Stereospecificity of Multiple Receptor Sites in a Labellar Sugar Receptor of the Fleshfly for Amino Acids and Small Peptides

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ABSTRACT N-Formylation and N-methylation of the α-amino group of L-phenylalanine result in extremely decreased responses of the labellar sugar receptor of the fleshfly, whereas the same structural alteration of L-valine hardly affects the response. Methyl esterification of the α-carboxyl group of phenylalanine, on the other hand, maintains the response to some extent, but similar treatment of valine completely diminishes the response. The aromatic structure in phenylalanine is not essential for stimulation. These results suggest a substantial difference in the stereospecificities and functional group specificities of the furnase (F) and aliphatic carboxylate (T) sites in the sugar receptor. The effect of small peptides on the sugar receptor was examined systematically. Their effectiveness depends mainly on the place of the constituent amino acids rather than on their composition, indicating the decisive role that certain aliphatic amino acids in the C-terminal position play in stimulation. Remarkable regularities in the stimulating effectiveness of small peptides exactly correspond to the stereospecificity of each receptor site. We propose two hypothetical models of the F and T sites, which involve three and two subsites, respectively, that are capable of hydrogen bond formation. The F and T sites also have a hydrophobic subsite that discriminates the R groups of the stimulants and a few spatial barriers.

INTRODUCTION

Ingestion of protein is indispensable to the reproductive activity of the adult fly. The fly discovers a protein source by detecting a protein itself, finding protein degradation products, or noticing some other accompanying substances. Dethier (1961) showed that the blowfly reacts to stimulation by certain proteins, especially brain-heart infusion, and that it discriminates between protein and carbohydrate by contact chemoreceptors and not by olfaction. However, he found no protein receptor. Wolbarsht and Hanson (1967) examined the stimulating effectiveness of 18 common amino acids and certain pure proteins, such as gelatin, casein, keratin, and bovine serum albumin, and concluded that they were equally ineffective. They suggested

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that the positive response to brain-heart infusion (a mixture of proteins, carbohydrates, and salts) was not from a protein degradation product, insisting on the necessity of a more rigorous series of experiments with chemically defined peptide reagents. This contradiction was partly resolved by an electrophysiological reinvestigation of stimulation by amino acids. Shiraishi and Kuwabara (1970), testing 19 L forms of amino acids instead of racemic mixtures, discovered a wide and complex series of responses to these compounds. They separated 19 amino acids into classes. Class 4 comprised six amino acids that stimulate the sugar receptor of the blowfly and fleshfly. Amino acids in class 1 stimulated no chemoreceptor cell, and those in class 2 nonspecifically inhibited the discharges from three kinds of chemosensory cells. Amino acids in class 3 stimulated the salt receptor. This conclusion was extended and generally confirmed by Goldrich (1973), who combined electrophysiological examination with behavioral studies. In the course of chemical treatment of the labellar sugar receptor of the fleshfly, Shimada (1975 *a*) found that a solution of pronase itself was highly effective as a stimulus. The adult fly, indeed, reacts with amino acids, which constitute a part of protein degradation products, but whether the fly can respond to peptides, the other part of protein degradation products, or to a protein molecule itself is an open question.

At least three different sites in the sugar receptor of the fleshfly (Evans, 1963; Shimada et al., 1974; Shimada, 1975b; Shimada, 1978; Shimada and Isono, 1978) have been documented. They are the pyranose site (P site), the furanose site (F site), and the third site (T site) for aliphatic carboxylate anion. In these studies six amino acids that stimulate the sugar receptor were classified into two groups according to the presence or absence of the inhibitory effect of pronase treatment. Valine, leucine, isoleucine, and methionine are in the first group, which reacted with the T site. Phenylalanine and tryptophan are in the second group, which reacted with the F site. That the α-amino group plays a differential role in stimulation was also suggested.

We report here the different, rigid stereospecificities of two of the sites, revealed by a study of the effectiveness of several amino acid derivatives, and the results of a systematic study of the relation of the structure of small peptides to their stimulating effectiveness. We also discuss the stereospecificity of the T site for small peptides and how the fly detects a protein source.

MATERIALS AND METHODS

Fleshflies (*Boettcherisca peregrina*), 4–5 days old, raised in our laboratory, were kept at room temperature on a 3% sucrose solution given freely just before the experiment. The chemosensory setae corresponding to the largest hair No. 10 on the left side of the labellum of *Phormia regina* Meigen (Wilczek, 1967) were fixed. Impulses were recorded from the sidewall of the setae (Morita and Yamashita, 1959). The methods have been described in detail (Shimada, 1978). The magnitude of the response was defined as the number of sugar spikes (largest impulses) recorded during a period of from 0.15 to 0.35 s beginning at the onset of the stimulus, that is, in the steady-state part of the response. Although the phasic part of the response (<0.1 s) is behaviorally significant, the steady-state part was used for a quantitative analysis of transducing mechanism of the sugar receptor because the impulse frequency in the steady state was proved to

be proportional to the amplitude of the receptor potential (Morita, 1972). The stimulating effectiveness of each chemical was always calculated relative to the control response of the same preparation to L-valine, one of the simplest amino acids able to stimulate the sugar receptor. Sugars were dissolved in redistilled water, and solutions of amino acids, their derivatives peptides, and pronase were made up in M/15 phosphate buffer (pH 7.2). We most frequently used a stimulus concentration of 0.01 M, at which the magnitude of the response to most stimulative amino acids reached near maximum but never went to excess. The ambient temperature was 22 ± 1°C, and the relative humidity was maintained at 62–80% throughout this work.

