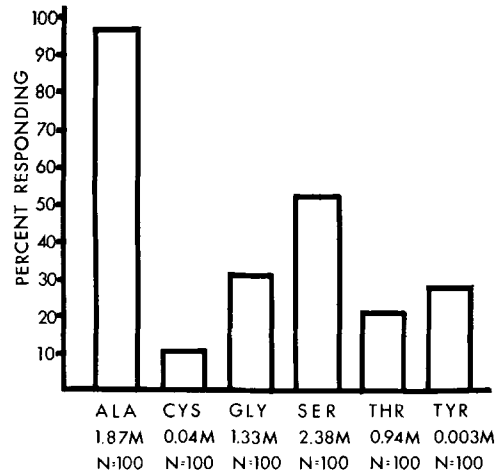
FIGURE 2. Responses of water-adapted flies to saturated solutions of class 1 amino acids (test B). Response levels below 86% are not significant ($P = 0.01$).
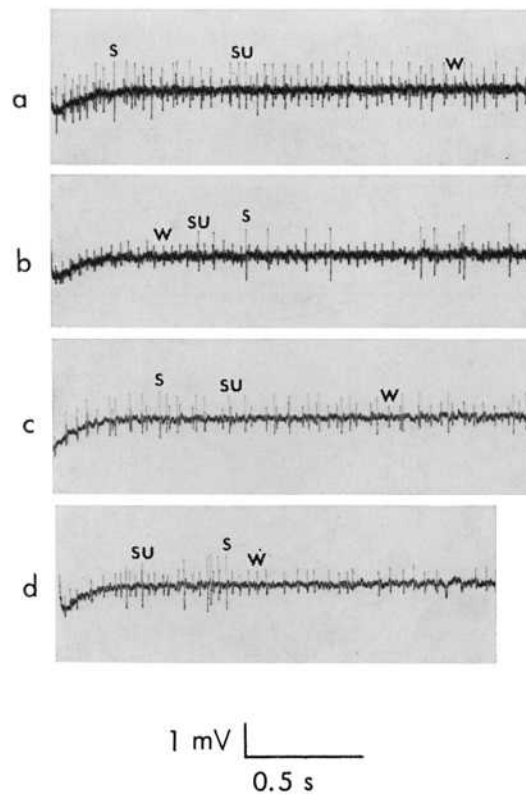

$$1 \text{ mV} \quad \rule{0pt}{0pt}$$

0.5 s

FIGURE 3. Response to L-alanine: (a), 0.5 M sucrose, largest hair no. 6, right labellum (r 6); (b), 0.9 M alanine, r 6; (c), 0.5 M sucrose, r 11; (d), 0.5 M alanine, r 11. w, water receptor; s, salt receptor; su, sugar receptor. Sucrose-fed flies.

sensitivity spectra of these receptors and the relationship between specific firing patterns and the feeding response.

Class 2 Amino Acids

Behavior suggested that inhibitory effects are not uniform within this group. Aspartic and glutamic acids did not interfere with responses to water, sucrose, or salt and their electrophysiological effects supported this observation. These acids differ from the others in the group in several properties: they are relatively insoluble in water, their isoelectric points are low (2.8 and 3.2, while the others' are above 7.0), and they are dicarboxylic. The percent response to dicarboxylic acids varies with both pH and chain length (Mc-Cutchan, 1969), and so these two acids should be expected to have complex effects. Although they did reduce the firing rate of all three cells, they produced complete inhibition in only 16 out of 101 cases (15 animals). Alone and in combination with sucrose both seemed to excite the salt cell. There were many cases of salt cell rebound ("off effect" mentioned by Shiraishi and Kuwabara), particularly when solutions less than saturated with respect to amino acid were used. Rebounds were also reported by Wolbarsht and Hanson (1967). This poststimulatory activity in the salt cell seems to signal behavioral rejection. When saturated acid solutions were tested, rebounds were less frequent and salt spikes appeared while the stimulus still touched the hair (Figs. 4 and 5). Mixtures of acid + NaCl were less stimulating to the salt receptor than NaCl but more stimulating than acid alone. In about 10% of trials using saturated solutions of these acids a fourth spike with characteristics
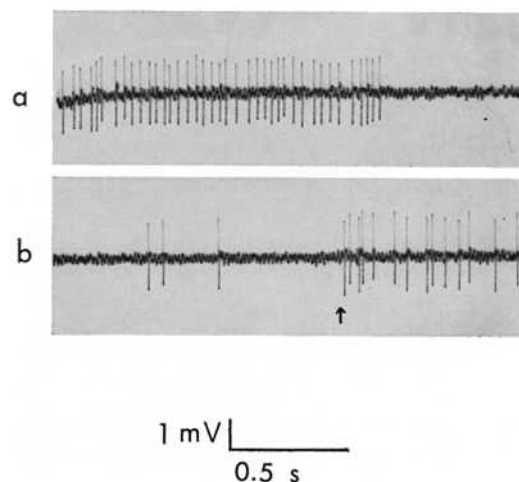

