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Quantitative Analysis of the Behavior of *Dictyostelium discoideum* Amoebae: Stringency of Pteridine Reception

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A convenient, sensitive, quantitative assay for the measurement of chemotaxis of populations of *D. discoideum* vegetative amoebae is presented. A strategy for determining the boundary of the bulk of a population of migrating amoebae was devised and is described. This assay employs a dynamic gradient and is independent of deaminase activity. Measurements of chemoattractant capabilities of various pteridines, folates, and mixtures of folate fragments are reported. 2-Amino 4-quinazolinone, a pterin analog without the pyrazine ring nitrogens, is chemotactic. Lumazine, deaminated pterin, inhibits chemotaxis towards pterin but not towards folic acid. Deaminofolic acid is a chemoattractant as are mixtures of lumazine plus aminobenzoylglutamic acid or deaminopteroic acid plus various amino acids. Separately, the components of these mixtures exhibit no ability to stimulate chemotaxis. These mixtures are of fragments that together comprise most of the folate structure. Our results are in accord with separate receptors for pterin vs. folic acid and with a high stringency for pterin reception but a relative tolerance for folate reception. The possibility of using such mixtures to investigate the requirements of various parts of the folate structure for competent signalling is discussed. Cell Motil. Cytoskeleton 51:39–48, 2002.

Key words: chemotaxis; Dictyostelium; population assay; signal reception; folates; pteridines; filopodia

INTRODUCTION

Movement and change of appearance are two hallmarks of behavioral responses. In amoebae, reorganization of the actin cytoskeleton is required for motility and generation of filopodia. Such responses are apparent in *D. discoideum* vegetative amoebae when presented with various pteridines. These predatory cells find bacteria for feeding by preferentially moving towards folic acid [Pan et al., 1972], which is secreted by bacteria.

Pterin, a portion of folic acid, is itself a chemoattractant for *D. discoideum* vegetative amoebae [Pan et al., 1975]. Both folic acid and pterin stimulate an increase in number and length of filopodia, thereby changing the appearance of these cells [Rifkin and Isik, 1984]. A variety of pteridines, all containing an intact pterin moiety, stimulate both chemotaxis [Pan et al., 1975] and size of the filopodial array [Rifkin and Wali, 1986] in the same concentration range.

Various components of effective chemotaxis include signal binding, g-protein activation, and signal transduction that leads to directed motility. Use of various pteridines with structural variations of the pterin moiety present an unclear picture as to specificity and consequences of pteridine reception by *D. discoideum* vegetative amoebae. For example, there appears to be disagreement about the competence of deaminofolic acid as a signal molecule [Pan and Wurster, 1978; Wurster and Butz, 1980; Van Haastert et al., 1982; De Wit and Bulgakov, 1986]. Treatment with other variations, e.g., a quinazoline derivative that stimulates filopodia [Rifkin

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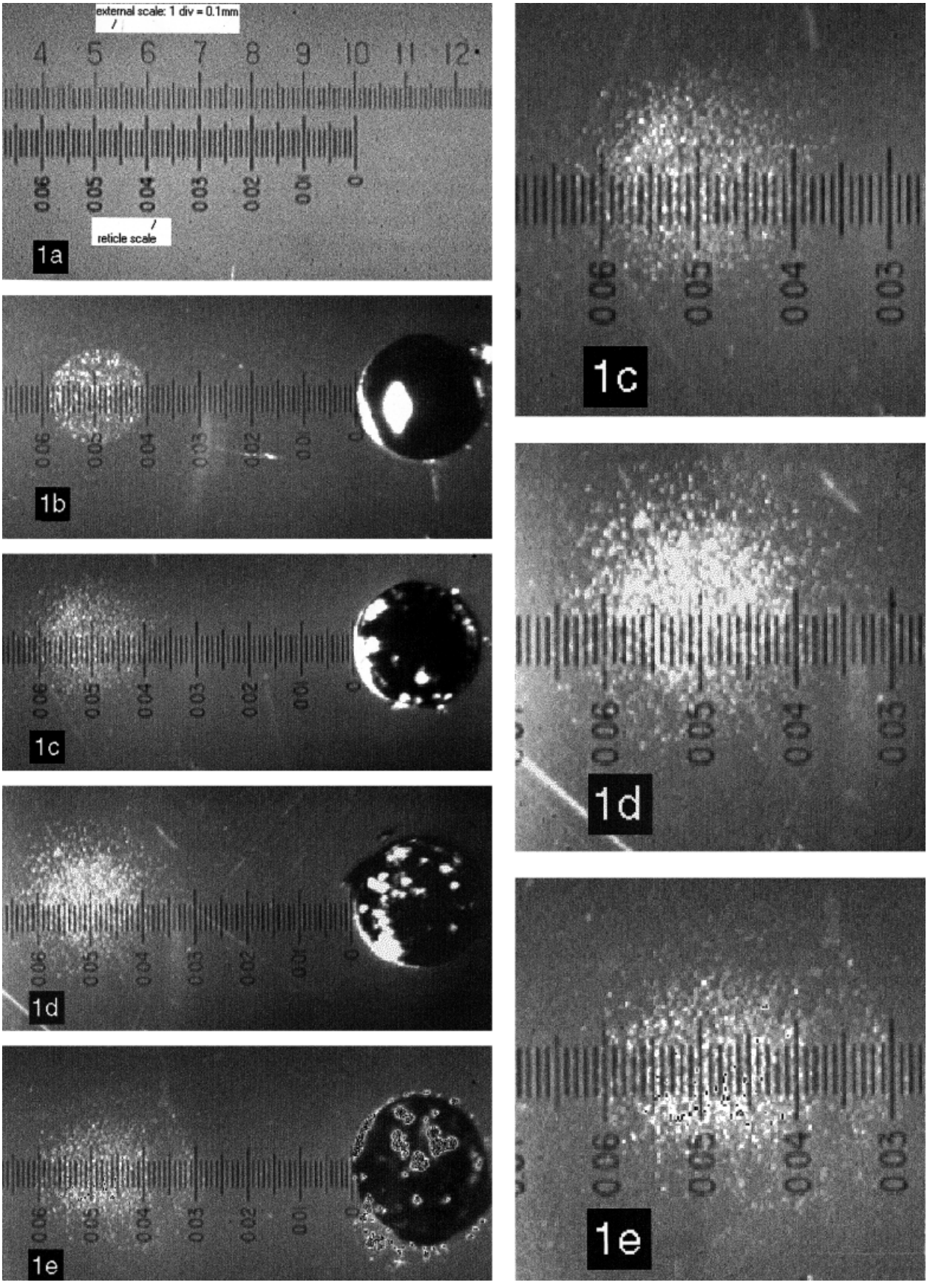


