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BINDING OF ISOVALERALDEHYDE, AN ATTRACTANT, TO ZOOSPORES OF THE FUNGUS *PHYTOPHTHORA PALMIVORA* IN RELATION TO ZOOSPORE CHEMOTAXIS

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SUMMARY

The interaction of isovaleraldehyde, an attractant, with zoospores of the fungus *Phytophthora palmivora* was investigated by using binding techniques. The amount of isovaleraldehyde bound diminished with time, an effect that may be related to sensory adaptation. In addition to non-specific binding, specific and saturable isovaleraldehyde binding was demonstrated. Cooperativity occurred at intermediate but not at low and high ligand concentrations. [^{3}H]isovaleraldehyde was displaced by unlabelled isovaleraldehyde and by a number of chemically related ligands known to be chemotactic agents. Their dissociation constants as calculated from the displacement experiments paralleled their potency as attractants and it is suggested that these attractants act through the same receptor. Some other chemotactic agents did not displace [^{3}H]isovaleraldehyde, and these presumably act through one or more other receptors.

INTRODUCTION

Swimming of zoospores of *Phytophthora palmivora* (Butler) Butler is influenced by environmental factors. Zoospores tend to swim upwards, i.e. to show negative geotaxis (Cameron & Carlile, 1977). This response, however, is unlikely to be a true sensory response; it is widespread in unicellular eukaryotic micro-organisms and is explicable in terms of purely mechanical interaction between the cells and the suspending medium (Carlile, 1980). Zoospores are repelled by various cations (negative chemotaxis). This appears to involve sensory transduction but not specific receptors; we believe that cations produce their effect on the cell by reducing the negative charge at the cell surface (Cameron & Carlile, 1980). Zoospores are attracted (i.e. show positive chemotaxis) to various organic compounds (Cameron & Carlile, 1978). In bacteria it is firmly established that chemoreceptors of high specificity are involved in positive chemotaxis (Carlile, 1980). In this paper we report the use of ligand-binding techniques to determine whether positive chemotaxis in a flagellate eukaryote is also mediated by specific receptors. Ligand-binding techniques are most efficient when

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the expected dissociation constant (K_D) of a ligand is in the range 10^{-10} to 10^{-6} M (Cuatrecasas & Hollenberg, 1976). In this study we therefore employed isovaleraldehyde, the most powerful attractant for *P. palmivora* so far found, which is able to attract zoospores at 10^{-6} M (Cameron & Carlile, 1978).

MATERIALS AND METHODS

Materials

All chemicals were of the highest purity commercially available and unless otherwise stated were used without further purification. Aldehydes, being of low stability, were purchased shortly before use and kept at 4 °C. Isobutyraldehyde on examination by nuclear magnetic resonance (n.m.r.) with a Varian T60 spectrometer was found to contain isobutyric acid. The aldehyde was distilled twice from the parent mixture under partial vacuum and condensed by means of an acetone/solid carbon dioxide mixture. The condensate was further purified by repeating the procedure. Subsequent n.m.r. analysis showed no peaks other than those of isobutyraldehyde.

Isoamyl isovalerate was prepared by reacting isovaleryl chloride with dry isoamyl alcohol, followed by redistillation. Ante-isovaleraldehyde (2-methylbutanal) was prepared from isoleucine by the method of Virtanen & Rautenan (1947). Chemicals were dissolved in the zoospore-suspending medium (DS, see below) before being applied to zoospores.