1 mV

0.5 s

FIGURE 4.   Salt fiber activity after removal of mixture less than saturated with respect to L-glutamic acid: (a), 0.5 M NaCl, r 7; (b), equal volumes 0.5 M NaCl and saturated glutamic acid, r 7 (record begins 2.5 s after stimulus onset). Starved fly.
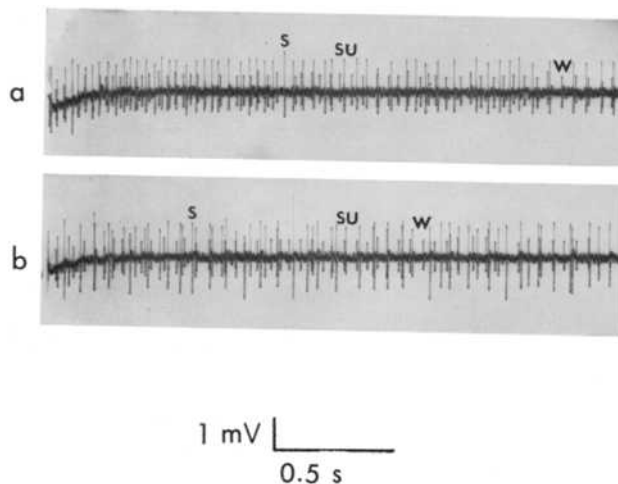
FIGURE 5.   Increased firing of salt fiber in presence of mixture saturated with respect to L-aspartic acid: (a), 0.5 M sucrose, r 1; (b), sucrose 0.5 M in saturated aspartic acid, r 1. Sucrose-fed fly.

corresponding to descriptions of the fifth cell or anion receptor (Steinhardt, 1965) could be distinguished. Sucrose mixtures seemed to increase the firing rate of this cell while NaCl mixtures decreased it.

The remaining acids in the class conformed to expectations. The percentage of water-deprived flies that accepted water was reduced from 98 to 70% or less ($P < 0.001$) by the addition of arginine-, histidine- or lysine-HCl. The rejection threshold for NaCl was dramatically raised: while no flies accepted NaCl 0.5 M in water, 60–80% drank NaCl 0.5 M in 1.0 M acid ($P < 0.001$). Responsiveness to sucrose was depressed. Only 10–30% of water-adapted flies responded to sucrose 0.015 M in 1.0 M acid; 88% accepted this concentration of sucrose in water ($P < 0.001$).

In order to determine if the chloride might contribute significantly to the effects of these three acids, tests were repeated with arginine and histidine free bases. While the hydrochlorides were effective only at 0.5 M or above, the free bases caused appreciable inhibition (water receptor, $P = 0.05$, sugar and salt receptors, $P < 0.001$) in the 0.1 M range (they are considerably less soluble). Furthermore, the hydrochlorides inhibited the water and salt cells to a greater degree than did the corresponding free bases (Figs. 6 and 7). This probably reflects the higher osmolarity of the NaCl-acid hydrochloride solutions as well as competition between Cl^- from the acid and salt for sites on the receptor membrane (Shiraishi and Kuwabara, 1970). Another factor might be the fifth cell, if it is responding to anions.