Figure 1.

and Wali, 1986], leads to questions of receptor stringency with regard to the pterin moiety.

To pursue these questions, we required quantitative measurements of amoeboid responses to even weak stimulants. Such an assay for characterization of filopodia of *D. discoideum* vegetative amoebae has been developed [Rifkin and Isik, 1984]. For chemotaxis measurement, we desired a dynamic-gradient method that was direct-reading, sensitive, quantitative, independent of deaminase activity, and was at the same time relatively quick and convenient. A variety of chemotaxis assays [e.g., Bonner et al., 1966; Segall et al., 1987; Fisher et al., 1989; Kuwayama et al., 1993] have been used but none satisfies our criteria. This study was undertaken to develop such an assay and to investigate questions regarding the stringency of pteridine reception.

MATERIALS AND METHODS

Culture and Harvesting of Vegetative Amoebae

Non-axenic *D. discoideum,* strain NC-4H, was co-cultured with *Escherichia coli,* strain B/r, in complete medium [Sussman, 1961] at 22°C in aerated suspension on a tilted-plane rotator in the dark. Vegetative amoebae were harvested in middle to late log phase (24–27 h after inoculation) by centrifuging the culture suspension at 50*g* for 3 min, aspirating off the supernatant fluid, and resuspending the softly sedimented cells in standard saline solution [Bonner, 1947]) buffered at pH 6.3 with 0.01M Na-K phosphate (PBSS) [Rifkin and Speisman, 1976]. This procedure was repeated once to provide harvested amoebae free of bacteria and with >99.99% of the original growth medium removed. Immediately after harvesting, amoebae were resuspended in PBSS to a concentration exhibiting 0.5 Abs @ 500 nm [Rifkin, 2001], determined by hemocytometer count to equal $\sim 7 \times 10^6$ cells/ml. This standardized suspension of harvested amoebae was then incubated under growth conditions for at least 10 min before initiating any experiment.

Filopodia

Filopodia of living *D. discoideum* vegetative amoebae were visualized with dark-field microscopy [Rifkin and Speisman, 1976]. Harvested cells were mixed with test substances, 100 μM in PBBS, and examined within 1 min under dark field illumination. The number and length of filopodia were compared to those of amoebae in PBSS. An increase in these parameters indicated an enlargement of the filopodial array [Rifkin and Isik, 1984].

Population Assay for Chemotaxis

Five-milliliter aliquots of 2.3% non-nutrient agar (B.B.L. Agar Agar), dissolved in PBBS, were poured into 50 × 9 mm petri plates (Falcon no. 1006). These assay plates, with tight fitting lids, were marked into labelled quadrants, stored at 5°C, and used within 3–15 days after preparation.