All the small peptides and most amino acid derivatives were purchased from Sigma Chemical Co., St. Louis, Mo. D-Glucose, D-fructose, and L-phenylalanine ethyl ester HCl were the products of Nakarai Chemicals Ltd., Kyoto, Japan. All the amino acids were obtained from Takara Kosan Co., Tokyo, Japan. *N*-Methyl-L-phenylalanine and *N*-methyl-L-valine were from Kokusan Chemical Works, Tokyo. β-Cyclohexylpropionic acid was the product of Tokyo Kasei, Co., Tokyo. Racemates of β-cyclohexyl-α-aminopropionic acid were a kind gift of Dr. K. Kabuto of Tohoku University.

TABLE I

RELATIVE RESPONSE TO FORMYLATED AMINO ACIDS

Formylated amino acids	Concentration	Relative response ± SD	No. tests*
	$M \times 10^3$		
Val	10	1.0	5
F-Val	10	0.91±0.17	5
Phe	10	1.41±0.36	5
F-Phe	10	0.34±0.17	5
Met	10	0.82±0.14	5
F-Met	10	0.87±0.14	5
Leu	10	1.12±0.26	5
F-Leu	10	0.96±0.25	5

* Refers to the number of multiple tests on plural flies. The number of tests never exceeds two on one fly on the same chemical. This applies to all the tables.

RESULTS

The complete ineffectiveness of 0.01 M β-phenylpropionic acid (3-phenylpropionic acid) reported by Shimada and Isono (1978) indicates that the α-amino group is essential for stimulation by phenylalanine, whereas similarity in the stimulating effectiveness between valine and isovaleric acid indicates that the α-amino group is not essential for stimulation by valine (Shimada and Isono, 1978). These differences between valine and phenylalanine in the importance of the α-amino group may constitute a significant difference between the T site and F site. To discover the intrinsically different structure-activity relationships between the two groups of amino acids, we examined the stimulating effectiveness of several amino acid derivatives. We then studied the effectiveness of 31 small peptides on the sugar receptor of the fleshfly.

Responses to Amino Acid Derivatives

Table I shows the stimulating effectiveness of N-formylated amino acids compared with the corresponding free amino acids. All the chemicals were

dissolved in M/15 phosphate buffer (pH 7.2). Relative response is the ratio of (response to each chemical):(response to 0.01 M valine). The first group of amino acids (valine, methionine, and leucine) showed no significant decrease in activity by formylation of the α-amino group, whereas N-formylated phenylalanine alone showed a remarkable decrease in activity, ~21% of that of free phenylalanine, one of the second-group amino acids. This drastic decrease in the activity of phenylalanine by formylation can be explained by the steric hindrance of the formyl group, though it is rather small, and suggests that the second-group amino acids stringently require the α-amino group. An alternative explanation is that this decrease arises from the disappearance of a positive charge from the protonated α-amino group of phenylalanine in the zwitterion form caused by formylation.

The results of our examination of the stimulating effectiveness of N-methylated amino acids are shown in Table II. At neutral pH, N-methylated amino acids are in the zwitterion form with a positive charge on the methylated amino group. Methylation of the α-amino group of phenylalanine again decreased the response remarkably. Methylation of valine, on the other hand,

TABLE II

RELATIVE RESPONSE TO N-METHYLATED AMINO ACIDS

N-Methylated amino acids	Concentration	Relative response ± SD	No. tests
	$M \times 10^3$		
Val	10	1.0	9
N-Me-Val	10	0.78±0.18	9
Phe	10	1.39±0.31	9
N-Me-Phe	10	0.16±0.14	9

induced no significant decrease. This indicates that the decrease in response by modification of the α-amino group of phenylalanine is not the result of the disappearance of a positive charge but that it originates in the steric hindrance.

Table III also shows a difference between valine and phenylalanine in the importance of the α-carboxyl group. Methyl esterification of the α-carboxyl group of valine markedly decreased the response, whereas the same structural alteration decreased the response to phenylalanine only slightly. The effectiveness of phenylalanine methyl ester clearly was maintained, which indicates that phenylalanine requires the carboxyl group somewhat less stringently. That the response remains also means that a negative charge on the ionized carboxyl group of phenylalanine is not necessary for stimulation. Ethyl esterification of phenylalanine further decreased the response, and *t*-butyl esterification resulted in the complete elimination of the response. Increase in the bulk of the alkyl group for esterification of the carboxyl group decreased the activity. This tendency indicates that there may be a steric hindrance of stimulation by esters of phenylalanine. Acid amide formation of the α-carboxyl group of both amino acids, on the other hand, resulted in complete elimination of the response.