Class 3 Amino Acids

88% of a sample of water-adapted flies responded to 0.015 M sucrose, while 68% responded to a mixture 0.015 M with respect to sucrose and 0.0625 M with respect to NaCl. Likewise, fewer than 70% responded to a solution 0.015 M with respect to sucrose and 0.5 M with respect to class 3 acid. Proline was significantly more effective than hydroxyproline ($P = 0.01$), as was reported from electrophysiology (Fig. 8).

Class 4 Amino Acids

With the exception of valine, these acids elicited responses indicative of sugar fiber stimulation. More than 38% (chance level) of water-adapted flies accepted saturated or 0.056 M solutions (response levels were not significantly different in the two situations). Leucine was the most stimulating, evoking 100% acceptance. The response to valine was less than 5% (Fig. 9). Further tests implicated the salt fiber in this case, since nearly 66% of thirsty flies drank NaCl 0.5 M in water but only 37% accepted NaCl 0.5 M in 0.056 M valine ($P < 0.001$).

Within the strict limits of the electrophysiological criterion I must conclude that that valine is nonstimulatory and noninhibitory for all three cells (66 trials, 15 flies). However, aqueous solutions of valine (0.5 and 0.056 M) reliably induced a rebound from the salt cell (Fig. 10). This was not seen when
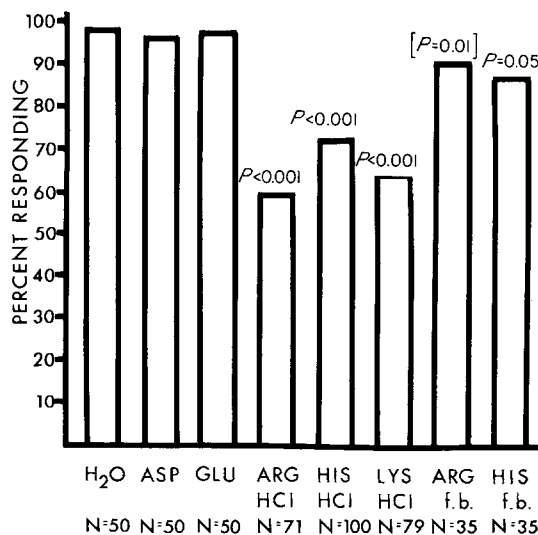

FIGURE 6. Responses of thirsty flies to saturated solutions of class 2 amino acids (test A). Probabilities in brackets refer to significance of differences between HCl and free base forms.
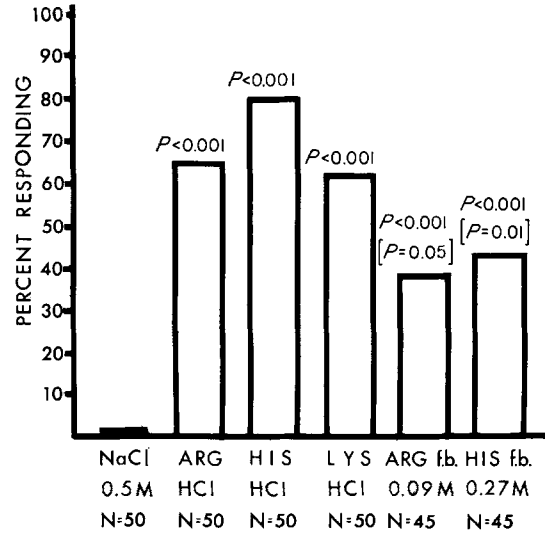
FIGURE 7.   Responses of thirsty flies to NaCl 0.5 M in 1.0 M class 2 amino acid (test C). Probabilities in brackets refer to significance of differences between HCl and free base forms.
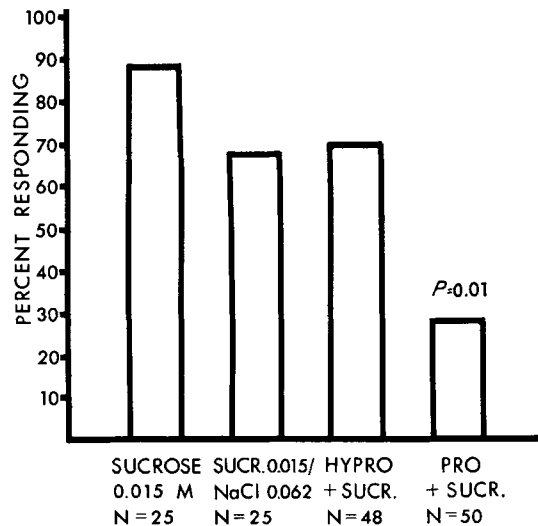