For quantitative measurement of chemotaxis, a micrometer disc (100 divisions, labelled every 10 divisions) was fitted into one ocular of a 7–40× variable-magnification binocular dissecting microscope. This microscope was calibrated by viewing an external standard micrometer and adjusting the magnification so that the divisions of the two scales matched. Routinely, we set 1 reticle division equal to 0.1 ± 0.01 mm (Fig. 1a). Suspended amoebae were deposited on the agar and all measurements of distances were made while viewing the preparations through this calibrated microscope, thus, allowing direct reading of distances to the nearest 0.1 mm.

Motility responses of populations of *D. discoideum* vegetative amoebae to putative chemoattractants were quantitatively measured with a modified small-population assay [adapted from Konijn, 1970; Varnum and Soll, 1981; Rossomando, 1987]. In the center of each quadrant, a sample well was made by removing a cylindrical plug of agar with a 2-mm diameter borer. Ten-microliters of a solution of test substance dissolved in PBBS, at 100 μM unless otherwise noted, was placed in a well. A 0.5-μl aliquot of suspended amoebae, $\sim 3.5 \times 10^3$ cells, was then placed on the agar surface, towards the outside of the plate, with 4 ± 0.1 mm between the nearest edges of the suspension and the well. Within 5 min, the suspension fluid was absorbed into the agar, leaving a spatially well-defined population of amoebae on the surface (Fig. 1b). Routinely, different test samples were placed in the different wells of a plate and 3 separate aliquots of suspended amoebae were placed radially, ~4 mm from each other, around each well.

Fig. 1. Videomicrographs pertaining to the quantitative population assay for chemotaxis of *D. discoideum* vegetative amoebae. The visible scale in all panels, marked every 10 divisions as "0". . ."0.06", is in one microscope ocular. **a:** View of an external micrometer scale with each division = 0.1 mm. The magnification was adjusted until the divisions of the two scales were in registry. Hence, in b–e, the dimension bar is the scale. c–e: Observations at 3.25 h after t_0 and chemotaxis is towards the right. **b:** View of a typical preparation at t_0. At the right, edge at "0.0", is a sample well filled with test solution. At the left, appearing as a disk of white points is a deposited aliquot of amoebae. Direct microscopic observation of this preparation exhibited an edge-to-edge distance between the well and the cell population as d_i = 4.0 mm. **c (and enlargement):** Migration of amoebae with PBBS in well and, by direct microscopic observation, d_f = 3.8 mm. These cells appear to have spread symmetrically from their original center. **d (and enlargement):** Pterin in well, d_f = 3.2 mm. **e (and enlargement):** Folic acid in well, d_f = 2.9 mm. Amoebae spread asymmetrically towards the well in response to pterin (d) and folic acid (e).

The initial distance (d_i) between each population and its associated sample well was recorded. The plates were then incubated in the dark at 22°C for 3.25 h after which the final distance (d_f) between the leading edge of the population and the well was recorded. The leading edge of a population of amoebae was at that nearest point to the well where there were >5 amoebae at a 0.1 mm interval (transect).

Inhibition Experiment

Amoebae were harvested as described but suspended in 100 μM lumazine in PBBS. To evaluate chemotaxis, the agar also contained 100 μM lumazine but the assays were performed as described in all other respects.

Data Analysis

All measurements of filopodial dimensions and chemotaxis were made in a single-blind manner with the observer not informed of the identity of the substance in a given sample experiment. Every experiment set included both a null control, PBBS, and a positive control, folic acid. For chemotaxis, the "taxis index" was calculated as (d_i-d_f). Data, the taxis indices of each population for different test substances in replicate experiments, were subjected to statistical analysis using SAS® programs (SAS Institute). Generalized linear models were used employing Bonferroni criteria to determine if means of taxis indices in different treatments were not significantly different from each other or from PBBS. Each table of results contained data of pooled experimental results containing at least two replicate runs. Means of taxis indices are reported with their respective standard errors of the mean (SEM).

Materials

Folic acid, pterin, pteroic acid, *p*-aminobenzoylglutamic acid (ABG), alanine, glycine, and glutamic acid were obtained from Sigma. 2-amino 4-quinazolinone (AQ) was a gift from J. Hynes. Deaminopteroic acid (DAP), or 2,4-dihydroxypteroic acid, was synthesized from pteroic acid [Wolf et al., 1947; Angier et al., 1952] and deaminofolic acid (DAFA), or 2,4-dihydroxyfolic acid, was synthesized from folic acid [Angier et al, 1952]. For both compounds, purity and homogeneity were verified by HPLC and key structural characteristics were verified by UV spectrophotometry.