Production of zoospores

P. palmivora was used under licence from the Ministry of Agriculture, Fisheries and Food and the strain was the one previously employed (Cameron & Carlile, 1978, 1980). Green bean agar was prepared by homogenizing 227 g Birds Eye frozen sliced green beans in a Sorvall Omnimixer. Glucose and agar (Difco Bacto) were added to give final concentrations of 0·2 % and 1·5 %, respectively, and the homogenate was made up to 1 litre with distilled water. The medium (pH *ca.* 6·5) was autoclaved at 120 °C for 20 min and poured into Petri dishes. After inoculation, cultures were maintained in the dark until, at *ca.* 6 days, mycelium covered the dish; they were then transferred to daylight (20–25 °C) in the laboratory. Zoosporangia were produced within 24 h and zoospores liberated by flooding the plates with 10 ml dilute salt (DS) buffer (K_2HPO_4, 13 mg; KH_2PO_4, 10 mg; $MgCl_2 \cdot 6H_2O$, 10 mg; $CaCl_2 \cdot 2H_2O$, 7 mg; distilled water to 1 litre; pH 5·8), incubating at 10 °C for 30 min and returning to room temperature. Abundant zoospores (*ca.* 10^7 ml^{-1}) were produced within 30 min, and were active for several hours.

Synthesis of [^3H]isovaleraldehyde

[^{3}H]isovaleraldehyde was prepared by a 1-step reductive deamination of DL-[4,5-^{3}H]leucine (30 Ci $mmol^{-1}$; Radiochemical Centre, Amersham) in the presence of excess ninhydrin (Virtanan & Rautenan, 1947). This reaction, the basis of the familiar ninhydrin method for amino acid determination, released the corresponding aldehyde at high yields (99 %). In the case of leucine this is isovaleraldehyde. The isovaleraldehyde formed was distilled over with the solvent water and collected in an acetone/solid carbon dioxide trap. The radiochemical purity of the isovaleraldehyde was checked by adding a sample of about 1 μl of the distillate to 200 μl of a 2 mg ml^{-1} solution of 2,4-dinitrophenyl-hydrazine (2,4-DNP) in ethanol and concentrated sulphuric acid in a ratio of 20 : 1, followed by *ca.* 0·5 μl of pure unlabelled isovaleraldehyde. An aliquot of this resultant solution was spotted onto a silica TLC plate (Polygram SilG, Camlab, Cambridge) and developed in ethyl acetate:petroleum ether 60–80 (2 : 1). Two control spots, one containing 2,4-DNP alone and the other 1 μl of 2,4-DNP-treated [^{3}H]leucine, were developed concurrently. The plate was cut up parallel to the solvent front into sections each representing 0·1 R_F unit and the pieces placed in scintillation vials, which were then filled with scintillant. Over 95 % of the counts were in the band corresponding to isovaleraldehyde 2,4-DNP adduct (R_F 0·8–0·9). No counts from the leucine control occurred in this region; leucine itself stayed at the origin.

Binding assays

Zoospore suspensions were filtered through Whatman no. 541 paper to remove sporangia and mycelial fragments, and stored briefly (5 min) on ice. An aliquot of the suspension was removed for estimation of spore density with a haemocytometer. The cell concentration was in the region $0{\cdot}5$–$1{\cdot}0 \times 10^7$ ml^{-1}. Total binding was measured by transferring 1 ml of cell suspension to 1·5 ml capped microcentrifuge tubes containing solutions of [^{3}H]isovaleraldehyde to give final concentrations of 1–1000 nM. Non-specific binding was measured by including 100 μM unlabelled isovaleraldehyde in the incubation mixture. After incubation at 0° C for 2 min, the cell suspensions were centrifuged for 30 s in a Beckman microfuge. The remaining solution was removed from the centrifuge tubes using a Pasteur pipette connected to a vacuum line. The pellets were rinsed twice with 1 ml ice-cold DS buffer. Further rinsing did not remove any more radioactivity. Three replicate incubations at each ligand concentration were carried out. The cell pellets were each dissolved in 0·3 ml of Packard Soluene 350:water (7:1) and the solution transferred to a scintillation vial containing 10 ml toluene plus scintillant and 5 ml 2-methoxyethanol. Specific binding was calculated by subtracting non-specific binding from total binding.

