Biochimica et Biophysica Acta 814 (1985) 214–226
Elsevier

BBA 72533

Relationships between the ligand specificity of cell surface folate binding sites, folate degrading enzymes and cellular responses in *Dictyostelium discoideum*

René J.W. de Wit, Roman Bulgakov, Johan E. Pinas and Theo M. Konijn

Cell Biology and Morphogenesis Unit, Zoological Laboratory, University of Leiden, Kaiserstraat 63, 2311 GP Leiden (The Netherlands)

(Received December 10th, 1984)

Key words: Cell-surface receptor; Folate analog; Ligand specificity; Folate degrading enzyme; Chemotaxis; (*D. discoideum*)

The affinity of four distinct types of folate binding sites and of two membrane-bound folate degrading enzymes for 15 folic acid derivatives was monitored. Apart from two types (A^H and A^L) of binding sites, all binding classes or enzymes show different affinity patterns. This strongly suggests the observed binding sites to be nonidentical to the membrane-bound folate deaminase or folate C_9-N_{10} cleaving enzyme. The analog specificity of the chemotactic response towards folates shows a strong resemblance to the specificity of binding to one of the binding classes: 'B-sites' ($p < 0.01\%$). Less or no correlation was observed between the other classes or the enzymes and the chemotactic response. This may indicate that the B sites are involved in the transduction of an extracellular signal to chemotactic cell movement. Folates elicit secretion of cAMP. Recently, the activity of several folate derivatives to evoke a cAMP response was studied (Devreotes, P.N. (1983) Dev. Biol. 95, 154–162). Comparison of this activity and the specificity of the binding sites in this study, suggests that the 'A-sites' are involved in the cAMP response.

Introduction

Since the discovery that folate is a universal chemoattractant in the cellular slime molds [1], chemotaxis towards folic acid has been regarded as a food-seeking device. This proposal was supported by the chemotactic activity for all species and the generally higher activity in the vegetative stage [2], during which the amoebae feed on bacteria by phagocytosis. When the food supply is depleted, starvation induces differentiation to an aggregation competent phase. Consequently the cells aggregate via chemotaxis towards a specific attractant or acrasin. Several recent reports indicate a role for folate or its derivatives during the interphase, i.e. between the vegatative and the aggregation competent stage, and during cell aggregation. (i) Folic acid pulses were found to stimulate development [3–5]. (ii) *Dictoystelium discoideum* secreted larger amounts of folates and pterins during the interphase [2,6,7] and (iii) the acrasins of *Dictyostelium lacteum* and *Dictyostelium minutum* were identified as a pterin derivative and a folate derivative, respectively [8,9]. These findings led us to investigate the folate chemoreceptor in *D. discoideum*. The binding affinity of folate derivatives to a cell surface binding site as reported previously [10,11] did not correlate to the chemotactic activity of these compounds;

Abbreviations: 2-deaminofolic acid, 2-deamino-2-hydroxyfolic acid; methotrexate, 4-amino-N_{10}-methylfolic acid; aminopterin, 4-aminofolic acid; D-N_{10}-methylfolic acid; N_{10}-methylpteroyl-D-glutamic acid. Enzymes: membrane-bound folate deaminase (EC 3.5.4.11), membrane-bound folic acid C_9-N_{10} cleaving enzyme (not classified).

0005-2736/85/$03.30 © 1985 Elsevier Science Publishers B.V. (Biomedical Division)

2-deaminofolic acid was shown to be a ligand equipotent to folic acid, while the chemotactic activity was at least 1000-fold less [12–14]. Further, antagonistic activity of 2-deaminofolic acid (up to 10^{-4} M) was excluded [15]. Thus to our view the studied binding site could not be the chemoreceptor. Using the deaminase inhibitor 8-azaguanine we were able to demonstrate two folate binding sites [16]. One type bound 2-deaminofolic acid and folic acid with equal high affinity, the other bound 2-deaminofolic acid at least 100-fold less than folic acid. The latter was proposed to be a better candidate for being the receptor involved in the processing of chemotactic signals.

In the preceding report [17] the binding of folate was studied in detail. Five classes of binding sites were detected. The A-sites correspond to the previously detected folic acid/2-deaminofolic acid non-selective sites [10,16]. Two interconverting subtypes with different affinity constants were observed. C-sites are identical to the folic acid specific sites as were described before [16]. Again, two subtypes exist: before the addition of ligand mainly rapidly exchanging (low affinity) C^F sites are present; addition of ligand induces a conformational change into a slower binding C^S type with a higher affinity. Finally, sites of the B type were described, showing relatively slow kinetics.

The present work deals with the ligand specificity of (i) four types of binding sites. The fast exchanging C sites were not studied, because of the impossibility of measuring competition at these sites without interference by the slow type of C sites, (ii) two membrane-bound folate degrading enzymes: folate deaminase [18] and a folate C_9-N_{10} cleaving enzyme [19], (iii) the chemotactic response, (iv) folate induced cAMP secretion [20].

Comparison of these specificity patterns suggests that the enzymes and the binding sites are nonidentical and that certain binding types are linked to specific cellular responses.

Materials and Methods

Cultures. Dictyostelium discoideum NC4(H) was cultivated on nutrient agar [21] together with *Escherichia coli* 281. Cells were harvested in the late logarithmic phase before clearing of the bacterial lawn occurred and washed three times by centrifugation at $150 \times g$ during 4 min in cold 10 mM sodium/potassium phosphate buffer (pH 6.5). The cell density was adjusted to $5 \cdot 10^7$ per ml and the suspension was aerated at 0°C during 5 min before use in binding or enzyme assays.