FIGURE 8.   Responses of water-adapted flies to mixtures 0.5 M with respect to class 3 amino acid and 0.015 M with respect to sucrose.

sucrose or salt mixtures were tested. The relationship between salt cell rebound activity and behavioral rejection warrants further study.

DISCUSSION

It is certain now that the blowfly can recognize and discriminate amino acids. There is no evidence for the existence of an independent amino acid receptor
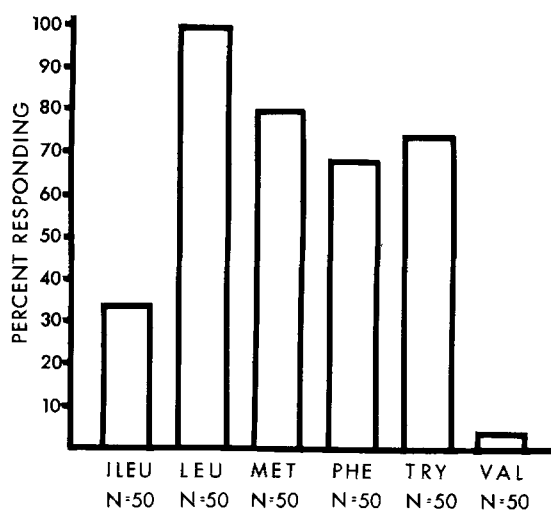
FIGURE 9. Responses of water-adapted flies to 0.056 M solutions of class 4 amino acids (test B). Levels above 38% are significant ($P = 0.05$).
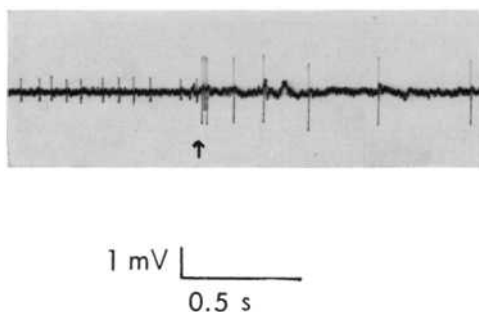

1 mV

0.5 s

FIGURE 10. Salt cell rebound after removal of 0.056 M L-valine, r 7. (Record begins 1.9 s after stimulus onset.) Watered fly.

since it has been shown that the fly's responses toward these acids are governed by the activities of the classical water, salt, and sugar receptors. Furthermore, acceptance and rejection of amino acids are related to the same electrophysiological responses as acceptance and rejection of all compounds previously tested: basically, activity in the water and/or sugar receptors mediates acceptance, activity in the salt fiber mediates rejection. Without data on actual intake and utilization one cannot determine whether the individual amono acids are what the fly actually detects in a protein source. But from the information at hand one can predict that combinations of amino acids or mixtures of amino acids + carbohydrates, salts, and/or other classes of compounds will produce complex inhibitions and synergisms at the receptor level which are probably the basis for the recognition of protein in naturally-occurring forms (e.g. decaying meat).