RESULTS AND DISCUSSION

Time course of isovaleraldehyde binding

Binding rates for ligands are usually very high and may approach the diffusion-controlled limit (Cuatrecasas & Hollenberg, 1976), with an association rate constant of *ca.* 10^8 mol s^{-1} for small molecules colliding with a large surface such as a cell membrane. Ligands with dissociation constants (K_D) of *ca.* 10^{-8} M will therefore have a dissociation rate constant of *ca.* 1 mol s^{-1} and less tightly bound ligands will have a faster off-rate. Equilibration is thus faster for less tightly bound ligands. Specific binding of [^{3}H]isovaleraldehyde was not observed, even at 0 °C, in experiments in which unbound ligand was removed by filtration, indicating that the volatile ligand had been released into the atmosphere prior to dissolving the filter in scintillant and suggesting a rapid off-rate for isovaleraldehyde. Furthermore, spores respond to isovaleraldehyde in chemotaxis assays at concentrations down to 10^{-6} M at source (Cameron & Carlile, 1978), which reduces to around 10^{-8} to 10^{-7} M at the cell surface (Cameron, 1979). For these reasons we do not expect a K_D of less than 10^{-8} M. Hence the time taken to reach equilibrium should be of the order of seconds and the amount of isovaleraldehyde bound to cells should have reached a maximum value well before 2 min (the minimum feasible contact time between free ligand and cells in our experiments), and longer periods of contact should give similar values.

Experiments carried out at 0 °C with 10 nM (Fig. 1A) and 230 nM (Fig. 1B) [^{3}H]isovaleraldehyde showed, however, that exposures to the ligand for longer than 2 min resulted in less isovaleraldehyde being bound. A trivial explanation for this result would be the release of isovaleraldehyde due to anoxia and resulting deterioration of the very dense zoospore suspensions employed. This can be excluded since the addition of ligand 5 min after preparing zoospore suspensions, instead of immediately, gave a similar time course for binding (Fig. 1B). The possibility that the isovaleraldehyde is rapidly metabolized can also be excluded; the zoospore phase lasts for a few hours only and physiological, ultrastructural and histochemical data (Bimpong,

1975) indicate that endogenous metabolism alone occurs. It would seem therefore that the decrease of isovaleraldehyde binding with time is due to changes in the properties of the cell brought about by isovaleraldehyde.

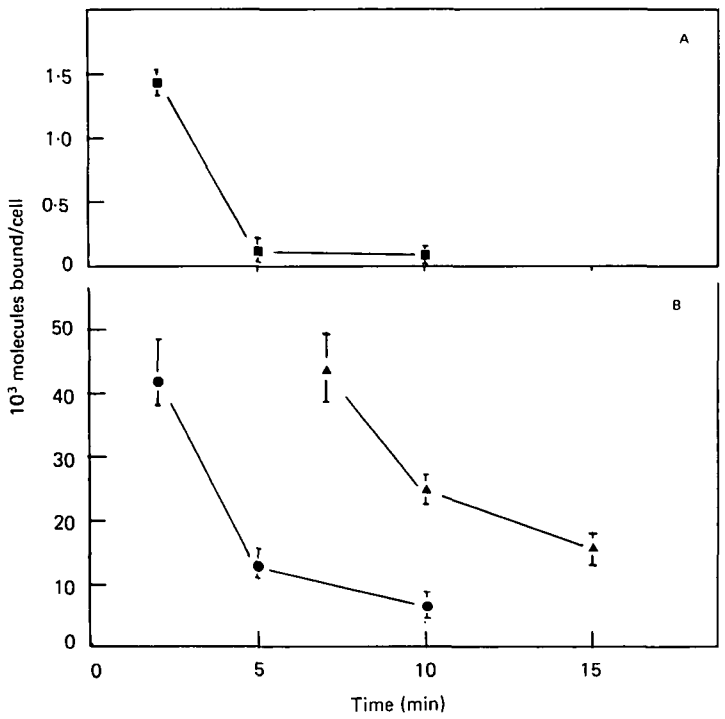

Fig. 1. Typical time course for binding of [^{3}H]isovaleraldehyde to zoospores. The specifically bound isovaleraldehyde was measured after incubation at 0 °C with zoospores for 2, 5 and 10 min. Each point is the mean of 3 replicate determinations with the bars indicating the range of the replicates. In A, 10 nM labelled isovaleraldehyde was added at zero time (■); in B, 230 nM at zero time (●), or at 5 min after harvesting (▲).