Chemicals. [7,9,3′,5′-^{3}H]Folic acid, [2-^{14}C]folic acid and [7,3′,5′-^{3}H]methotrexate were purchased from Amersham Radiochemical Centre. 2-Deamino[7,9,3′,5′-^{3}H]folic acid was prepared by enzymatic deamination as described previously [16]. N_{10}-Methyl[7,3′,5′-^{3}H]folic acid, unlabeled N_{10}-methylfolic acid, D-N_{10}-methylfolic acid and 10-deazafolic acid were prepared by alkaline hydrolysis from the respective 4-amino derivatives (methotrexate, D-methotrexate and 10-deazaaminopterin) [22]. The products were purified by RP-18 HPLC and identified by their ultraviolet spectra [23]. Unlabeled 2-deaminofolic acid was prepared enzymatically and purified as in Ref. 15. Folic acid was purchased from BDH Biochemicals (Poole, U.K.), pteroic acid from Lederle Laboratories (American Cyanamid Co., Pearl River, NY), tetrahydrofolic acid and 8-azaguanine from Fluka AG (Buchs SG, Switzerland) and aminopterin, methotrexate, D-methotrexate, pterin, *p*-aminobenzoylglutamic acid from Sigma (St. Louis, MO). 10-Deazaaminopterin and 5-deazafolic acid were gifts from Drs. J.R. Piper and R.D. Elliott, respectively, of the Southern Research Institute, Birmingham, AL; 9-methylfolic acid was generously supplied by Dr. J. Upeslacis, American Cyanamid Co., Pearl River, NY. 2′,5′-diazafolic acid was a gift of Dr. I. Sekikawa, Institute of Immunological Sciences, Hokkaido University, Sapporo, Japan. 5,8-Dideazafolic acid was purchased from Dr. J.B. Hynes, Department of Pharmaceutical Chemistry, College of Pharmacy, Medical University of South Carolina, Charleston, SC. Silicon oil AR 20 and AR 200 were from Wacker Chemie (Munich, F.R.G.). Tetrahydrofolic acid was dissolved in buffer containing 1 mM dithiothreitol within 10 min before use. Stock solutions of poorly soluble analogs (pterin, pteroic acid) were kept in 10 mM phosphate buffer (pH 8). Since 4-amino derivatives are easily hydrolyzed to 4-hydroxy derivatives (which were over 100-fold more active in some cases) these compounds were analyzed by HPLC. If necessary the compounds were purified. The detection limit for

4-hydroxy contaminants was approx. 0.05%. If that amount is present and if the 4-amino compound does not bind to a given site, the contaminant will cause the 4-amino compound to seem 2000-fold less effective than the contaminant ($\delta\Delta G = 12$ kJ · mol^{-1}). Thus, if a larger difference in $\delta\Delta G$ data is obtained, e.g. between aminopterin and folic acid, activity of aminopterin may entirely be due to contamination by folic acid.

Binding assay. Cells were prepared as described above. For measurement of A and C sites the final cell density during incubation was $3.3 \cdot 10^7$ ml^{-1}, for the B sites $1 \cdot 10^8$ ml^{-1}. In all cases 0.33 mM 8-azaguanine was present. The incubations were performed in harvesting buffer at 0°C. Degradation of radioligand or competing folate (derivative) due to deamination or C_9-N_{10} cleavage was always less than 5% during incubation.

Protocol for measurement of competition at the A sites: 67 nM or 670 nM 2-deamino[^{3}H]folic acid was incubated in the presence of various concentrations of competing analog for 60 s. During this period the 150 μl samples were layered on top of 180 μl silicon oil (AR 20/AR 200, 11 : 4, v/v) and 10 μl 10% sucrose in 1.6 ml centrifugation tubes. At 60 s the samples were centrifuged at $10\,000 \times g$ for 15 s in a swing-out rotor. The tubes were frozen in liquid nitrogen and the tips containing the pellets were cut off. Radioactivity was measured after addition of 1.5 ml Instagel (Packard). In this way the two types of A sites were not measured separately; however, different affinity of a compound for the two types should yield a biphasic inhibition curve. Such curves were not observed, indicating small or no differences in affinity of the tested compounds for the two A types.

Protocol for the B sites: 10 nM [^{3}H]folic acid was incubated in the presence of various competitor concentrations for 120 s. After incubation, a 60 s chase was performed by addition of the 150 μl sample to 1050 μl of harvesting buffer containing 20 μM unlabeled folic acid. This aliquot of buffer was previously layered on top of the silicon oil and kept at 0°C. The centrifugation was done as described above.

Protocol for the C^S sites: 1.85 nM or 18.5 nM N_{10}-methyl[^{3}H]folic acid was incubated in the presence of 3.3 μM 2-deaminofolic acid and various concentrations of competing analog for 60 s. During this incubation to equilibrium samples were layered on top of the silicon oil and treated further as described above.

Assay of membrane-bound folic acid specific deaminase. Final concentrations were: $3.3 \cdot 10^6$ cells/ml, 67 nM [^{14}C]folic acid (100 cpm/pmol), 67 μM pterin, various concentrations of folate analogs, in 10 mM phosphate buffer (pH 6.5). Incubation was for 4 min at 0°C, during which 30% of the substrate was deaminated. The membrane-bound enzyme was reported to be specific for folic acid, while at least one of the extracellular enzymes also accepted pterin as substrate [18]. Therefore pterin was added in order to suppress extracellular deaminase activity, which might have been secreted during incubation. Incubation was terminated by adding 500 μl ice-cold suspension of SP-Sephadex (cation exchange resin) in 10 mM HCO_2NH_4 (pH 2.0) (The volume of the settled resin was 50% of the total volume). After mixing and centrifugation at $8000 \times g$ for 1 min, radioactivity was determined in 250 μl supernatant containing the product 2-deamino[^{14}C]folic acid.

Assay of the membrane-bound folate C_9-N_{10} cleaving enzyme. Final concentrations were: $3.3 \cdot 10^7$ cells/ml, 67 nM [^{14}C]folic acid (100 cpm/pmol), 0.33 mM 8-azaguanine, various concentrations of folate analogs, 1 mM EDTA, 1 mM dithiothreitol, in 10 mM phosphate buffer (pH 6.5). Incubation was for 30 min at 0°C, during which about 30% of the folic acid was split into pterin-6-carboxaldehyde and *p*-amino-benzoylglutamic acid. After 30 min the newly secreted extracellular folate cleaving activity was only 10% of the membrane-bound activity. In the assay of membrane-bound C_9-N_{10} cleaving enzyme, a significant fraction of deamination of the tested folates could not be prevented. Despite the presence of 8-azaguanine, up to 40% was converted. Since 2-deaminofolic acid showed about the same affinity as folic acid, we concluded that deamination had no effect on binding to the C_9-N_{10} cleaving enzyme and that this degradation would not affect the results. Every 3–5 min the samples were mixed to prevent sedimentation of the cells. Incubation was terminated by addition of 500 μl ice-cold Dowex (AG1-X2) anion exchange resin

suspension in 10 mM ammonium acetate (pH 5.0) 1 mM dithiothreitol and 20% ethanol (The settled particles occupied 50% of the total volume). Samples were mixed and centrifuged at 8000 × *g* for 2 min, 250 µl was taken of the supernatant, containing the ^{14}C-labeled product, and counted.