Aromatic Structure in Phenylalanine not Essential for Stimulation

The stimulating effectiveness of racemates of β-cyclohexyl-α-aminopropionic acid (CAPA), is shown in Table IV. The structure of cyclohexane is intermediate between the structure of the R groups of the first- and second-group amino acids. We determined the classification of CAPA by testing the effectiveness of β-cyclohexylpropionic acid. The replacement of the α-amino group of CAPA with a hydrogen decreases its effectiveness drastically, which indicates that the α-amino group is essential for stimulation by CAPA. This agrees well with phenylalanine's stringent requirement for the α-amino group. The most conclusive evidence for the correspondence of CAPA to phenylala-

TABLE III
RELATIVE RESPONSE TO AMINO ACID DERIVATIVES

Chemicals	Relative response ± SD	No. tests
Val	1.0	8
Val-O Me	0.09±0.14	8
Val-NH_2	0±0	8
Phe	1.44±0.30	8
Phe-O Me	0.56±0.08	8
Phe-O Et	0.33±0.24	8
Phe-O-*t*-But	0±0	4
Phe-NH_2	0±0	8

The concentration of all chemicals was 0.01 M.

TABLE IV
RELATIVE RESPONSE TO AMINO ACID DERIVATIVES

Chemicals	Relative response ± SD	No. tests
Val	1.0	8
Phe	1.86±0.62	8
β-Cyclohexyl-α-amino-propionic acid	1.28±0.35	8
β-Cyclohexylpropionic acid	0±0	4

The concentration of all chemicals was 0.01 M.

nine was obtained from the pronase treatment experiments. Pronase, a powerful protease, selectively digests the T site protein in the sugar receptor under limited conditions (Shimada and Isono, 1978). Thus, if treatment with pronase eliminates the response to CAPA, it would indicate that CAPA reacts with the T site, whereas if treatment has no effect on the response, as was the case with valine, it would indicate that CAPA reacts with the F site. Because the pronase treatment had almost no effect on the response to CAPA, we place CAPA into the second group, which reacts with the F site. This classification provides further evidence that aromatic structure in phenylalanine is not essential for stimulation.

Stimulating Effectiveness of Small Peptides

Fig. 1 shows responses of a chemosensory seta of the fleshfly to various stimuli. At least three types of spikes can be distinguished. Spikes from the sugar receptor cell of the fleshfly are the largest among all receptor cells. The

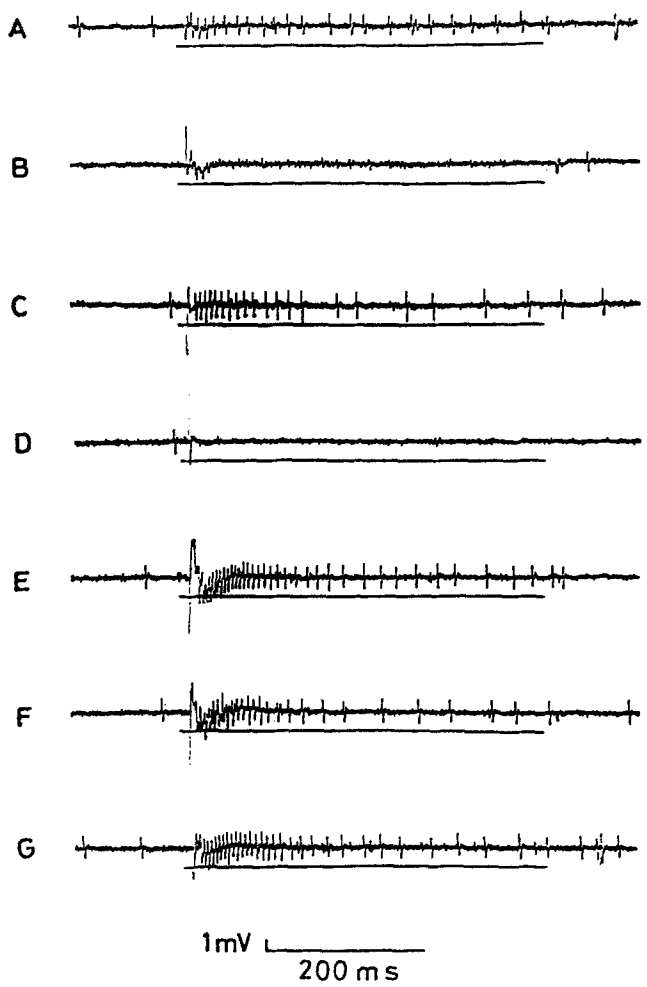


FIGURE 1. Responses to typical dipeptides. *A*, 0.5 M NaCl; *B*, water only; *C*, 0.01 M Phe-Val; *D*, 0.01 M Val-Phe; *E*, 0.01 M Glu-Val; *F*, 0.01 M Val; *G*, 0.01 M Phe. All chemicals except NaCl were dissolved in M/15 phospate buffer (pH 7.2). The line under each record indicates the length of stimulation.