RESULTS

Edge of Population of Migrating Amoebae

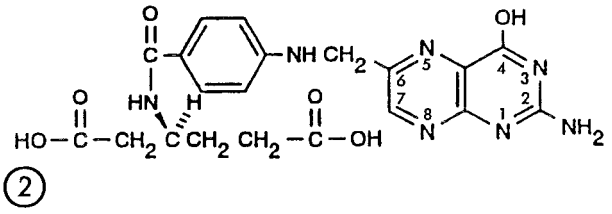
Determination of d_f required being able to identify the edge of a population of migrating amoebae. A number of treatments were prepared with either PBBS or folic acid in the sample wells and d_i = 4.0 mm in all cases. After 3.25-h dark incubation, the preparations were examined by counting the number of amoebae at each 0.1-mm interval out from the edge of the well (Fig. 3). For both the null and positive controls, the number of amoebae ranged from none to a few in much of the space between the well and the more dense part of a population of amoebae. We did not consider these as being part of the bulk of a population. However, as soon as there were >5 amoebae at any 0.1-mm transect, the number of amoebae very quickly increased in each succeeding 0.1-mm interval until there were too many to easily count. Therefore, we decided that the effective boundary of an overwhelming majority or bulk of a population of cells was at that nearest distance to a well at which there were >5 amoebae. These data yielded a distance from the sample well to the population edge of ~3.7 mm for PBBS and ~3.0 mm for folic acid. These values are consistent with the other values presented in this report.

Migration of Amoebae in the Presence of PBBS

All experiments with PBBS in the well revealed a small and random dispersion of amoebae after the 3.25-h dark incubation. This movement was symmetrical about the center of the deposited aliquot of cells and was not directed towards the sample well (Fig.1c). In this example, the edge of the population had advanced to the 3.8-mm transect, thus, yielding a taxis index value = (4.0–3.8) or 0.2. We ascribed no chemotaxis significance to such a non-directed >0 value for taxis index in response to PBBS and regard it as a measure of motility background or noise. However, such data were used in the statistical analyses in all associated experimental treatments to compare the taxis index of any other substance with the value for the null control in that experimental set and, further, to determine if there was any significant difference between responses to a test substance and to the null control.

Migration of Amoebae in the Presence of Pteridines

Pterin (100 μM) or folic acid (100 μM) was placed in a sample well. After 3.25 h, the preparations were viewed. With pterin in the well (Fig. 1d), microscopic examination revealed that the population edge had advanced to the 3.2-mm transect yielding a taxis index of 0.8 (4.0–3.2). With folic acid in the well (Fig. 1e), the population edge had advanced to the 2.9-mm transect, yielding a taxis index = 1.1 (4.0–2.9). In addition and typically, this folic acid preparation exhibited a region behind the population edge that appeared to be have fewer amoebae in it. We ascribe this to the action of folate deaminase [Pan and Wurster, 1978] produced by the leading cells. This diminishes the concentration of folic acid in their wake.



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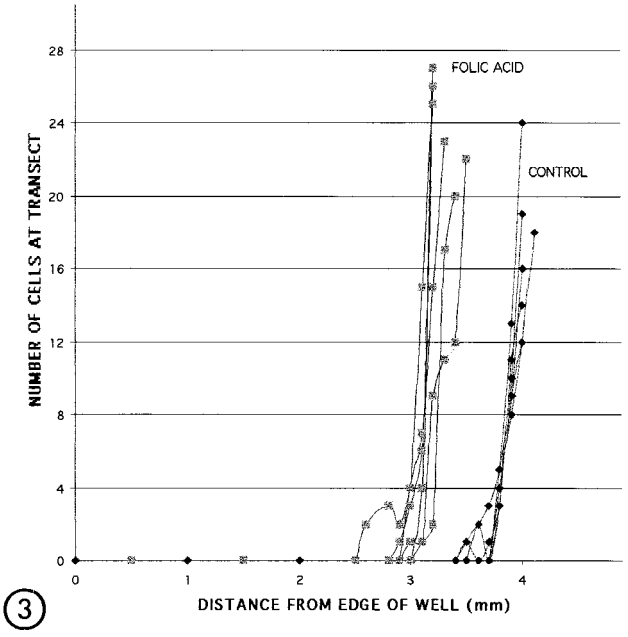

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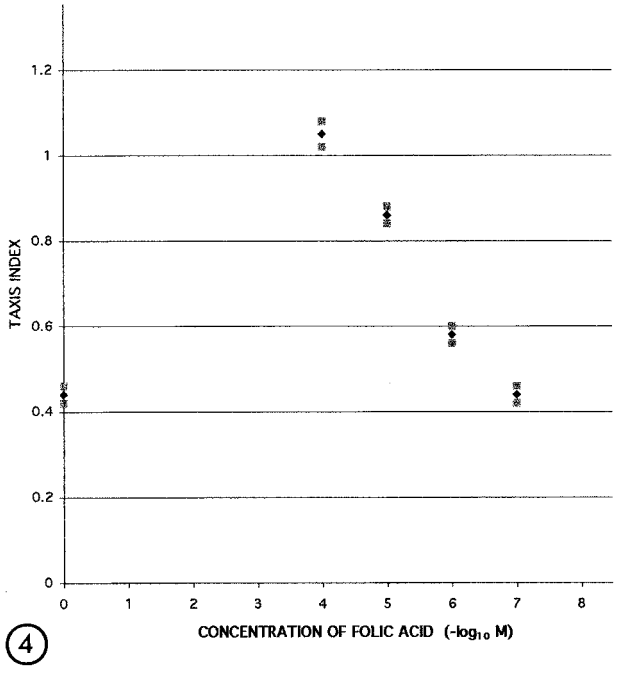

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Figures 2–4.