Uptake of folic acid and methotrexate. Final concentrations were: $3.3 \cdot 10^7$ cells/ml, 67 nM [^{3}H]folic acid or [^{3}H]methotrexate (6000 cpm/pmol), in 10 mM phosphate buffer (pH 6.5). Blank samples were incubated in the presence of 20 µM folic acid or methotrexate. Incubation was at 20°C for up to 30 min, mixing the samples every 3–5 min. Incubation was terminated by adding 20 µM folic acid or methotrexate to prevent binding of the labeled compounds to cell surface binding proteins. After 60 s samples were centrifuged as in the binding assay. After centrifugation through silicon oil, the supernatants and the pellets of several samples were used to identify the labeled compounds present in the medium and in the cells after incubation. The supernatant was analyzed immediately by HPLC. The pellet was freeze-thawed three times and centrifuged at 8000 × *g* for 1 min, after which the supernatant was analyzed by HPLC anion exchange. Column: Partisil PXS 10/25 SAX (Whatman). Mobile phase: 0.1 M NH_3 solution, 0.2 M NaCl, 20% (v/v) 1-propanol, 10% CH_3CN, adjusted to pH 5.3 with CH_3COOH.

Chemotaxis. The small population assay was used [24], which involved deposition on hydrophobic agar of two small, closely spaced, droplets, one containing cells and the other a test substance. The response was considered positive, when more than twice as many cells were pressed against the edge closest to the attracting drop as against the opposite side. The threshold concentration for activity of a compound was the concentration at which 50% of the responding populations was found positive. In order to minimize variability due to differences in developmental state, folic acid was included in each experiment as a reference compound. All obtained threshold values were then related to that of folic acid. The cells were starved for 30–60 min on the agar at 20°C prior to the assay.

Standardization. In order to compare the I_{50} values (concentration of analog yielding 50% inhibition) that were obtained for the enzymes and binding sites to the R_{50} values (concentration of analog yielding 50% response) for chemotaxis the following standardization was used [25]

$$\delta\Delta G = -RT \ln \frac{I_{50}\ (\text{folic acid})}{I_{50}\ (\text{derivative})}$$

I_{50} may be replaced by R_{50}. Thus, the affinity or activity of a derivative relative to folic acid is transformed into the free enthalpy scale. A 10-fold increase in the I_{50} of an analog corresponds to a $\delta\Delta G$ of 5.2 kJ · mol^{-1} if determined at 0°C, but 5.6 kJ · mol^{-1} at 20°C. Generally H-bonds have an energy content of 10–25 kJ · mol^{-1}, while ionic interactions amount to 25 or more kJ · mol^{-1}.

Results

Folate binding sites

The inhibition of radioligand binding to the four main folate binding classes by folate derivatives is shown in Fig. 1. The A^H and A^L sites were measured with 67 nM as well as 670 nM 2-deamino[^{3}H]folic acid. The contribution of binding to the other types of sites was less than 1%. At 67 nM 2-deamino [^{3}H]folic acid 20% of the bound radioligand will be contributed by A^L sites, at 670 nM 2-deamino [^{3}H]folic acid 63%. This may be calculated from the binding parameters [17]. The remaining part reflects the A^H sites. If a derivative should have clearly different affinities for the two A sites, the competition curve as shown in Fig. 1a should be biphasic, or at least non-parallel. In Fig. 1a no sign of biphasic inhibition is observed for any folate derivative. Thus, each compound has a characteristic affinity for A sites, irrespective of whether binding occurs at A^H or A^L.

It is essential to demonstrate that all compounds tested compete for identical sites when the binding data are used to describe the interaction between folic acid and the binding site (cf. Discussion). Therefore, inhibition was monitored at two radioligand concentrations in order to determine whether the observed inhibition was competitive or not. For all compounds, the concentration resulting in 50% inhibition (I_{50}), was increased to the same extent by raising the 2-deamino[^{3}H]folic acid concentration from 67 nM to 670 nM. As a consequence, the $\delta\Delta G$ values of all derivatives are

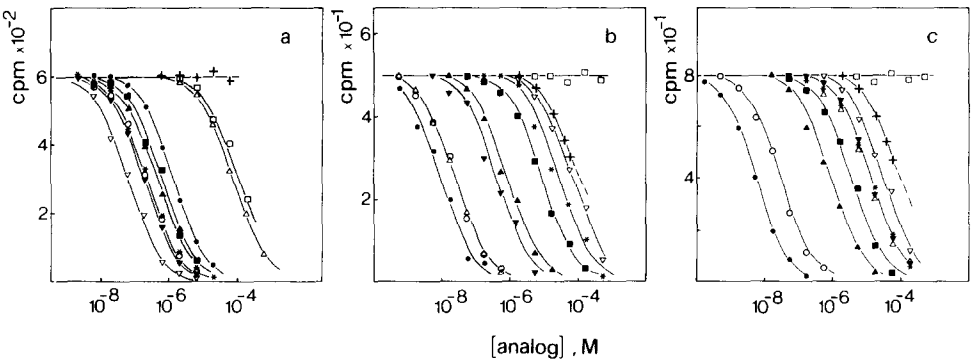

Fig. 1. (a) Competition of folate derivatives for binding of 67 nM 2-deamino[^{3}H]folic acid at the A sites. (b) Competition for the B sites at 10 nM [^{3}H]folic acid. (c) Competition for the C^S sites at 1.85 nM N_{10}-methyl[^{3}H]folic acid. Dashed lines indicate the expected inhibition at concentrations above the solubility limit of pterin in order to obtain I_{50} values. The data points were calculated from duplicate determination in three independent experiments. Symbols: (○) FA: (▼) DAFA; (▽) AP; (*) MTX; (■) 5-DFA; (▲) THF; (◇) 9-MFA; (+) pterin; (●) 10-MFA; (◆) 10-DFA; (△) 2′,5′-DFA; (⬮) PA; (⊕) D-10-MFA; (□) pABGA. For abbreviations see Table I.

TABLE I

RELATIVE ENERGIES FOR CELL SURFACE PROTEINS AND CHEMOTACTIC ACTIVITIES OF FOLATE DERIVATIVES

The binding increments ($\delta\Delta G$) are relative to folic acid (FA) which is set at 0. The absolute data for folic acid: A sites, half-maximal binding ($K_{0.5}$) = 140 nM; B sites, K_d = 17 nM; C^S sites, K_d = 15 nM; folate deaminase, K_m = 120 nM: folate C_9-N_{10} cleaving enzyme, K_m = 63 nM; chemotaxis, R_{50} = 100 nM. A large variation in this R_{50} value was observed in ten independent experiments, probably caused by differences in the developmental state of the cells. All R_{50} values were in the range 30–300 nM ($\delta\Delta G \pm 3$ kJ·mol^{-1}). By relating the activities of the analogs to that of folic acid, the standard deviations in those data (four experiments) were lowered to 2 kJ·mol^{-1}. The standard deviations in data of binding sites and enzymes generally are 1 kJ·mol^{-1} or less (three experiments). Abbreviations: DAFA, 2-deaminofolic acid (2-deamino-2-hydroxyfolic acid); FA, folic acid; MTX, methotrexate (4-amino-N_{10}-methylfolic acid); AP, aminopterin (4-aminofolic acid); PA, pteroic acid; THF, tetrahydrofolic acid; 5-DFA, 5-deazafolic acid; 5,8-DDFA, 5,8-dideazafolic acid; 9-MFA, 9-methylfolic acid; 10-MFA, N_{10}-methylfolic acid; D-10-MFA, D-10-methylfolic acid (N_{10}-methylpteroyl-D-glutamic acid); 10-DFA, 10-deazafolic acid; 2′,5′-DFA, 2′,5′-diazafolic acid; pABGA, *p*-aminobenzoylglutamic acid.