smallest and medium spikes are from the water and salt receptor cells, respectively (Shiraishi and Kuwabara, 1970). That the largest spikes are from the fifth cell (Dethier and Hanson, 1968) may be ruled out here because of the regular spike patterns of the response and the clear response-concentration

relationships over the concentration range of 0.0001–0.02 M (cf. Fig. 2; this possibility is discussed below). Fig. 1 *A* shows the record of the response to 0.5 NaCl in water. Most trains of the medium spikes represent a typical response of the salt receptor. Fig. 1 *B* shows the record of the stimulation of the water receptor by redistilled water. The trains of the smallest water receptor spikes are barely observable. The record in Fig. 1 *C* shows the response to 0.01 M L-phenylalanyl-L-valine in M/15 phosphate buffer (pH 7.2). The largest sugar spikes can easily be identified by comparing their height with that of the medium spikes in Fig. 1 *A*. Under the conditions of these experiments, phosphate buffer alone is barely able to stimulate the water receptor. This may be one of the first demonstrations of the stimulative effect of a small peptide on the sugar receptor of the fleshfly. Fig. 1 *D* shows the record of the response to 0.01 M L-valyl-L-phenylalanine in the phosphate buffer. The complete ineffectiveness on the sugar receptor was recorded. The activity of the water receptor also was thoroughly depressed. The record in Fig. 1 *E* shows the response to 0.01 M α-L-glutamyl-L-valine, which is the most stimulative of the various dipeptides examined. Fig. 1 *F* shows the response to 0.01 M L-valine in the phosphate buffer, and Fig. 1 *G* shows the response to 0.01 M L-phenylalanine in the phosphate buffer. The last two records are shown as the control response of the sugar receptor.

Comparison of Stimulating Activities of Various Peptides

DIPEPTIDES WHOSE CONSTITUENT AMINO ACIDS ARE STIMULATIVE We first studied the specificity of stimulation by peptides by testing a number of dipeptides composed of five stimulative amino acids in class 4. All chemicals were dissolved in M/15 phosphate buffer and the concentrations were kept 0.01 M. The data in Table V reveal striking regularities in the relationship of structure to activity. The stimulating effectiveness of a given peptide depends on the place of its constituent amino acids rather than its composition. L-Phenylalanyl-L-valine, for example, is stimulative, whereas L-valyl-L-phenylalanine is completely ineffective. Amino acids in the C-terminal position, compared with those in the N-terminal position, must play a decisive role in stimulation by dipeptides. Once the C-terminal position was occupied by aromatic amino acids of the second group, the dipeptides showed no noticeable response, whereas they were effective to an appreciable extent when the C-terminal position was occupied by the aliphatic acids of the first group. This specificity seems to be largely independent of amino acids in the N-terminal position, as far as the data in Table V are concerned. However, compared with the response to the constituent amino acids alone, the general tendency toward a somewhat decreased response was observed.

SMALL PEPTIDES COMPOSED OF GLYCINE AND STIMULATIVE AMINO ACIDS Glycine is a representative of the amino acids in class 1 that is unable to stimulate any receptor cell of the labellar chemosensory seta (Shiraishi and Kuwabara, 1970). Table VI shows the stimulating effectiveness of small peptides with the sequence Gly-X, Gly-Gly-X, or X-Gly (X = amino acids in class 4). As was the case with the peptides shown in Table V, amino acids in

TABLE V

RELATIVE RESPONSE TO DIPEPTIDES

Chemicals	Relative response ± SD	No. tests
Val	1.0	25
Leu	1.00±0.18	5
Phe	1.09±0.44	26
Trp	0.90±0.34	6
Val-Val	0.69±0.20	8
Val-Leu	0.35±0.19	8
Val-Phe	0±0	8
Val-Trp	0±0	6
Phe-Val	0.72±0.16	8
Phe-Leu	0.35±0.15	8
Phe-Phe	0±0	8
Leu-Val	0.29±0.29	5
Leu-Leu	0.15±0.20	5
Leu-Phe	0.02±0.04	5
Leu-Trp	0±0	6
Trp-Leu	0.53±0.20	6
Met-Val	0.73±0.12	5
Met-Leu	0.57±0.14	8
Met-Phe	0±0	5

All chemicals were dissolved in M/15 phosphate buffer (pH 7.2). The concentration of all chemicals was 0.01 M.

TABLE VI

RELATIVE RESPONSE TO PEPTIDES

Chemicals	Relative response ± SD	No. tests
Val	1.0	26
Leu	1.00±0.18	5
Phe	1.09±0.44	26
Trp	0.90±0.34	6
Gly-Val	0.85±0.13	8
Gly-Leu	0.53±0.16	8
Gly-Phe	0±0	8
Gly-Trp	0±0	6
Val-Gly	0.03±0.08	5
Leu-Gly	0±0	5
Phe-Gly	0.70±0.12	5
Gly-Gly-Val	0.21±0.31	5
Gly-Gly-Leu	0.42±0.24	5
Gly-Gly-Phe	0±0	5

The concentration of all chemicals was 0.01 M.

the C-terminal position play a decisive role in stimulation. According to whether the C-terminal position is occupied by first- or second-group amino acids, they become stimulative or unstimulative, respectively. Furthermore, when unstimulative glycine is located at the C-terminal position, they are

naturally unstimulative because of the role of the C-terminal amino acids. L-Phenylalanylglycine is the only exception. It is stimulative in spite of the occupation of the C-terminal position by glycine. The apparent contradiction will be mentioned below, after the discussion of pronase treatment (Table IX). The stimulating effectiveness of tripeptides with the sequence Gly-Gly-X is lower than that of dipeptides with the sequence Gly-X, which can be explained as an extension of the general tendency of a decreased response to dipeptides as compared with the component amino acids alone.