Experiments at room temperature failed to show isovaleraldehyde binding; presumably at this temperature changes in cell properties took place more rapidly so that the ability to bind ligand had been lost within 2 min. All subsequent experiments involved 2 min exposure to ligand at 0 °C; hence they reflect cell and receptor behaviour following 2 min exposure to the relevant ligand concentration, and not equilibrium values. This renders estimates for numbers of molecules bound per cell as deduced from Scatchard plots (Scatchard, 1949) tentative and necessitates a different approach, as used below, for the calculation of dissociation constants.

The response to a continuing stimulus of constant intensity diminishes with time in many sensory systems. Sensory adaptation permits sensitivity over a very wide range of stimulus intensities, and enables an organism to respond to a change in environment rather than to constant conditions (Carlile, 1980). The decrease in the ability of zoospores to bind isovaleraldehyde with continuing exposure to isovaleraldehyde could constitute such a mechanism of sensory adaptation. However, in the particular

case of *P. palmivora* zoospores, the dimunition of the response may be due instead to morphological changes elicited by the attractant. The attraction response is followed within 10 min by spore encystment, which involves loss of motility and the synthesis of a cell wall, and later by hyphal germination if conditions are favourable (Bartnicki-Garcia, 1973).

The concentration dependence of isovaleraldehyde binding

The specific binding curve obtained when [^{3}H]isovaleraldehyde is incubated with zoospores is approximately sigmoidal, whereas non-specific binding of isovaleraldehyde is linear (Fig. 2). A linear relationship was obtained when [^{3}H]leucine

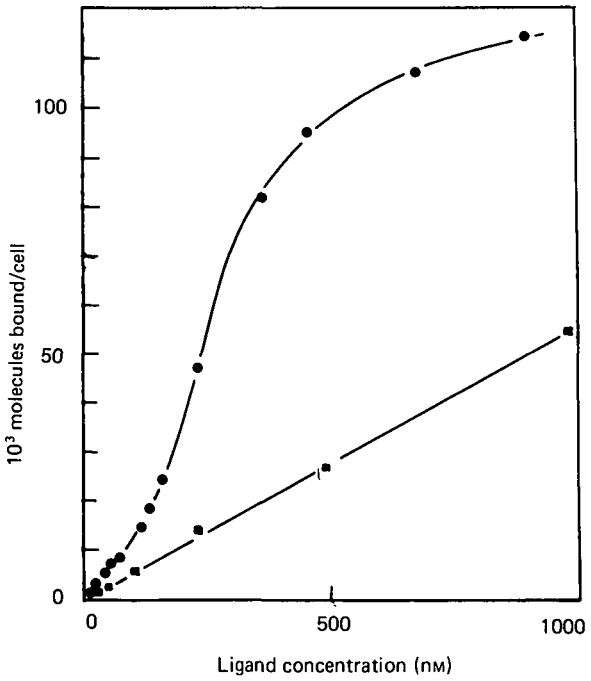

Fig. 2. Ligand concentration dependence of binding of [^{3}H]isovaleraldehyde to zoospores. Specific binding (●) of labelled isovaleraldehyde was measured after 2 min incubation with zoospores at 0 °C. Non-specific binding under the same conditions is also shown (■). Each point is a mean of 3 replicate determinations.