Compound	Binding or activity increment $\delta\Delta G$ (kJ·mol^{-1})					
	Binding sites			Folate deaminase	Folate C_9-N_{10} cleaving enzyme	Chemotaxis
	$A^H + A^L$	B	C^S			
FA	0	0	0	0	0	0
DAFA	−0.5	6.0	14	5.2	2.2	7.0 [a]
AP	−3.1	18	16	9.6	18	18
MTX	0.0	16	14	9.6	≥ 24	14
5-DFA	2.1	13	11	3.6	17	14
5,8-DDFA	0.5	13	13	n.d.	n.d.	n.d.
THF	1.5	7.7	7.7	−1.6	5.4	8.3
9-MFA	3.6	13	12	3.6	13	15
Pterin	≥ 17	18	18	≥ 19	≥ 22	5.6
10-MFA	4.4	−2.4	−3.8	0.9	13	−2.9
10-DFA	7.3	5.1	6.8	5.2	14	8.3
2′,5′-DFA	13	0.1	13	n.d.	n.d.	2.6
PA	11	11	10	14	9.6	11
D-10-MFA	12	5.0	4.2	3.4	n.d.	5.1
pABGA	14	≥ 24	≥ 21	≥ 19	23	≥ 28

[a] The R_{50} value of 2-deaminofolic acid (DAFA) was calculated assuming a maximal response level of 50% of that of folic acid.

independent of the radioligand concentrations, indicating that the inhibition is competitive. Table I presents the mean $\delta\Delta G$ values of the determination at high and low radioligand concentrations.

The inhibition of [^{3}H]folic acid binding to the B sites by the analogs is shown in Fig. 1b. In this case [^{3}H]folic acid was used at only one concentration (10 nM), since the ratio between the binding level and the blank value was rather unfavorable: 50 ± 6 cmp binding and 24 ± 3 cpm blank, respectively. Increasing as well as lowering of the radioligand concentration yielded even worse ratios and thus more difficulties in measuring the binding level accurately. However, it has already been shown that three derivatives (folic acid, N_{10}-methylfolic acid and 2-deaminofolic acid) apparently compete for identical B sites [17]. It was therefore assumed that also inhibition of [^{3}H]folic acid binding to the B sites by other analogs would be competitive.

The inhibition of N_{10}-methyl[^{3}H]folic acid binding to C^S sites was studied at 1.85 nM and 18.5 nM radioligand (Fig. 1c). As with the A sites the calculated $\delta\Delta G$ values (Table I) were independent of the radioligand concentration, suggesting competitive inhibition.

Folate degrading enzymes

Folate degrading enzymes are found in the extracellular medium as well as on the cell surface. The enzymatic activity in the medium was removed by washing. Secretion of enzyme activity during incubation of radioactive folic acid with the washed cells could not be prevented. However, as reported before [18], extracellular folate deaminase(s) also accept pterin as substrate, while the membrane-bound deaminase is specific for folate. It is thus possible to inhibit newly secreted extracellular activity with a high concentration of pterin (67 μM). The extracellular folate C_9-N_{10} cleaving enzyme could not be specifically inhibited. Fortunately, the rate of secretion of this enzyme at 0°C was low, yielding only 10% of the membrane-bound activity at the end of the incubation (30 min).

In Table I the inhibition constants of the folate derivatives for these two cell surface enzymes are shown. These K_i values are determined by the association and dissociation rate constants (k_1 and k_{-1}, respectively) as well as the rate of conversion of substrate into product (k_2) according to $(k_{-1} + k_2)/k_1$. Therefore, detailed interpretation of the affinity in terms of interaction between substrate and active site is not allowed. This is in contrast with the binding sites, where only the binding constants k_{-1} and k_1 determine the affinity of a compound and detailed interpretation is possible.

Chemotaxis

Dose-response curves of the chemotactic activity of the folate derivatives are shown in Fig. 2a. The concentrations inducing half maximal response (R_{50}) were related to that of folic acid and transformed into the free enthalpy scale (Table I). One of the compounds, 2-deaminofolic acid, showed a nonparallel dose response curve, since the apparent maximum response was always lower than that of folic acid. This compound was never observed to induce a positive response in more than about 50% of the populations (Fig. 2b). Thus, in the range of 10 μM–10 mM a constant submaximal chemotactic response was obtained. if 0.3 μM or 1 μM folic acid (inducing 75–90% response) was mixed with 2-deaminofolic acid concentrations in the range mentioned above, the response to this mixture was approx. 60%, which is significantly lower than to folic acid alone. Apparently, 2-deaminofolic acid acts as a partial antagonist of folic acid. If the R_{50} value for 2-deaminofolic acid is determined assuming that a 50% response is the maximum, a $\delta\Delta G$ value of 7.0 kJ · mol^{-1} is ob-

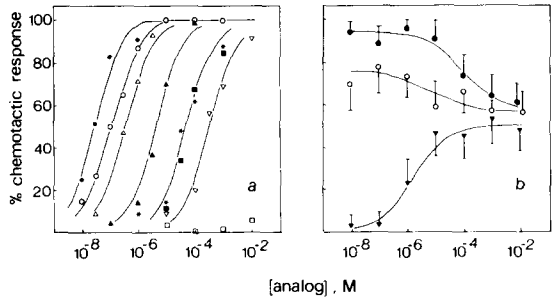

Fig. 2. (a) Dose response curves of the chemotactic activity of folate derivatives. The symbols are as in Fig. 1. (b) Chemotactic response to 2-deaminofolic acid (▼) and to mixtures of various concentrations of 2-deaminofolic acid with 0.3 μM folic acid (○) or 1 μM folic acid (●). The data are calculated from four independent experiments.

tained (Table I). It is not clear whether this approach is valid; therefore, the chemotactic activity of 2-deaminofolic acid is omitted from the calculation of correlation coefficients between binding and chemotactic data.

