REJECTION THRESHOLDS OF THE BLOWFLY FOR A SERIES OF ALIPHATIC ALCOHOLS

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INTRODUCTION

The categories of compounds that are known to be taste substances with respect to insects are limited in number. Chief among them are mineral acids, acetic acid, inorganic salts, quinine hydrochloride, and some sugars. The sugars and dilute solutions of certain salts are accepted while salts at higher concentrations and acids are generally rejected (1–13). Formidable barriers to the testing of organic compounds have been their inherent toxicity, their insolubility in non-toxic solvents, or their odors. The latter tend to confuse the issue by stimulating olfactory or common chemical sensilla.

To circumvent these difficulties in order to survey the organic field and ascertain which if any of these compounds are capable of stimulating gustatory receptors advantage was taken of the proboscis response of blowflies. As Minnich (14–18) first demonstrated and others (19–23, 13) confirmed, many butterflies, muscoid flies, and honeybees bear contact chemoreceptors on the tarsi. When these receptors are stimulated with certain sugars, notably sucrose in supraliminal concentrations, the insect responds by extending its retractible mouthparts. Thus extension of the proboscis may be taken as an index of reception. By adding various amounts of test substances to sugar it becomes possible at some point to prevent a positive proboscis response. In this manner rejection thresholds may be determined for acids and salts.

Successful extension of this technique to organic compounds, many of which are adequate olfactory stimuli, was achieved by extirpation of olfactory receptors. Frings (24) had demonstrated by conditioning experiments that the antennae and labella are the sole loci of olfactory end-organs in the blowfly *Cynomyia cadaverina*. A simple check in the form of observations of the behavior of flies toward the odor of acetic acid, ethyl alcohol, amyl alcohol, amyl acetate, and *iso*-amyl salicylate before and after combined antennectomy and labellectomy indicated that in the blowfly *Phormia regina* also the sole loci of olfactory end-organs are the antennae and labella. Thus by utilizing antennectomized-labellectomized blowflies the obstacle posed by odors was removed and the way opened to ascertaining the stimulating effect of water-soluble organic compounds by mixing them with sugar solutions and determining a rejection threshold. Moreover, the use of receptors not associated

with the mouth permits the testing of many compounds which might otherwise have toxic effects.

This technique has provided opportunities for correlating chemical structure and properties with stimulative efficiency by making possible studies of the sensory responses of insects to homologous series of chemical compounds.

Materials and Methods

The experimental procedure which was followed was essentially that of Minnich (18) and Frings (24). 1 to 3 day old adult blowflies (*Phormia regina* Meigen) from a standard culture were anesthetized with carbon dioxide. They were then suspended by fastening the wings to a glass rod with paraffin. Flies so mounted, if fed daily, suffer no loss in longevity and if freed at any time are still capable of normal reproduction. After receiving 0.1 M sucrose to repletion they were reanesthetized and the antennae and labella removed. This eliminated any response to odors but did not alter the thresholds of non-odorous substances. Thus the threshold for sucrose of 181 normal flies was 0.019 M ± 0.005 (standard deviation) prior to operation, and of 122 of the same individuals after operation it was 0.017 M ± 0.013.

On the day following operation the flies were given their fill of distilled water, after which a series of concentrations of the compound to be tested was offered. All test solutions were prepared with 0.1 M sucrose as a base. Each fly was tested with a given compound twice daily and subsisted until death on a water diet only. A test consisted of offering the insect each solution of a series consecutively in ascending order of concentration. The flies responded to acceptable solutions, as to plain sugar water, by lowering the proboscis when the tarsi were brought in contact with the fluid. Failure to lower the proboscis was interpreted as a rejection. When a negative response was obtained, the next lower concentration was offered again as assurance that refusal was truly rejection and not a result of fatigue or some other factor of behavior. In no instance were the mouthparts permitted to touch the solution. Following each test the legs were washed in distilled water. A minimum interval of 15 minutes elapsed between trials. By this method rejection thresholds were determined for a series of aliphatic alcohols.