DIPEPTIDES WITH THE SEQUENCE X-Val Experiments were performed with five typical dipeptides with the sequence X-Val (X = amino acids in class 1, 2, or 3). L-Alanine and L-tyrosine are amino acids in class 1 that are unable to stimulate any chemoreceptor cell. L-Glutamic acid and L-lysine are the amino acids in class 2 that nonspecifically inhibit the activity of three kinds of receptor cells. L-Proline in class 3 can stimulate the salt receptor (Shiraishi and Kuwabara, 1970). As shown in Table VII, most dipeptides (an exception is L-lysyl-L-valine) can stimulate the sugar receptor. This can also be explained by the role of valine in the C-terminal position mentioned in the previous section. The stimulating effectiveness of L-prolyl-L-valine also agrees well with the role of valine, since proline alone stimulates the salt receptor. L-

TABLE VII

RELATIVE RESPONSE TO DIPEPTIDES

Chemicals	Relative response ± SD	No. tests
Val	1.0	5
Ala-Val	0.55±0.10	5
Tyr-Val	0.60±0.19	5
Glu-Val	1.44±0.20*	5
Lys-Val	0.11±0.25	5
Pro-Val	0.90±0.12	5

The concentration of all chemicals was 0.01 M.

* Significantly different from Val ($P < 0.01$).

Lysyl-L-valine, which is exceptionally ineffective, has a positive charge on the R group of lysine, whereas the most stimulative amino acid, α-L-glutamyl-L-valine, has a negative charge on the R group of glutamic acid. The ineffectiveness of L-lysyl-L-valine, therefore, may result from its positive charge on the R group of lysine, since a steric factor may be neglected due to the relatively similar size of the two R groups. The high effectiveness of α-L-glutamyl-L-valine may contradict the general tendency of decreased response. Even in this case, however, the decisive role of valine in the C-terminal position can be maintained, since α-L-glutamyl-L-alanine is almost ineffective as judged from the results in Table VIII. It is ineffective because of the occupation of the C-terminal position by unstimulative alanine. Negatively charged glutamyl residue may enhance somewhat the efficacy of the stimulus. The character of the enhancement was investigated by comparing the response-concentration relationship of α-L-glutamyl-L-valine and valine.

Response-Concentration Relationship of Glu-Val and Val

Fig. 2 shows the results of a comparative study on the response-concentration relationship with α-L-glutamyl-L-valine and valine in the same preparation. Each circle indicates the mean value of the responses of six preparations at a given stimulus concentration. The concentration-response relationship for α-L-glutamyl-L-valine is largely similar to that observed with valine, which suggests that the two response functions are essentially the same. However, at higher concentrations (>0.0025M), the magnitudes of the responses to valine are always smaller than those to α-L-glutamyl-L-valine. The size of this

TABLE VIII

RELATIVE RESPONSE TO DIPEPTIDES

Chemicals	Relative response ± SD	No. tests
Val	1.0	8
Glu-Val	1.35±0.26	8
Glu-Ala	0.14±0.16	8

The concentration was 0.01 M.

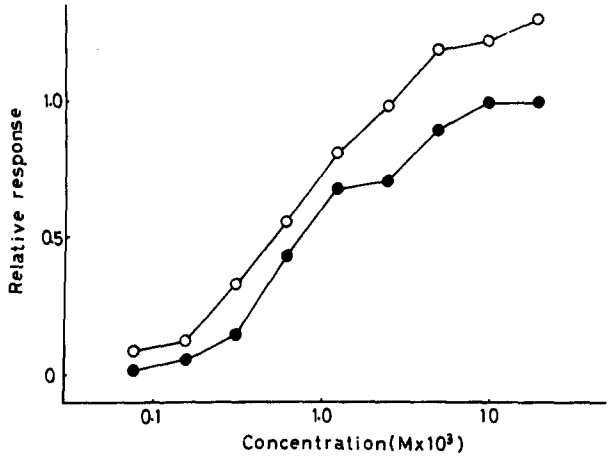


FIGURE 2. Responses to Val and Glu-Val of various concentrations obtained from the same chemosensory setae. ●, L-Valine; ○, α-L-glutamyl-L-valine. Each circle indicates the mean value of the responses of six preparations at a given stimulus concentration.

difference is not always statistically significant due to the large individual variations among preparations. The difference between the response functions lies in the magnitude of maximum responses rather than in K_b, the concentration of stimuli at which the magnitude of the response is one-half of the maximum. The maximum response to α-L-glutamyl-L-valine is ~1.3 times larger than that to valine. In these separate experiments the mean response magnitudes for 0.01M α-L-glutamyl-L-valine (Fig. 2, Tables VII and VIII) are 1.23, 1.44, and 1.35, respectively. Perhaps negatively charged glutamyl residue enhances the efficacy of stimulation rather than changing the affinity to the

receptor site, assuming that the K_b was simply related to the dissociation constant of a receptor site–stimulant complex.