Relationships between the specificity of binding sites, enzymes and chemotaxis

In theory, folate binding might be caused by membrane-bound enzymes, provided that the affinity for the substrate and the number of sites per cell are sufficiently high for detection of binding above the blank value (non-specific binding). The present data on the ligand specificity of the binding sites and the substrate specificity of the enzymes, however, suggest these sites to be nonidentical. As shown in Table II not more than a slight correlation was observed between the specificity profiles and in case of a correlation the slope was clearly different from 1. As argued before, the A^H and A^L sites should not differ much in their ligand specificity. As is obvious from Table II, also B and C^S sites are similar. Only the $\delta\Delta G$ values of 2-deaminofolic acid and 2',5'-diazafolic acid for these binding sites are significantly different.

When the chemotactic sensitivity of the cells for the analogs is compared to the affinity of these compounds for the binding sites, the data for 2-deaminofolic acid should be excluded, since the chemotactic response to this derivative is a result of agonistic as well as antagonistic effects. In other words, the antagonistic property of 2-deaminofolic acid may have affected the determination of the R_{50} value. Also pterin should be excluded, since this compound is thought to elicit a chemotactic response via a pterin specific receptor. If the remaining compounds are compared with respect to their chemotactic activity and their binding to the A, B of C^S sites (Fig. 3), it is clear that a high correlation ($p < 0.01\%$) exists with the B sites, less with the C^S sites but no correlation with the A sites (Table II). In addition, a weak correlation was observed between chemotactic activity and affinity for both membrane-bound folate degrading enzymes.

These data are suggestive of a role of the B sites in the transduction of a folate signal into the chemotactic response. However, as a consequence of the similarity between B and C^S sites involvement of the latter in the chemotactic response should not be excluded.

Uptake of folates

Since the B sites (and possibly C^S sites) appear

TABLE II

CORRELATION BETWEEN SPECIFICITY OF BINDING SITES, ENZYMES AND THE CHEMOTACTIC RESPONSE

The values shown are the slopes from linear-regression analysis of the $\delta\Delta G$ values when the parameters at the top of the table are plotted versus the parameters at the left. Correlation coefficients (r) were calculated from $\delta\Delta G$ values of Table I. The chemotactic $\delta\Delta G$ values of 2-deaminofolic acid and pterin were omitted from calculations, as were the $\delta\Delta G$ values preceeded by $\geq$. Significance was determined using a r_{test} table.A high significance in combination with a slope of 1 should indicate similarity (or a relationship) between the tested parameters.

		Slope				
		Binding sites		Folate deaminase	Folate C_9-N_{10} cleaving enzyme	Chemotaxis
		B	C^S			
Binding sites	A^H and A^L	−0.50 [a]	−0.20 [a]	0.15 [a]	0.61 [a]	−0.46 [a]
	B	–	0.71 [c]	0.44 [b]	0.53 [b]	0.95 [d]
	C^S	–	–	0.45 [b]	0.27 [a]	0.92 [c]
Folate deaminase		–	–	–	0.46 [a]	0.80 [b]
Folate C_9-N_{10} cleaving enzyme		–	–	–	–	0.70 [b]

[a] Significance of less than 80%.
[b] Significance between 80% and 99%.
[c] Significance between 99% and 99.9%.
[d] Significance of 99.9% or more.

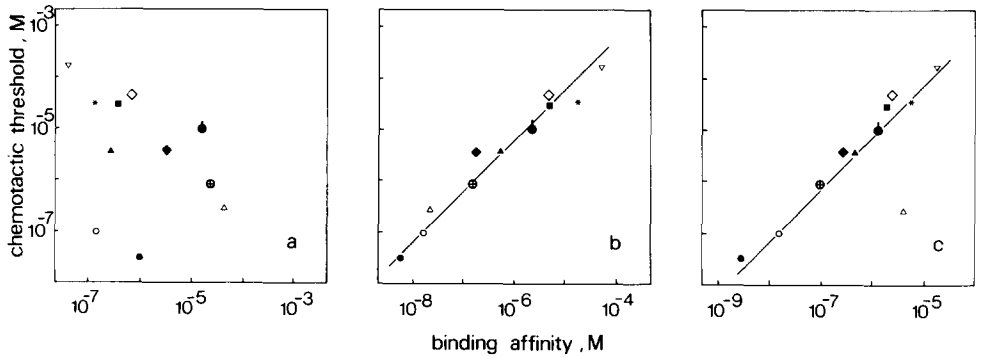

Fig. 3. Comparison of binding data of folate analogs to their chemotactic activity. (a) A sites. (b) B sites. (c) C^S sites. The symbols are as in Fig. 1.

to be linked to the chemotactic response, the question arises what may be the physiological function of the A sites. One possibility is that these sites are carrier proteins for the uptake of folate from the medium. Therefore, the uptake of two potent ligands for the A sites (folic acid and methotrexate) was studied. [^{3}H]Folic acid was completely deaminated within 1 min incubation, and the C_9-N_{10} cleaving enzyme further degraded the formed 2-deamino[^{3}H]folic acid within 10 min. Despite these degradations the rate of uptake of radioactive material by the cells was constant during 30 min (Fig. 4a). Therefore, the internalized radioactive compound(s) were analyzed by HPLC. After correction for non-specific binding of labeled compounds to the cell surface, a compound which behaved chromatographically like lumazine-6-carboxaldehyde, was found inside the cells Fig. 4b). The presence of pterin-6-carboxaldehyde could not be excluded as judged by this chromatography system, but the fact that folic acid and pterin were completely deaminated within 1 min of incubation makes the constant uptake of pterin-6-carboxaldehyde during 30 min unlikely. The rate of uptake of this degradation product of folic acid was 2.8 fmol/10^7 cells per min.

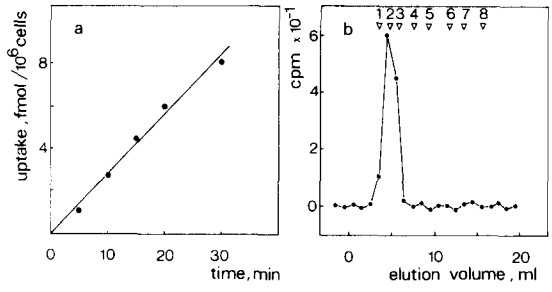

Fig. 4. (a) Uptake of [^{3}H]folic acid at 20°C. As stated in the text, the internalized compound may be [^{3}H]lumazin-6-carboxaldehyde, which has a lower specific activity than the undegraded [^{3}H]folic acid. It was assumed that all of the tritium on C_7 was retained during degradation, but that C_9 lost 50% of the original amount (one of the two hydrogen atoms is removed). Thus 41.5% of the tritium content of [^{3}H]folic acid should be present. (b) Analysis by HPLC anion exchange of the radioactive compound taken up by t_0 cells during 30 min of starvation at 20°C. Reference compounds: 1, pterin-6-CH_2OH; 2, pterin-6-CHO and lumazin-6-CHO; 3, *p*-aminobenzoic acid; 4, lumazin-6-COOH; 5, pterin-6-COOH; 6, 2-deaminofolic acid; 7, folic acid; 8, *p*-aminobenzoylglutamic acid.