Sugar was weighed into 100 ml. volumetric flasks to which were added a measured amount of the test substance and double distilled water. From this mixture a series of concentrations was made by diluting with 0.1 M sucrose. The following grades of alcohols were employed:

Alcohol	Formula		Grade
Methyl	CH_3OH	B. P. 64.5–65°	Eastman
Ethyl	CH_3CH_2OH		Eastman
n-Propyl	$CH_3CH_2CH_2OH$	B. P. 96–98°	Eastman
iso-Propyl	$(CH_3)_2$ CHOH	98–99 per cent	Eastman
n-Butyl	$CH_3(CH_2)_2CH_2OH$	B. P. 116–118°	Merck reagent
iso-Butyl	$(CH_3)_2CHCH_2OH$	B. P. 106–108°	Eastman
sec-Butyl	$CH_3CH_2CHOHCH_3$	B. P. 99–101°	Eastman
tert-Butyl	$(CH_3)_3COH$	M. P. 23–25°	Eastman
n-Amyl	$CH_3(CH_2)_3CH_2OH$	B. P. 136–139°	Eastman
iso-Amyl	$(CH_3)_2CHCH_2CH_2OH$	Analytical reagent	Mallinckrodt
sec-act-Amyl	$CH_3(CH_2)_2CHOHCH_3$		Eastman

Alcohol	Formula		Grade
tert-Amyl.....	$CH_3CH_2C(CH_3)OHCH_3$		Eastman
n-Hexyl.....	$CH_3(CH_2)_4CH_2OH$	95 per cent B. P. 155–158°	Eastman practical
sec-n-Octyl.....	$CH_3CHOH(CH_2)_5CH_3$		Merck reagent
Allyl	$CH_2{:}CH\cdot CH_2OH$	B. P. 95.5–97°	Eastman

All experiments were run at temperatures of 25–30°C.

RESULTS

The results of the tests are summarized in Table I, together with the values of several properties of the alcohols which are of interest in the interpretation

TABLE I

Comparison of Rejection Thresholds and Various Physical Properties of the Aliphatic Alcohols

Alcohol	Mean threshold molarity $m \pm \sigma m$	No. of tests n	Boiling point	Melting point	Vapor pressure at 30°C.	Molecular surface spherical	Molecular surface cylindrical	Molecular moment	Distribution coefficient water-oil at 25°C.	Standard free energy $(F_2^0)_w - F_2$ cals.	Activity coefficient f_2^0
			°K.	°K.	mm. Hg	Å²	Å²	$\mu \times 10^{18}$			
Methyl.....	11.3 ± 0.54	76	337.7	175.3	160	79.13	125.77	1.78	103.6	240	1.51
Ethyl.....	3.2 ± 0.13	57	351.5	155.8	79	101.55	148.70	1.85	28.3	740	3.48
iso-Propyl.....	2.1 ± 0.08	66	353.3	184.1	70	121.71	—	—	—	—	—
tert-Butyl.....	1.9 ± 0.08	66	355.8	298.6	64	139.58	—	1.84	—	—	—
Allyl.....	0.92 ± 0.04	55	369.6	144.1	27	112.39	160.93	—	—	1100	—
n-Propyl.....	1.3 ± 0.02	84	370.2	146.1	27	114.39	169.49	1.98	6.28	1500	12.5
sec-Butyl.....	1.1 ± 0.02	67	372.7	184.1	24	137.36	—	1.95	—	—	—
tert-Amyl.....	0.38 ± 0.013	50	374.9	261.2	22	156.45	—	—	—	—	—
iso-Butyl.....	0.66 ± 0.014	58	381.4	165.1	15.5	133.16	—	2.07	1.70	—	—
n-Butyl.....	0.64 ± 0.022	131	390.7	183.6	9.0	137.16	190.86	2.06	—	2280	46.5
sec-act-Amyl.....	0.22 ± 0.016	50	392.4	—	8.2	156.45	—	—	—	—	—
iso-Amyl.....	0.10 ± 0.007	50	403.5	155.9	5.5	153.68	—	2.10	0.471	—	—
n-Amyl.....	0.10 ± 0.007	56	411.0	194.6	5.5	153.38	212.06	2.15	—	3190	219.0
n-Hexyl.....	0.012 ± 0.0008	67	430.3	221.5	3.3	168.66	233.10	—	—	4030	903.0
sec-n-Octyl.....	0.0021 ± 0.0002	50	451.6	234.5	1.2	193.35	—	—	—	—	—
	r	=	−0.98	−0.34	+0.95	−0.86	−0.96	−0.90	+0.98	−0.91	−0.99