Concentration Effects on Chemotaxis to Folic Acid

Chemotactic responses of *D. discoideum* vegetative amoebae to various concentrations of folic acid were measured (Table I). These results demonstrate that the threshold concentration for folic acid chemotaxis was $\geq 10^{-7}$M. A plot of these data (Fig. 4) is consistent with a logarithmic relationship between concentration and chemotactic response.

TABLE I. Effect of Concentration of Folic Acid on Chemotaxis of *D. discoideum* Amoebae

Treatment	No. of populations	Mean taxis index (±SEM)	Bonferroni test*
Folic acid @ 100 μM	43	1.05 ± 0.03	A
Folic acid @ 10 μM	37	0.86 ± 0.02	B
Folic acid @ 1 μM	35	0.58 ± 0.02	C
Folic acid @ 0.1 μM	39	0.44 ± 0.02	D
PBBS	47	0.44 ± 0.02	D

*α = 0.05; $t_{critical}$ = 2.90; minimum significant difference = 0.07. Means with same letter are not significantly different.

Chemotaxis Towards a Pterin Analog

AQ is a pterin analog with carbon substituted for the nitrogens at positions 5 and 8 (Fig. 2). Computer-assisted structural analysis of AQ (W. Hersh, personal communication) revealed that pterin and AQ are of the same size and planar geometry. AQ stimulates filopodial size and number [Rifkin and Wali, 1986]. AQ (100 μM) in sample wells generated a taxis index of 0.47, a value significantly different from PBBS but significantly less than 0.73, the average for pterin in other experiments.

Fig. 2. Chemical structure of folic acid. The atomic positions within the pterin moiety are numbered. Pterin consists of only the double-ring moiety while the usual deamination occurs at position 2.

Fig. 3. Chemotaxis assays were prepared with either 100 μM folic acid or PBBS in the wells. After 3.25 h in the dark at 22°C, the number of amoebae of each migrating population were counted at each 0.1 mm interval (transect) from the edge of well towards the population of cells. With either treatment, there was a varying small number of amoebae in the space between the well and the bulk of a population of amoebae. But as soon as there were >5 amoebae at any transect, the density of amoebae rapidly increased to uncountable levels. Therefore, we decided that the edge of a population of amoebae was at that nearest distance to a well at which there were >5 amoebae.

Fig. 4. Chemotaxis of *D. discoideum* vegetative amoebae towards folic acid at various concentrations, 100, 10, 1, and 0.1 μM, in the sample well. The concentration levels are indicated as their negative log values. Chemotaxis of these cells appears to have an inverse linear relationship to the log of folic acid concentration, i.e., taxis = k (-log [folic acid]).

TABLE II. Effect of 2-Amino 4-Quinazolinone (AQ) on Chemotaxis of *D. discoideum* Amoebae

Treatment	No. of populations	Mean taxis index (±SEM)	Bonferroni test*
Folic acid	15	1.09 ± 0.04	A
AQ	18	0.47 ± 0.04	B
PBBS	33	0.38 ± 0.02	C

*$\alpha = 0.05$; $t_{critical} = 2.47$; minimum significant difference = 0.07. Means with same letter are not significantly different.

Microscopic examination of responses to AQ revealed an asymmetric directed pattern of migrating amoebae and our data (Table II) are consistent with a chemoattractant capability for AQ.

Preferential Inhibition by Lumazine on Chemotaxis

Measurement of chemotaxis of amoebae that were pretreated with lumazine revealed differences between responses to folic acid vs. pterin (Table III). Pretreatment with lumazine did not affect chemotaxis towards folic acid but did inhibit chemotaxis towards pterin. The taxis index for lumazine itself was not significantly different from PBBS.

TABLE III. Effect of Lumazine on Chemotaxis of *D. discoideum* Amoebae Towards Pterin and Folic Acid

Treatment	No. of populations	Mean taxis index (±SEM)	Bonferroni test*
Folic acid	18	0.92 ± 0.02	A
Folic acid with lumazine[a]	12	0.97 ± 0.02	A
Pterin	19	0.82 ± 0.03	B
Pterin with lumazine[a]	15	0.57 ± 0.02	C
Lumazine	15	0.43 ± 0.03	D
PBBS	74	0.43 ± 0.02	D
PBBS with lumazine[a]	27	0.39 ± 0.02	D

[a]Amoebae harvested into suspended in PBBS with added 100 μM lumazine; agar contained 100 μM lumazine. This amounts to pretreating amoebae before they come in contact with test substance.
*$\alpha = 0.05$; $t_{critical} = 3.17$; minimum significant difference = 0.06. Means with same letter are not significantly different.