Methotrexate was resistant to deamination and cleavage of the C_9-N_{10} bond during 30 min of incubation. This compound was not found inside the cells in detectable levels after 30 min (< 0.05 fmol/10^7 cells per min).

Apparently, folic acid and methotrexate are not internalized by *D. discoideum* cells. A mechanism for the uptake of lumazine-6-carboxaldehyde may be present, however, with only a low capacity. The absence of a folate specific uptake system is not unlikely, since the activity of folate degrading enzymes is too high to allow efficient uptake of folic acid.

Discussion

A general property of membrane-bound receptors is the presence of multiple binding classes of

which at least some are interconvertable by addition of ligand [30], guanine nucleotides [30,31] drugs [32] or compounds affecting membrane fluidity [33]. In the accompanying report multiple folate binding sites on the cell surface of *D. discsoideum* cells have been demonstrated [17]. These types were divided into three classes. The A sites are the most abundant class to which approx. 99% of the sites belong. Two interconvertible types with different affinity constants were detected (A^H, A^L). B sites were observed to release the ligand more slowly and are assumed to be formed from another binding class after the addition of ligand. About 0.3% of the total number of sites belongs to this class. The C sites exist in two forms, of which the faster exchanging form is present before and shortly after addition of ligand. Formation of the slower type is induced by ligand binding. About 1% of the sites contribute to the C class.

Recently, two distinct types of the oligopeptide receptor of leukocytes were proposed to transduce a receptor type-specific response [33]. Furthermore it is generally accepted that the distinct forms of the adrenergic receptor reflect different states of receptor-effector (e.g. GTP binding protein) coupling [30] and possibly the process of 'induced fit' [34].

Using a selected series of folate derivatives we have attempted to demonstrate the following properties of the folate binding sites. (i) The binding types are not identical to the active sites of two membrane-bound folate degrading enzymes *. (ii) The three main binding classes (A, B and C) each recognize folic acid in a different way, confirming that these sites indeed are nonidentical. (However, interconversion between these types is not excluded). (iii) The chemotactic response seems to be transduced by the B sites. (iv) Carrier mediated folate uptake is not evident. Apparently, none of the observed sites corresponds to a carrier protein.

An omission in the present work is the ligand specificity of the C^F sites. As argued before [17] it is extremely difficult to obtain data for the C^F type without significant interference by C^S. Since C^F and C^S are interconvertable and show a similar specificity for four analogs (folic acid, N_{10}-methylfolic acid, 2-deaminofolic acid and methotrexate) [17], not much merit was expected from a detailed study of the specificity of C^F.

Interactions between folic acid and its binding sites

Using the binding affinity of the folate analogs for a site an image of the essential interactions between folic acid and its site may be derived. A decrease in binding energy ($\delta\Delta G$) as a result of modification in the structure of folic acid is thought to reflect the weaker interaction between the binding site and the modified functional group. Obviously, derivatives should be selected in each of which the modification is limited to one atom or functional group as much as possible. This approach has been commonly used for the mapping of cAMP binding sites in a variety of proteins [26–28].

The possible interactions between folic acid and the A sites are shown in Fig. 5a. None of the modifications in the pterin moiety resulted in a significantly decreased binding energy. Thus, the following possible interactions may be excluded. Substitution of 2-NH_2 by a keto function (2-deaminofolic acid) prevents the H-bond donation of the amino group and, by tautomeric coupling, also prevents N_1 to be a H-bond acceptor. When the C_4-keto function is replaced by amino (aminopterin), the H-bond accepting property of the C_4-keto groups is inhibited as well as the H-bond donation by N_3 (tautomerism). Substitution of N_5 or N_8 by carbon (5-deazafolic acid; 5,8-dideazafolic acid) prohibits H-bond formation with this atom. In tetrahydrofolic acid the H-bond acceptance of N_5 and N_8 is absent, furthermore the pyrazine ring it not aromatic (not flat). Apparently this ring is not bound by e.g. aromatic stacking interactions. Introduction of a methyl group on C_9 (9-methylfolic acid) results in a minor decrease in binding energy, suggesting only a slight steric hindrance at this position. Summarizing, the pterin moiety is not recognized by H-bonds and

* Binding of folic acid to the catalytic sites of membrane-bound folate degrading enzymes might well escape detection. The deaminase activity is approx. 20 pmol/10^7 cells per min; assuming that the molecular activity of this deaminase is 10^3 molecules per enzyme-molecule per min (which is a low estimate), the number of sites per cell is approx. 1000. With a K_m of 10^{-7} M, binding to these sites will be only 0.6% of the binding to the A sites. The folate cleaving enzyme has a lower activity of 0.2 pmol/10^7 cells per min and may therefore be present in still lower numbers per cell.

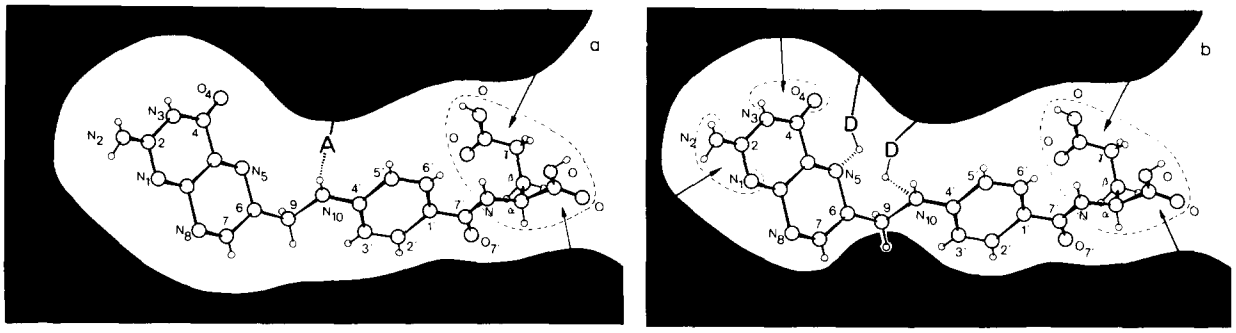

Fig. 5. (a) Possible interactions between folic acid and the binding sites of the A type. The arrows indicate interactions which could not be addressed to one atom of folic acid, but ony to a certain region. (b) Interactions between folic acid and the site of the B or C^S type. Folic acid is drawn in a conformation reported for folic acid dihydrate crystals [38]. D: H-bond donator, A: H-bond acceptor. The backbone of the binding protein (black region) is drawn arbitrarily, except in the vicinity of C_9 in the B or C^S site where little free space is available.