Classification of Dipeptides with Respect to Pronase Treatment

We finally examined the effect of pronase treatment of the sugar receptor cell on the response to typical dipeptides to determine which site they reacted with, the F site or the T site. If the treatment decreased the response to a dipeptide, in the same way as that to valine, we concluded that it reacts with the T site. It was believed to react with the F site if the treatment had no effect on the response, as was the case with phenylalanine. The results are shown in Table IX. Pronase treatment was limited to 2.5 min. The responses were measured within 20 min after the treatment, i.e., before any recovery might be expected. Control response indicates the mean value of the magnitude of the response to each dipeptide before pronase treatment. Relative response is the mean value for the ratio of (magnitude of response to each dipeptide after pronase treatment):(magnitude before treatment). Three dipeptides, L-phenylalanyl-L-valine, α-L-glutamyl-L-valine, and L-prolyl-L-valine, behaved like valine and exhibited a complete loss of acitivity. We conclude that they react with the T site in the same way as valine. The specific reaction of L-phenylalanyl-L-valine with the T site indicates that valine in the C-terminal position plays a decisive role, since phenylalanine alone reacts with the F site, rather than the T site. In spite of a contradiction to the general tendency of decreased response to dipeptides, the reaction of highly effective α-L-glutamyl-L-valine with the T site gives additional support to the decisive role of valine in the C-terminal position. L-Prolyl-L-valine reacts with the T site regardless of the effectiveness of proline on the salt receptor. Thus, the role of valine in the reaction with the T site corresponds well to the major role of aliphatic amino acids in the C-terminal position in stimulation, as was mentioned in the previous sections. The specific reaction with the T site, therefore, is the primary process in the molecular mechanism of stimulation by small peptides. L-Phenylalanyl-glycine and phenylalanine react with the F site. In this case, phenylalanine in the N-terminal position plays a decisive role. This may explain why L-phenylalanylglycine is stimulative in spite of the occupation of the C-terminal position by the unstimulative glycine. It may

TABLE IX

RELATIVE RESPONSE AFTER PRONASE TREATMENT

Chemicals	Control response	Relative response ± SD	No. tests
Phe	10.0	0.86±0.06	5
Phe-Gly	6.4	0.91±0.16	5
Val	7.8	0.02±0.05	5
Phe-Val	6.2	0±0	5
Glu-Val	10.4	0.04±0.08	5
Pro-Val	7.4	0±0	5

The concentration of all chemicals was 0.01 M.

also be consistent with the loose specificity in stimulation of the carboxyl group of phenylalanine shown in Table III.

DISCUSSION

The study of the stimulating effectiveness of amino acid derivatives has made clear the existence of certain intrinsic differences in structural requirements for stimulation between the two groups of amino acids. The ineffectiveness of N-formylated and N-methylated phenylalanine indicates the important role of the α-amino group and of the presence of a rigid spatial barrier on the receptor site near the group, and suggests that a positive charge on the protonated α-amino group of the second-group amino acids has no relevance for stimulation. On the other hand, modification of α-amino group of the first-group amino acids did not induce any appreciable decrease in the response, which suggests that the α-amino group and the absence of a spatial barrier around it play an insignificant role. With regard to the α-carboxyl group, methyl esterification and acid amide formation of valine completely diminished the response, whereas methyl esterification of phenylalanine maintained the response to an appreciable extent, in contrast to complete elimination by its acid amide formation. The gradual decrease in the response as a result of ethyl and *t*-butyl esterification indicates the presence of a steric barrier near the carboxyl group of phenylalanine. That the response remains indicates that a negative charge on the ionized carboxyl group of phenylalanine is not essential for stimulation. According to our preliminary experiments, both DL-valine hydroxamate and DL-phenylalanine hydroxamate are almost completely ineffective at neutral pH. At neutral pH they are in the ionized form, which also suggests that a charge factor of the ionized carboxyl group, i.e., coulomb force interaction, does not contribute to stimulation by either group of amino acids. However, we cannot neglect the possibility that the coulomb interaction is a secondary factor in complex formation between stimulants and receptor sites, inasmuch as it may support their access to the site. The primary factor in complex formation, therefore, may be hydrogen bond formation, modified by steric hindrance and hydrophobic interaction.

Most dipeptides (L-phenylalanylglycine was an exception) were classified as belonging to the first group of amino acids, because of the decisive role of aliphatic amino acids in the C-terminal position in stimulation and because of the inhibitory effect of pronase treatment. The nature of the stimulating effectiveness of small peptides can easily be explained by the different steric requirements for the two groups of stimulative amino acids. L-Phenylalanyl-L-valine, for example, is stimulative because it satisfies only the steric requirement for valine, one of the first-group amino acids; valine in the C-terminal position has a free α-carboxyl group and a modified α-amino group by a peptide bond formation. The modification, corresponding to N-formylation, should not essentially inhibit the stimulating effectiveness, but the effectiveness may suggest the presence of a large space toward phenylalanine in the N-terminal position. Phenylalanine in the N-terminal position has a free α-amino group and a carboxyl group blocked by peptide bond formation. The blockade, which largely corresponds to the acid amide formation of phenylalanine,

should result in the complete elimination of response (Table III). In contrast, L-valyl-L-phenylalanine is completely unstimulative because it does not satisfy the steric requirement for either the first group or the second. Both blocking the α-carboxyl group of valine and modification of the α-amino group of phenylalanine by peptide bond formation in the dipeptide should result in the complete elimination of the response. This interpretation is valid for most of the peptides examined.