Preferential Inhibition by Lumazine on Filopodia

Examination by dark-field microscopy of amoebae in PBBS or in PBBS with 100 μM lumazine revealed no difference in the size or number of filopodia. Both folic acid and pterin stimulate enlargement of the filopodial array [Rifkin and Isik, 1984; Rifkin and Wali, 1986]. However, amoebae pretreated with lumazine did not respond to pterin. In contrast, pretreatment with lumazine had no effect upon the ability of folic acid to stimulate the filopodial array (Table III).

TABLE IV. Chemotaxis of *D. discoideum* Amoebae Towards Folic Acid, Pterin, and Deaminofolic Acid (DAFA)

Treatment	No. of populations	Mean taxis index (±SEM)	Bonferroni test*
Folic acid	78	1.02 ± 0.02	A
Pterin	93	0.70 ± 0.02	B
DAFA	101	0.54 ± 0.02	C
PBBS	147	0.31 ± 0.02	D

*$\alpha = 0.05$; $t_{critical} = 2.66$; minimum significant difference = 0.05. Means with same letter are not significantly different.

Chemotaxis Towards Deaminofolic Acid

DAFA (100 μM) in sample wells generated a taxis index = 0.54 (Table IV), less that for pterin and greater than for PBBS. Microscopic examination of responses to DAFA revealed an asymmetric directed pattern of migrating amoebae and our data are consistent with a conclusion that deaminofolic acid is a chemoattractant for *D. discoideum* vegetative amoebae.

Chemotaxis Towards Fragments of Folic Acid

This report demonstrates that deaminofolic acid is a chemoattractant for *D. discoideum* vegetative amoebae (Table IV) and confirms that lumazine is not a chemoattractant [Pan et al., 1975]. ABG represents most of the non-pterin portion of folic acid. We investigated the effect of a mixture of 100 μM lumazine plus 100 μM ABG, upon migration of these amoebae. This mixture generated a taxis index = 0.47, an effect not significantly equal to that of PBBS (Table V). Lumazine and ABG individually produced taxis indices not significantly different from each other or from PBBS. Our data demonstrate that this mixture, lumazine + ABG, is a chemoattractant for *D. discoideum* vegetative amoebae.

D. discoideum vegetative amoebae are chemotactic towards pteroic acid [Pan et al., 1975]. This result to-

TABLE V. Effect of *p*-Aminobenzoylglutamic Acid (ABG) on Chemotaxis of *D. discoideum* Amoebae Towards Folic Acid, Pterin, and Lumazine

Treatment	No. of populations	Mean taxis index (±SEM)	Bonferroni test*
Folic acid	81	1.08 ± 0.03	A
Folic acid + ABG	38	1.02 ± 0.04	A
Pterin	93	0.75 ± 0.02	B
Pterin + ABG	109	0.79 ± 0.02	B
Lumazine + ABG	78	0.47 ± 0.02	C
Lumazine	54	0.39 ± 0.01	D
ABG	101	0.31 ± 0.01	D
PBBS	147	0.34 ± 0.01	D

*$\alpha \pm 0.05$; $t_{critical} = 3.14$; minimum significant difference = 0.10. Means with same letter are not significantly different.

TABLE VI. Chemotaxis of *D. discoideum* Amoebae Towards Deaminopteroic Acid (DAP), Glutamic Acid, Alanine, Glycine, and Combinations of These

Treatment	No. of populations	Mean taxis index (±SEM)	Bonferroni test*
Folic acid	77	0.98 ± 0.02	A
DAP + alanine	29	0.47 ± 0.02	B
DAP + glycine	26	0.47 ± 0.03	B
DAP + glutamic acid	22	0.48 ± 0.03	B
DAP	82	0.40 ± 0.01	C
Alanine	21	0.31 ± 0.02	C
Glutamic acid	20	0.35 ± 0.03	C
Glycine	21	0.34 ± 0.02	C
PBBS	87	0.39 ± 0.01	C

*$\alpha = 0.05$; $t_{critical} = 3.11$; minimum significant difference = 0.01. Means with same letter are not significantly different.

gether with the data in Table V suggested a possibility that mixtures of other portions of the folate molecule might stimulate chemotaxis. To determine the requirements of the glutamate end of folic acid, we investigated the chemotactic capabilities of mixtures of DAP with various amino acids. In this experiment, DAP was mixed with glutamic acid, alanine, or glycine, each at 100 μM. Taxis indices (Table VI) for these mixtures averaged 0.47, greater than for PBBS. DAP and these amino acids individually did not stimulate chemotaxis. Their taxis indices were not significantly different from each other or PBBS. These results demonstrated that these mixtures are chemoattractants and microscopic examination of responses to these mixtures revealed an asymmetric directed pattern of migrating amoebae. These data are consistent with a model in which the presence of some amino acid is necessary but glutamic acid per se is not a stringent requirement.