stacking interactions with its pyrazine ring. However, removal of the pterin moiety of the folic acid molecule (*p*-aminobenzoylglutamic acid) yields a significantly decreased binding energy (14 kJ · mol^{-1}). Therefore, the pterin moiety should interact with the binding site, e.g. by stacking of an aromatic amino acid of the binding protein with the pyrimidine ring or by other hydrophobic interactions. Changes in the *p*-aminobenzoylglutamic acid part of the molecule always resulted in weaker binding. Methylation of N_{10} (N_{10}-methylfolic acid) as well as replacement of N_{10} by carbon (10-deazafolic acid) caused a decrease in binding energy of 4–7 kJ · mol^{-1}, probably reflecting the donation of a weak H-bond by N_{10}-H. However, steric effects of the N_{10}-methyl group or of the bond angles and lengths in 10-deazafolic acid might also have caused the reduced affinities. Introduction of two nitrogen atoms in the benzene ring (2′,5′-diazafolic acid) severely reduced the binding affinity. Particularly the polarization of the benzene moiety in stacking interactions is changed by this modification. Removal of the L-glutamic acid residue (pteroic acid) caused a significant drop in binding energy (11 kJ · mol^{-1}), while replacement by D-glutamic acid (D-N_{10}-methylfolic acid) yielded a decrease of 7.6 kJ · mol^{-1} (i.e. the difference between D-N_{10}-methylfolic acid and N_{10}-methylfolic acid). Obviously, the glutamate is recognized in a stereospecific manner, indicating that at least two groups of the amino acid interact with the binding site (whatever these interactions may be). Pterin did not bind in detectable amounts to the A site, again indicating the essential role of the *p*-aminobenzoylglutamic acid moiety.

The possible structure of the B sites is presented in Fig. 5b. The most striking difference with the A sites is the highly specific recognition of the pterin moiety. H-bonds are thought to occur with 2 NH_2 and/or N_1 (weak), N_3 and/or C_4-keto (strong), and N_5. The space at position C_9 in the binding site is very limited since 9-methylfolic acid showed a remarkably low affinity. N_8 is not expected to be involved in H-bond formation, since tetrahydrofolic acid (in which N_5, N_8 and the aromaticity are changed) and 5,8-dideazafolic acid are ligands not worse than 5-deazafolic acid, in which only N_5 is modified. A complicating factor is the chemical lability of tetrahydrofolic acid. Dithiothreitol was added to prevent oxidation to 7,8-dihydrofolate and eventually to folic acid. However, degradation of tetrahydrofolic acid might still have caused overestimation of the real affinity. Also in contrast to the A sites N_{10}-methylfolic acid is bound with higher affinity than folic acid, probably as a result of a higher electron density at N_{10} and thus a stronger H-bond accepting capacity. The low affinity of 10-deazafolic acid is consistent with this model. The binding of the benzene moiety is not affected by introduction of two nitrogen atoms in the ring (2′,5′-diazafolic acid), either indicating that no interaction with this site occurs or that positive and negative effects on the affinity are balanced. The stereospecific recognition of the

glutamic acid residue is similar to that of the A sites. Pterin is bound by the B sites with a reduced energy of 18 $kJ \cdot mol^{-1}$. Since the loss in binding energy due to the absence of glutamate (pteroic acid, 11 $kJ \cdot mol^{-1}$) and N_{10} (10-deazafolic acid, 5.1 $kJ \cdot mol^{-1}$) may almost account for the absence of the whole *p*-aminobenzoylglutamic acid moiety (pterin, 18 $kJ \cdot mol^{-1}$), no further interactions between the site and the *p*-aminobenzoylglutamic acid moiety are expected. This is consistent with the unimpaired affinity of 2′,5′-diazafolic acid.

As stated before, the C^S sites show a ligand specificity similar to that of the B sites. Only the affinity of 2-deaminofolic acid as well as 2′,5′-diazafolic acid for the C^S sites was significantly lower. This may reflect a stronger H-bond with 2-NH_2 and/or N_1 and probably the altered polarization of the benzene moiety resulting in weaker stacking interactions with the site. In this case, the summed binding increments of pteroic acid, 10-deazafolic acid and 2′,5′-diazafolic acid (29.8 $kJ \cdot mol^{-1}$) do not equal the increment of pterin (18 $kJ \cdot mol^{-1}$), implying that either molecular modifications are not independent or that one of the binding increments does not reflect an essential interaction between folic acid and the site. The first possibility may be true since 2′,5′-diazafolic acid has a lower electron density on N_{10} than folic acid, and thus a weaker H-bond accepting potency [29]. Also in 10-deazafolic acid this H-bond is affected. The energy of this bond (6.8 $kJ \cdot mol^{-1}$) however, cannot fully explain the discrepancy. Therefore, the second alternative may contribute too. Possibly, the benzene moiety of folic acid is only weakly bound by a stacking interaction; introduction of the nitrogen atoms changes the polarization and thus may not only eliminate the original weak affinity, but also create a repulsion between the analog and the C^S site. Besides this possible difference in the vicinity of the benzene moiety, the proposed structure of the C^S site is identical to that of the B site (Fig. 5b).

The B sites being involved in the chemotactic response raises the question of the role of the A and C type. A well studied cellular response to cAMP and folic acid is the synthesis and secretion of cAMP [4,35]. The potency of several folates and pterins to elicit cAMP secretion in *D. discoideum* has been studied recently [20]. It was observed that at 10^{-4} M 5,8-dideazafolic acid, 5,8-dideazaisofolic acid and 7,8-dihydrofolic acid are at least half as effective as folic acid (10^{-4} M folic acid is a non-saturating concentration). Apparently, N_5, N_8 and the position of N_{10} are not essential for the induction of the cAMP response. On the other hand, introduction of a formyl group on N_5 of tetrahydrofolic acid (folinic acid) or of four glutamic acid residues (folate tetraglutamate) rendered the compound much less active. The latter two compounds or comparable analogs were not tested in the present study, but a conclusion may be drawn from the high activity of the first three. The N_5 position is essential for binding to the B and C^S sites, whereas for the A sites modification of N_5 as well as N_8 is allowed. In addition the positions of C_9 and N_{10} are unimportant for the cAMP response. For the B and C sites, the environment of C_9 does not even tolerate introduction of a methyl group. In contrast, this is unimportant for the A sites. It would thus be unlikely that either the B or the C sites transduce a folate signal to a cAMP response. Moreover, the A sites may be involved in this process. This is confirmed by the finding that methotrexate is a potent and reversible antagonist of folate elicited cAMP secretion. At 10^{-4} M this analog blocked the response to 10^{-4} M folic acid. Again, this is suggestive of the A sites for which methotrexate is an excellent ligand. If the antagonistic potency resides in the 4-NH_2 group, aminopterin should also be an antagonist, which is in agreement with its low activity.