of the data. Boiling and melting points on the Kelvin scale were obtained by adding 273.1° to the Centigrade values given in the Handbook of Chemistry and Physics (25). Vapor pressures at 30°C. were determined from the nomogram constructed by Thomson (26). Molecular areas for spherical and cylindrical surfaces were computed from the molecular volumes, the latter having been derived from the formula

$$V = \text{molecular weight/density} \times 6.06 \times 10^{23}.$$

Molecular weights and densities were taken from the Handbook (25). In calculating the cylindrical surfaces for the *pri-n*-alcohols, the dimensions and procedure given by Langmuir (27) were followed. Molecular moments are

from the paper by Smyth (28), water-cottonseed oil distribution coefficients from Wroth and Reed (29), standard free energies and activity coefficients from Butler *et al.* (30). The standard free energy of allyl alcohol was determined by subtracting 400 calories, the approximate decrease due to the presence of the double bond (31), from the value given for *n*-propyl alcohol by Butler *et al.*

The product-moment coefficients of correlation, r, were calculated from the formula $r = \Sigma xy/\sqrt{(\Sigma x^2)(\Sigma y^2)}$, where x and y are the differences between each pair of statistics and the respective means. For these calculations the logarithms of the mean threshold concentrations were paired with the logarithms of the several sets of physical data. The r values are thus measures of the validity of the hypotheses

$$\log \text{threshold} = k_1 \log X_1 + a,$$
$$\log \text{threshold} = k_2 \log X_2 + b, \text{ etc.},$$

where X_1, X_2, etc., represent the several series of values for the physical properties.

DISCUSSION

The high degree of correlation (see Table I) found between the mean concentrations of the alcohols at rejection threshold and such properties as boiling point, vapor pressure, molecular surface, molecular moment, water-oil distribution coefficients, standard free energies, and activity coefficients leaves little doubt that the experiments dealt primarily with the receptor cells rather than with some other link in the complex which intervened between presentation of the stimulus and the response on which the measurements depended.

If the *pri-n*-alcohols alone are considered, the results approximate to a Traube series (32), in which the effective concentrations are related as $1:3^{-1}:3^{-2}\ldots 3^{1-n}$, where n is the number of carbon atoms in the chain. Actual values from our data are $1:3.49^{-1}:2.98^{-2}:2.60^{-3}:3.26^{-4}:3.92^{-5}.\,.:3.51^{-7}$ (the last figure is for *sec-n*-octyl alcohol). The discrepancies from a constant ratio are significant, but of about the same order of magnitude as those noted with a variety of other material (33).

Conformity to this type of pattern indicates that stimulation of the tarsal receptors was not dependent on osmotic pressure nor on rate of molecular diffusion in solution, which decrease as the series is ascended. The relationship between stimulatory efficiency (1/threshold) and vapor pressure is also inverse. However, simple correspondence between stimulating power and molecular weight or number of CH_2 groups is refuted by the results with the several isomeric alcohols tested, since the mean concentrations at rejection threshold were significantly different for all pairs with the same number of carbon atoms except for two (*iso*-butyl and *n*-butyl, *iso*-amyl and *n*-amyl). The possible criticism that the various isomers might not constitute a homo-

geneous system from the point of view of the receptors is answered, we believe, by the correlations shown in Table I, even though it be granted that these are subject to correction because the physical data are incomplete in a number of instances and on account of the presence of 0.1 M sucrose in the test solutions.