DISCUSSION

Chemotaxis Assay

The goals of developing an assay for chemotaxis towards even weakly stimulating compounds and towards compounds that are insensitive to deaminase activity have been achieved. Both the cellophane-square assay [Bonner et al., 1966] and the agar block assay [Kuwayama et al., 1993] rely upon cellular deaminase to create concentration gradients. Our assay was able to demonstrate, with statistical significance, chemotaxis towards weak, even deaminated, stimulants. We also realized the further goals of having the assay direct-reading and relatively quick, The inclusion of a calibrated reticle in an ocular provided measurements directly at 0.1-mm levels and entire assays including measurements of multiple plates were completed within 4 h.

The original determination that folic acid is a chemoattractant for *D. discoideum* vegetative amoebae [Bonner et al., 1970; Pan et al., 1972] used the cellophane-square assay, a method requiring measurements extending to >4 h after harvesting of amoebae into nutrient-free saline. Furthermore, it is generally known that vegetative amoebae of the non-axenic strain used in this study, NC-4H, retain their responsiveness to folate even beyond 6 h post-harvesting and, during a starvation period of >10 h, these amoebae slowly gain a cyclic AMP response while slowly losing the folate response (J. T. Bonner, personal communication). Hence, we regard the <3.5-h post-harvesting period during which we observed and measured chemotaxis towards pteridines as valid for observation of folate-related chemotaxis and that these results are physiologically valid.

Our assay allowed the determination of responses by amoebae in a dynamic gradient generated by diffusion of test substances. Although a physiologically high concentration of test chemoattractant was placed in a sample well, 10–100 times greater than optimal in the case of folic acid, diffusion of test substances out into the agar generated a dynamic concentration gradient. We suggest that as the amoebae migrated from their initial position, they eventually met a threshold level of signal moving towards them and were then oriented to move up the gradient. Incidental long-term observation, beyond 7 h after t_{zero}, of some plates with folic acid as test substance, revealed that amoebae had begun to scatter widely over the agar. This observation is consistent with an equilibration of the chemoattractant gradient in the agar but occurred very much after the time period during which our measurements were taken. Furthermore, for all chemotactic samples tested, we were measuring the distinct movement of $>10^2$ amoebae, which were at the leading edge of the deposited population (see Fig. 1c–e). Hence, regardless of the processing of signal, e.g., endocytosis, deamination, etc., by amoebae as contact is made, these cells are still influenced by fresh signal diffusing towards them. The asymmetric pattern produced by a population of amoebae exhibiting chemotaxis refers to the distribution of cell migration from the edge of the deposited mass, comparing regions nearest to vs. farthest from the source of the test substance.

Folic acid has an MW = 441 and pterin has an MW = 163. Could differential diffusion of test substances have led to distinct levels of motility responses? A conjugate of folic acid-lactalbumin-FITC is a very much larger molecule, MW = ~15,000, and has an exposed native pterin. This probe was used in a semiquantitative version of this chemotaxis assay and evoked a level of chemotaxis similar to pterin in the same time interval [Rifkin, 2001]. Hence, we conclude that the

aqeuous space of the agar gel is not differentially restrictive to diffusion of the various substances reported in this study and that any differences in motility from control levels are primarily due to amoeboid responses.

Tables I–VI exhibit data from pooled replicate experiments. Differences of taxis indices for the same compounds used in different experimental sets were within 1 standard deviation of each other. Thus, this methodology generated results that are statistically reproducible.

The concentrations reported are those of substances when placed in sample wells. Clearly, the amoebae were responding to lower concentrations because diffusion had not yet reached equilibrium within the time frame of these experiments. The incubation interval of 3.25 h was determined empirically from observations of amoebae migrating in response to folic acid. At less than 2.5 h, there was a barely detectable directed asymmetry in the migration pattern. This indicated that a threshold concentration had not yet reached the cells. At longer than 4.5 h, amoebae appeared to be scattered in various directions, suggesting that a greater than threshold level of folic acid had passed the front-most cells probably creating confusion amongst the amoebae regarding the gradient. After 10 h, amoebae were generally distributed over the agar indicating that an equilibrium distribution of folic acid had been achieved.

Another particular goal was to determine responses from average amoebae, i.e., those typically found within the bulk of a population of cells, rather than some very small number of the fastest moving amoebae. The problem of determining the boundary of any population, in order to avoid non-typical isolated individuals, is of significance in field studies, as in "Where is the edge of a forest?" or "Where is the edge of a herd?" A search of the literature did not yield any objective and convenient quantitative techniques to answer such questions. Consequently, we developed the methodology described in this report (Figs. 1 and 3).