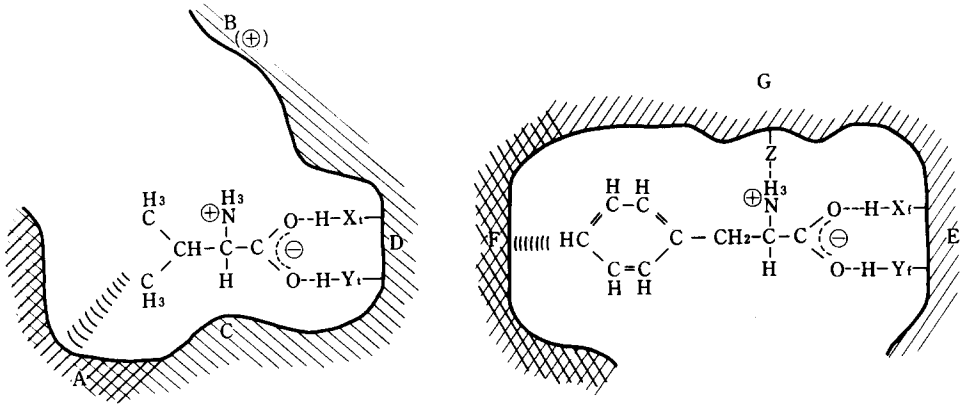
L-Phenylalanylglycine is exceptionally stimulative, in spite of a phenylalanine carboxyl group blocked by peptide bond formation. It is possible, however, for the free α-carboxyl group of unstimulative glycine in the C-terminal to make up for the modified carboxyl group of phenylalanine. According to the three-dimensional molecular model of the dipeptide, the carboxyl group of glycine can occupy almost the same position as that of phenylalanine. A less stringent requirement for the carboxyl group of phenylalanine as well as a loose structural requirement for the bulky phenyl group due to stimulative effect of CAPA allow an anomalous conformation. The carboxyl group of valine in the case of L-phenylalanine-L-valine, on the other hand, may not occupy the same position because of the steric hindrance resulting from the R group of valine.

The general tendency toward a decreased response to peptides indicates that there may be a certain limitation to the large space toward the N-terminal amino acid. Contrasting results from α-L-glutamyl-L-valine and L-lysyl-L-valine suggest further that the space may be more or less positively charged.

To find some clues to more efficient stimulants and to summarize the results obtained so far, it is useful to consider the stereospecificities of the receptor sites and the models of their substantial structure. The consideration is based primarily on the reasonable assumption of a conformational complementarity between stimulants and receptor sites according to the classic "lock and key" theory. In relation to peptide as well as amino acid stimulation, there are at least two receptor sites, the F and T sites, in the sugar receptor of the fleshfly. We conclude that most of the small peptides and the first-group amino acids react with the T site. L-Phenylalanylglycine, as well as phenylalanine, tryptophan, and CAPA, respond to the F site. On the basis of the results of the present work, we propose a model of the essential structure of the two receptor sites (Fig. 3).

The T site is a system of at least three subsites, whereas the F site is a four-subsite system with a few spatial barriers. (*a*) At either site, the carboxyl group of stimulants forms two hydrogen bonds with two subsites. A carboxyl group can easily form two hydrogen bonds, as is well known from the formation of a stable dimer of formic and acetic acids (Pauling, 1950). Both subsites here serve as a proton donor. Simultaneous formation of two hydrogen bonds may be one of the initial and essential mechanisms of the "sweet" taste sensation of the fly. Acid amide formation of the stimulants causes complete elimination of the response because, due to the predominant character of its amino group as a proton donor, it destroys one of the two essential hydrogen bonds. Methyl esterification in itself may not completely destroy the hydrogen bonds, since

it still leaves two oxygens as a proton acceptor, but it may cause some steric hindrance to stimulation. Because of the small space around the subsites (Xt, Yt) at the T site, there is complete steric hindrance, whereas at the F site it is only partial because of the quite large space around the subsite Yf. Esterification of the stimulant with a bulky group such as *t*-butyl (Table III), however, causes complete hinderance. (*b*) Subsite Z in the F site forms a hydrogen bond with the α-amino group of stimulants. Replacement of the amino group with a hydrogen atom might destroy the hydrogen bond and would result in complete elimination of the response in the case of β-phenylpropionic acid. The small space around Z does not allow even as small a modification of the amino group as N-formylation and N-methylation. On the contrary, there is no specific subsite in the T site for the α-amino group of stimulants, but there



T site F site

FIGURE 3. Proposed model of the two receptor sites. The T site and the F site involve three subsites (A, Xt, and Yt) and four subsites (F, Xf, Yf, and Z), respectively. C, D, G, and E indicate the spatial barriers in the receptor sites (see text).