(30 Ci mmol^{-1}) was incubated with zoospores, implying that this non-attractant shows only non-specific binding. A Scatchard plot for specific isovaleraldehyde binding yields a 3-phase curve (Fig. 3): a steep negative slope at low ligand concentrations, a positive slope at moderate concentrations, and a less steep negative slope at high concentrations. Such a 3-phase curve is unusual, but has been reported by Thom, Cox, Safford & Rees (1979) for the binding of concanavalin A to fibroblasts. A Hill plot of the data shows a Hill constant of 1 for low and high concentrations, indicating that at these concentrations binding sites do not interact. At moderate

concentrations, however, a Hill constant of 2 is obtained. This implies cooperativity between sites, the binding of ligand molecules enhancing the ability of other sites to bind further ligand molecules (Gutfreund, 1972). This effect has been reported often in protein–ligand binding studies (e.g. see Frieden & Colman, 1967). The negative slope yielded in a Scatchard plot at high ligand concentrations can be extrapolated to the ordinate to give a tentative value of $1{\cdot}4 \times 10^5$ ligand molecules bound per cell. This first phase of binding, a precooperative phase, is unusual. A similar phase was

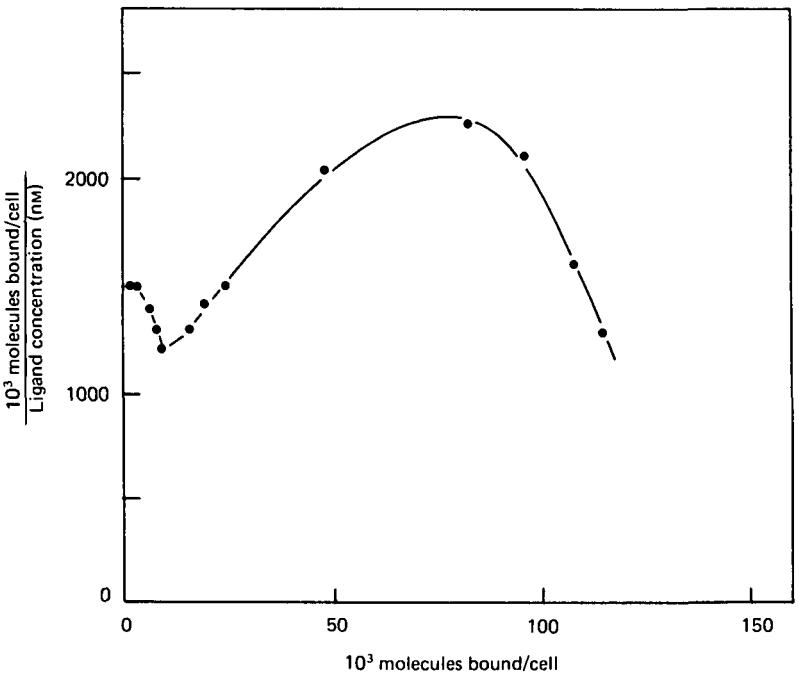

Fig. 3. A Scatchard plot of the specific binding data from Fig. 2. Extrapolation to the ordinate indicates the number of binding sites for isovaleraldehyde per cell.

reported by Thom *et al.* (1979), investigating the interaction of concanavalin A with fibroblasts. These workers suggested that the initial phase of binding caused a physical rearrangement of the membrane receptors so as to allow cooperative binding of the polyvalent concanavalin A. An alternative explanation for our results is that the first phase represents a separate set of high affinity receptors.

The specificity of isovaleraldehyde binding in relation to zoospore chemotaxis

The specificity of isovaleraldehyde binding was investigated by measuring the inhibition of binding of a low concentration (10 nM) of [^{3}H]isovaleraldehyde by various ligands compared to the binding of unlabelled isovaleraldehyde. The displacement of [^{3}H]isovaleraldehyde by various concentrations of isovaleric acid, valeraldehyde and unlabelled isovaleraldehyde is shown in Fig. 4. The dissociation constant of these ligands has been estimated (Table 1) from the concentration of unlabelled ligand