The grouping of amino acids according to electrophysiological effects does not coincide with any classification according to their structure (Shiraishi and Kuwabara, 1970) or physical properties. Other types of compounds have exhibited this perplexing deficiency. The stimulating effectiveness of salts for the cation receptor depends on the anion as well as the cation, particularly at submaximal stimulus concentrations (Steinhardt, 1965). The hierarchy of cation or anion effectiveness does not correlate with their properties, except that the more stimulating cations have high ionic mobilities. The stimulating ability of aliphatic compounds bears some relation to their water solubilities, although some other characteristic seems to be important as well (Dethier and Chadwick, 1950). The carbohydrates are also troublesome. Extensive work has led to the conclusion that there must be at least two sites at the molecular level of the sugar receptor (glucose and fructose subunits) (Morita and Shiraishi, 1968; Omand and Dethier, 1969). On the basis of response curves for mixtures Shiraishi and Kuwabara (1970) determined that phenylalanine, which stimulates the sugar receptor (class 4), has greater affinity for the fructose site. Similar investigation of the other class 4 acids will further elucidate the molecular nature of the receptor sites.

All five of the highly acceptable class 4 amino acids are essential for insects generally (*Phormia* does not require methionine). Of the nonstimulatory class 1 acids only two are essential (glycine, threonine); of the inhibitory class 2 acids three are essential (arginine, histidine, lysine); and of the salt-cell-stimulating class 3 acids neither is essential (*Phormia* does require proline) (Chapman, 1969). However, this information applies to larval growth. The amino acid requirements for oocyte development have not been determined for *Phormia*. It would be interesting to see whether the adult female shows amino acid preferences in correlation with cycles of yolk deposition. The fact that the chemoreceptors can discriminate among the amino acids means that the problem of protein recognition and intake regulation might possibly reduce to that of amino acid recognition and regulation.

Discrepancies between the results of this study and the previous one may be due to procedural differences. Flies used in the former investigation were 6–10 days old and must have been fed carbohydrate. Behavioral thresholds to sugars and salts are lowest and highest respectively when *Phormia* are 3–4 days old. Omand (1971) has reported that the sensitivity of the receptors in the largest labellar hairs is greatly altered by the nutritional history of the fly, those of a fully fed fly producing at least 50% fewer impulses to water, salt, and sugar. In the original study information on the sugar receptor was obtained from a flesh fly and, although the receptors of this fly appear to have pH-inhibition and threshold characteristics similar to those of *Phormia*, pooling data from the two species may not be wise in this case. Furthermore, although the present study did not provide sufficient data on each hair to justify definite

conclusions, there are indications that responses differ even among the largest hairs. For example, valine was tested on all 11 hairs (right and/or left side); salt cell effects were found in hairs 7–11 only (see Fig. 1). Alanine was tested on all hairs except number 4. Salt cell responses were evenly distributed, but sugar spikes appeared only in hairs 6–11. This phenomenon demands closer attention. Omand (1968) found that these hairs differ in sensitivity to water, salt, and sugar according to their position on the labellum. Unless responses to all compounds are investigated in this manner, the importance of spatial summation at a higher level may be underestimated and much information contained in the receptor activities will be missed.

The behavioral studies gave some insight into the effects of amino acids on the shorter hairs and interpseudotracheal papillae. It can be assumed that an acid excites or at least does not inhibit these receptor populations if a significant proportion of positive responses culminated in full extension of the proboscis or actual feeding. The class 1 acids evoked full extensions. Histidine free base, hydroxyproline, and the class 4 acids elicited feeding. However, the other amino acids whose various stimulus mixtures caused positive responses (arginine free base, class 2 hydrochlorides, proline) did not educe full extensions and apparently inhibit some or all of the smaller receptors. This may provide additional support for Dethier's (1961) finding that protein inhibits medium hairs.

The significance of the data presented here for the problem of protein recognition cannot be assessed as yet. It will require further electrophysiological analysis of responses of all receptor populations to amino acids, peptides, and mixtures and intake studies that relate the sensory information to the animal's metabolism.

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