is a huge space, B, toward the group. It can accomodate more than one amino acid residue of a small peptide. It may be positively charged, according to the stimulating effectiveness of α-L-glutamyl-L-valine and to the ineffectiveness L-lysyl-L-valine. The T site can discriminate D and L forms of the aliphatic amino acids by the presence of the spatial barrier C, since the α-amino group of D-amino acid is hindered by it at the corresponding position of the α-hydrogen of L-amino acids. (*c*) The A and F subsites may play an important role in the discrimination of the R groups of amino acids between the two groups. Subsite A has a certain size and hydrophobic space that give it the ability to reject R groups as small as those of glycine and alanine, as well as relatively hydrophilic serine and threonine. Subsite F may have a large space and a loose structure that allow it to accomodate such bulky and various R groups as those of phenylalanine, tryptophan, and CAPA. (*d*) The F site can

accomodate L-phenylalanylglycine, but it does not allow, for example, L-phenylalanyl-L-valine, because the R group of valine causes steric hindrance due to the spatial barrier E.

Here we must mention the relation between the F site structure and the previously proposed basic structure of monosaccharides in the furanose form that is essential for stimulation (Shimada et al., 1974; Shimada, 1975 *b*). The four essential subsites in the F site model may not easily accomodate the monosaccharide in the furanose form with its two hydroxyl groups and R_2 residue. This difficulty may require a complete revision of the previously proposed basic structure through a more rigorous series of experiments with various derivatives of furanose, which may suggest the presence of an independent site, other than the furanose site, specific for the second group of amino acids, though various chemical treatments always change the response to them in parallel with fructose. Here we document for the first time in multicellular animals multiple receptor sites with rigid stereospecificity for amino acid and peptide stimulation. Those of unicellular organism have been reported by Koshland (1977). The discovery of the two receptor sites, the T and F sites, led to the discovery of a substantial difference in structural requirements for stimulation among class-4 amino acids that are all hydrophobic and similarly stimulative to the sugar receptor. The difference corresponds exactly to that of the two receptor sites and reveals conversely, the full character of each site. This may be a typical example of a substantial difference in a group of chemicals as similar as class-4 amino acids, which were put into the same group because of their apparent similarity of physiological response.

A stereospecificity for peptide stimulation as strict as that of these two receptor sites has rarely been reported in the taste of animals. It seems remarkable that all amino acids that can stimulate the sugar receptor of the fly taste bitter to man (Shiraishi and Kuwabara, 1970). The bitterness of peptides as well as amino acids is attributable to hydrophobic amino acids, regardless of amino acid sequence (Matoba and Hata, 1972). Furthermore, Ney (1971) has proposed a quantitative method that adequately predicts whether a peptide will taste bitter or not by calculating hydrophobicity from amino acid composition. In contrast, the stimulating effectiveness of small peptides on the sugar receptor of the fly depends strictly on amino acid sequence (unstimulative L-valyl-L-phenylalanine vs. stimulative L-phenylalanyl-L-valine). Exceptionally rigid stereospecificity for L-aspartyl dipeptides has been revealed by Mazur et al. (1969), who reported the accidental discovery that L-aspartyl-L-phenylalanine methyl ester has a pronounced sweet taste. There has, however, been no report of substantial differences in the group of L-aspartyl dipeptides.

As far as the present results are concerned, the multiple sites probably do not involve responses of the fifth cell (Dethier and Hanson, 1968). This exclusion is supported by the following observations. First, the stimulative amino acids and small peptides examined so far showed clear response-concentration relationships over the concentration range of 0.0001–0.02 M (cf. Fig. 2). Dethier and Hanson (1968), on the other hand, observed that the

excitability of the fifth cell commonly appeared in the 0.1–0.2 M range and at higher concentrations. Second, most stimulants in phosphate buffer (pH 7.2) evoked regular patterns of impulse discharge (Fig. 1). If the impulses are from different cells, i.e., the sugar cell and the fifth cell, overlapping or irregular spike patterns should be observed, even if the fifth cell gives a spike with an amplitude similar to that of the sugar cell. The phosphate buffer may inhibit the supernumerary spikes from the fifth cell.

Our results may throw light on the question of flys' ingestion of protein. The fly can discover a protein source by detecting degradation products, small peptides, and certain amino acids. There may be little possibility for the fly to detect a protein molecule itself due to the rigid stereospecificity of the two receptor sites. It may recognize the terminal amino acid of the protein molecule, if any. The possibility that flys recognize an aromatic amino acid in the N-terminal position may be discarded by considering the fact that L-phenylalanyl-L-valine does not react with the F site because of the limitation of looseness in the carboxyl group of phenylalanine. On the other hand, the T site may react with the protein molecule by recognizing the aliphatic amino acid in the C-terminal position. This possibility may be fruitless because of the general tendency toward decreased response. It indicates the presence of some limitation for the large space toward the N-terminal amino acids. The space may not accomodate a molecule as large as protein. According to our preliminary experiments, the remarkable effectiveness of the pronase solution results from certain amino acids and small peptides in the degradation products. The protein molecules cannot react with both sites because of their rigid stereospecificity and the limited space of the T site. We cannot neglect the possibility of the presence of a new fourth site specific for protein molecule.

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