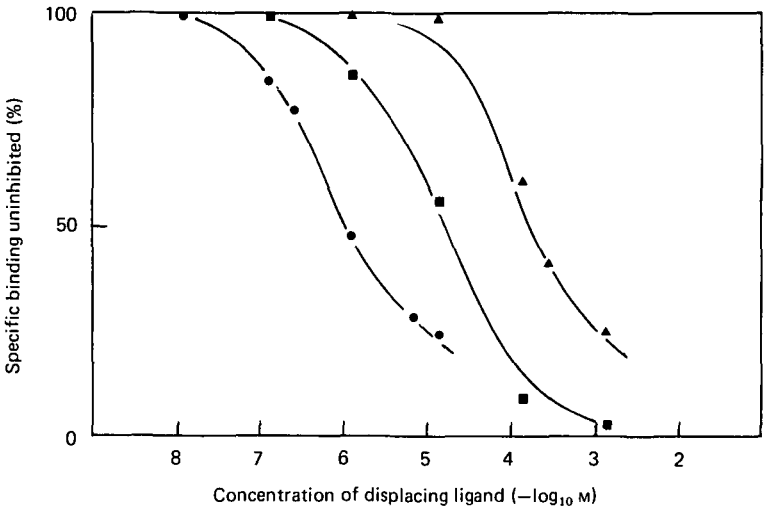

Fig. 4. Displacement of [^{3}H]isovaleraldehyde by unlabelled isovaleraldehyde (●), valeraldehyde (■) and isovaleric acid (▲). Zoospores were incubated in the presence of 10 nM-[^{3}H]isovaleraldehyde and the indicated concentration of a second ligand.

Table 1. *Binding of ligands to the isovaleraldehyde receptor*

Ligand	Dissociation constant (μM)	Chemotactic threshold* (μM)
Isovaleraldehyde	1	1
Ante-isovalderaldehyde	3	10
Valeraldehyde	20	100
Isobutyraldehyde	50	100
Isocaproic acid	100	100
Isovaleric acid	200	500
Hexanal	> 1000†	—‡
Heptanal	> 1000†	—‡
Octanal	> 1000†	—‡
Isoamyl alcohol	No binding§	100
Isoamyl amine	No binding§	100
Asparagine	No binding§	250
Isoamyl isovalerate	No binding§	1000
Leucine	No binding§	—‡
β-ionone	No binding§	—‡

* Determined as described by Cameron & Carlile (1978).

† Partial displacement of [^{3}H]isovaleraldehyde by 1000 μM ligand; higher concentrations not tested.

‡ Chemotaxis not observed.

§ No displacement of [^{3}H]isovaleraldehyde by 10000 μM ligand.

(IC_{50}) required to displace 50% of the specifically bound [^{3}H]isovaleraldehyde using the equation:

$$K_D \text{ inhibitor} = IC_{50}\left(1 + \frac{\text{concentration of ligand}}{K_D \text{ ligand}}\right).$$

For displacement of [^{3}H]isovaleraldehyde by unlabelled isovaleraldehyde this becomes $K_D = IC_{50}/(1+(10\text{ nM}/K_D))$, which simplifies to $K_D = IC_{50} - 10$ nM, which from Fig. 4 gives the K_D for isovaleraldehyde as *ca.* 1 μM. From this value the dissociation constants of other displacing ligands can be calculated. Apart from unlabelled isovaleraldehyde, 5 compounds: ante-isovaleraldehyde, valeraldehyde, isobutyraldehyde, isocaproic acid and isovaleric acid, were able to displace most or all of the [^{3}H]isovaleraldehyde. All these were chemotactic, with their chemotactic thresholds approximately paralleling their effectiveness in replacing isovaleraldehyde. It is suggested, therefore, that they act through the same receptor. Hexanal, heptanal and octanal at high concentrations are able partially to displace [^{3}H]isovaleraldehyde, but chemotaxis to these compounds has not been demonstrated; any chemotactic threshold would probably be at toxic levels. Isoamyl alcohol, isoamylamine, asparagine and isoamyl isovalerate are chemotactic but do not displace [^{3}H]isovaleraldehyde, and hence probably act through a different receptor or receptors.

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