From the facts determined the logical inference is that some property shared by the entire series and varying with molecular structure in an orderly manner is concerned intimately with the stimulatory process. The nature of the correlations shown in Table I makes it obvious that this property is dependent upon surface energy relationships, but it would be fruitless at this time to attempt to define the exact mechanism of its operation. We know nothing of the molecular structure of the receptor surface, and while the results reported here indicate that its properties are very similar to those of other cell membranes throughout the animal and plant kingdoms, we are not even certain that penetration of the surface is essential to stimulation. Theoretically at least, simple accumulation of lipoid-soluble substances in the cell membrane might so alter the properties of the latter as to cause excitation, the result possibly of a change in permeability to water or to ions. In fact, Wigglesworth observed the formation of water droplets on the cuticle of insects immersed in oil (34), and Hurst states that immersion of blowfly larvae in mixtures of kerosene and ethyl alcohol results in a visible outrush of water (35). Even finely ground, chemically inert dusts have been shown to increase the permeability of insect cuticle (36) and of some model membranes to water, in some cases, it is believed, through adsorption of lipid molecules and consequent disruption of the surface structure (37).

But the alcohols have been found actually to enter other types of cells with much the same order of effectiveness as observed here for sensory stimulation (33). Also, the order of stimulative efficiency of a number of fatty acids for the tarsal receptors of *Phormia*, the data for which will be reported elsewhere, is the same as that of their penetration into other types of cells, for example into the mantle cells of *Chromodoris* (38). A definite correlation may thus be argued between rate of penetration and stimulative efficiency, but this does not prove the one to be the cause of the other, since the forces which favor penetration of these compounds into cells are in general the same as those which would facilitate their accumulation at an oil-water or lipoprotein interface. More direct evidence for or against penetration, as well as additional knowledge of the nature of the receptive surface and of what constitutes excitation will be required to settle this question.

Whatever the exact mechanism of their action, the majority of the data in the now rather extensive literature show that the physiological activity of the alcohols (and of many other compounds) runs parallel with the rate of their adsorption at a lipid-water interface or with their distribution between lipid and water. The diversity of the phenomena investigated, which, in addition

to sensory responses of several types, include narcosis, lysis of red cells, toxicity to various kinds of organisms, streaming of plant protoplasm, etc., suggests that what has been measured in most cases is the rate of access of the compounds to the system, rather than their final interaction with the process under observation. The demonstration that the contact chemoreceptors of insects respond in a similar manner to the alcohol series again emphasizes the fact that ready access to the cell is of primary importance in determining what substances will be effective in stimulation. This finding has an obvious application in the development of improved insect repellents, and it is highly probable that similar considerations apply in regard to the activity of insecticides (39–41).

With modifications of the technique used in these studies it will now be possible to survey other series of homologous compounds, so as to develop additional correlations between chemical structure and stimulatory effect. The knowledge accumulated in this way should not only be of immediate service in dealing with problems of insect control, but should contribute also to the solution of the broader problems of chemoreception in general.

SUMMARY

Series of concentrations of 15 aliphatic alcohols were presented in 0.1 M sucrose to the tarsi of antennectomized-labellectomized blowflies (*Phormia regina* Meigen). With the *pri-n*-alcohols the mean concentrations at rejection formed a Traube series. When the rejection thresholds for all the alcohols tested were compared with their boiling points, vapor pressures, molecular surface areas, molecular moments, water-cottonseed oil distribution coefficients, standard free energies, and activity coefficients, a very high degree of correlation was found in each case. It is concluded that the limiting process which was measured is concerned with the receptor cells rather than with some other element in the complex response. Stimulative power was evidently not dependent on osmotic pressure nor on rate of molecular diffusion in solution, and the correlation with vapor pressure was inverse. It is judged that surface energy relationships are concerned in stimulation, but the exact mechanism cannot be defined until more is known about the structure of the sensory surface and about the process of excitation. The physiological activity of the alcohols is related more closely to the ease with which they gain access to the cell than to their chemical interaction with cellular constituents.

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