Stringency of Pterin Reception

AQ stimulates both filopodia [Rifkin and Wali, 1986] and chemotaxis (Table II). These results are consistent with the premise [Rifkin and Wali, 1986] that common pteridine signals initiate a reorganization of the actin cytoskeleton that leads in parallel to enlargement of the filopodial array and to directed motility. AQ has a very similar geometry and contains the 2-amino 4-hydroxy groups that pterin does. However, AQ is a much weaker but statistically significant stimulant. Our results and the evaluation of chemotaxis to a number of substituted pterins [Pan et al., 1975] suggest that both the 2-amino and the 4-hydroxy substitutions and the planar geometry are essential.

Our results (Table III) that lumazine inhibits pterin stimulation of chemotaxis but not that of folic acid are consistent with other studies [Van Haastert et al., 1982; DeWit and Van Haastert, 1985] that show that pterin and folic acid have separate receptors. Most side group additions at the pterin 6-position, even large ones as in folic acid, appear to have little effect on chemotactic potency. However, the addition of electrophilic groups at position 6, the absence of pyrazine ring nitrogens in AQ, or a side-group at the 7- position [Pan et al., 1975] greatly reduces or abolishes potency. These observations are consistent with a model in which a cellular response to a pterin signal requires a signal molecule with a very particular distribution of charge over the pterin moiety. All of these observations are consistent with high stringency for pterin reception.

Stringency of Folate Reception

Previous work [Pan and Wurster, 1978], using the cellophane-square assay [Bonner et al., 1966] reported that DAFA was not a chemoattractant. However, that assay starts with an equilibrated distribution of pteridine in the agar and involves a deaminase made by the amoebae to cleave the 2-amino group from pteridines. This deamination then creates a gradient that causes chemotaxis. But, DAFA is already deaminated and, thus, no gradient would have been made. Our results (Table IV), using a method in which a dynamic gradient is generated by diffusion of test samples, demonstrated that DAFA is a weak but measurable chemoattractant. This observation is consistent with the observations that DAFA binds to folate receptors [Wurster and Butz, 1980] and that DAFA stimulates cGMP levels, generally considered indicative of initiation of signal transduction [De Wit and Bulgakov, 1986]. Our observation that DAFA elicits chemotaxis suggests that reception of folic acid, >2.5 larger than pterin, might be less stringent. Could a combination of folate fragments be used to investigate the requirements for the various parts of the folate molecule necessary to achieve cellular responses?

Tables V and VI display the results of presenting mixtures of fragments that are separately inactive. A mixture of lumazine and aminobenzoyl glutamic acid (ABG) produces a statistically significant stimulation of chemotaxis (Table V). It is intriguing that pterin plus ABG, constituting most of the native folic acid molecule, does not produce chemotaxis greater than pterin alone and we are pursuing this question with a more intensive study. Pteroic acid is a chemoattractant for *D. discoideum* vegetative amoebae [Pan et al., 1975]. Deaminopteroic acid (DAP) is as expected not a chemoattractant (Table VI). However, a mixture of DAP with glutamic acid, alanine, or glycine does stimulate measurable chemotaxis (Table VI). These results are consistent with a model of folate reception that is less stringent than for

pterin reception. Preliminary studies (not reported) indicate that a mixture of lumazine plus *p*-aminobezoic acid, without a glutamate moiety, does not stimulate chemotaxis.

What is the nature of the folate receptor? It appears to have at least a requirement for some pteridine, not necessarily native pterin, and for a component that represents most of the rest of a folate. This component includes a terminal group that may be an amino acid. The results in Tables V and VI also support the strategy of using DAP in mixtures to those properties of the glutamate end of folates that are necessary to stimulate Dictyostelium amoebae. Is an amino acid required? Are there size and charge requirements for this moiety in order to achieve chemotaxis? The efficacy of mixtures of DAP and a variety of compounds is currently being studied. Furthermore, given the relative difficulty of synthesizing various pteridine derivatives, this demonstration that chemotaxis can be elicited by some mixtures of non-tactic compounds may allow a more detailed description of the pteridine receptors.

CONCLUSIONS

Lumazine inhibits pterin-stimulated chemotaxis but not stimulation by folic acid in *D. discoideum* vegetative amoebae. These behavioral results are consistent with separate pterin and folic acid reception. The chemoattractant capability of aminoquinazolinone is consistent with a high stringency for pterin reception. Chemoattractant capabilities of deaminofolic acid and mixtures of lumazine plus aminobenzoylglutamic acid or deaminopteroic acid plus various amino acids support a model of relative tolerance for folate reception. Mixtures of some non-tactic compounds, representing fractions of native and modified folic acid, can elicit chemotaxis.

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