Pterin (derivatives) may also elicit cAMP secretion as well as chemotaxis, but several lines of evidence suggest this signal to be transduced by specific pterin receptors. (i) Pterin induces a chemotactic response at concentrations which do not result in binding to the folate binding sites, (ii) 6-aminopterin antagonizes the chemotactic response towards pterin but not to folic acid in *D. discoideum* [15]. (iii) Methotrexate antagonizes the cAMP response to folic acid but not to pterin [36].

The conclusions that the A and B sites are linked to the cAMP secretion response and to the chemotactic response, respectively, leaves the C sites without physiological role. Recent evidence, however, suggests that during incubation with

ligand C^S sites are converted to B sites and that upon removal of ligand B sites change into C^F sites. Thus, B and C sites seem to be distinct states of one receptor (manuscript in preparation). This conclusion is supported by the similar ligand specificity of these sites as shown in this study.

With 2-deaminofolic acid being a partial (ant)agonist for chemotaxis the existence of antagonists in all three classes of stimuli (cAMP, folic acid and pterin) has been demonstrated in *D. discoideum*. In a recent study a physiological mechanism for this partial (ant)agonism was proposed [37]. However, further research of the properties of these antagonists at the level of the binding site is required for the understanding of the molecular mechanism. Also earlier reports presented data concerning the chemotactic activity of 2-deaminofolic acid. In each case neither a response nor antagonism was observed [10,12–15]. The present work shows that 2-deaminofolic acid is a partial agonist. The low response level might easily have escaped detection in previous studies. The same is true for the antagonism, since in our hands the response to folic acid was only inhibited from 75–90% of the maximal response to 50–60%.

Formerly, in folate binding studies by other investigators, the A sites were detected of which several properties were compared to the results of studies of chemotaxis [10,11]. Presently, it is shown that the A sites were abusively supposed to be identical to the chemotactic receptors. Therefore, we are now engaged in a study of the time-course of each of the binding sites during the development of *D. discoideum* cells in relation to the chemotactic sensitivity and the cAMP response. Furthermore, it is being investigated whether the cells secrete folic acid or a derivative during their development.

Together, these approaches should yield information about the physiological role of folate(s) during the development of *D. discoideum*.

Acknowledgement

Peter Van Haastert is gratefully acknowledged for his valuable contribution to this work in many discussions.

References

1 Pan, P., Hall, E.M. and Bonner, J.T. (1972) Nature New Biol. 237, 181–182
2 Pan, P., Hall, E.M. and Bonner, J.T. (1975) J. Baceriol. 122, 185–191
3 Wurster, B. and Schubiger, K. (1977) J. Cell Sci. 27, 105–114
4 Wurster, B., Schubiger, K. and Brachet, P. (1979) Cell Differentiation 8, 235–242
5 Bernstein, R.L., Rossier, C., Van Driel, R., Brunner, M. and Gerisch, G. (1981) Cell Differentiation 10, 79–86
6 Tatischeff, I. and Klein, R. (1982) FEBS Lett. 138, 265–269
7 Gerisch, G., Blank, G., Schweiger, M., Fuchs, D., Hausen, A., Reibnegger, G. and Wachter, H. (1982) in Biochemical and Clinical Aspects of Pteridines (Wachter, H., Curtius, C. and Pfleiderer, W., eds.) pp. 253–256, De Gruyter, Berlin and New York
8 Van Haastert, P.J.M., De Wit, R.J.W., Grijpma, Y. and Konijn, T.M. (1982) Proc. Natl. Acad. Sci. USA 79, 6270–6274
9 De Wit, R.J.W. and Konijn, T.M. (1983) Cell Differentiation 12, 205–210
10 Wurster, B. and Butz, U. (1980) Eur. J. Biochem. 109, 613–618
11 Nandini-Kishore, S.G. and Frazier, W.A. (1981) Proc. Natl. Acad. Sci. USA 78, 7299–7303
12 Van Haastert, P.J.M., Pasveer, F.J., Van der Meer, R.C., Van der Heijden, P.R., Van Walsum, H. and Konijn, T.M. (1982) J. Bacteriol. 152, 232–238
13 Kakebeeke, P.I.J. (1980) Thesis, University of Leiden, The Netherlands
14 Pan, P. and Wurster, B. (1978) J. Bacteriol. 136, 955–959
15 Van Haastert, P.J.M., De Wit, R.J.W. and Konijn, T.M. (1982) Exp. Cell Res. 140, 453–456
16 De Wit, R.J.W. (1982) FEBS Lett. 150, 445–448
17 De Wit, R.J.W. and Van Haastert, P.J.M. (1985) Biochim. Biophys. Acta 814, 199–213
18 Wurster, B., Bek, F. and Butz, U. (1981) J. Bacteriol. 148, 183–192
19 De Wit, R.J.W., Van der Velden, R.J. and Konijn, T.M. (1983) J. Bacteriol. 154, 859–863
20 Devreotes, P.N. (1983) Dev. Biol. 95, 154–162
21 Konijn, T.M. and Raper, K.B. (1961) Dev. Biol. 3, 725–756
22 Seeger, D.R., Cosulich, D.B., Smith, J.M., Jr. and Hultquist, M.E. (1949) J. Am. Chem. Soc. 71, 1753–1758
23 Cosulich, D.B. and Smith, J.M., Jr. (1948) J. Am. Chem. Soc. 70, 1922–1926
24 Konijn, T.M. (1970) Experientia 26, 367–369
25 Hoppe, J., Freist, W., Marutzky, R. and Shaltiel, S. (1978) Eur. J. Biochem. 90, 427–432
26 Jastorff, B., Hoppe, J., Mato, J.M. and Konijn, T.M. (1979) in Molecular Mechanisms of Biological Recognition (Balaban, M., ed.), pp. 107–117, Elsevier Biomedical Press, Amsterdam and New York
27 De Wit, R.J.W., Hoppe, J., Stec, W.J., Baraniak, J. and Jastorff, B. (1982) Eur. J. Biochem. 122, 95–99
28 Van Haastert, P.J.M. and Kien, E. (1984) J. Biol. Chem. 258, 9636–9642

29 Nakahara, Y., Sekikawa, I. and Kakimoto, S. (1975) J. Heterocycl. Chem. 12, 1073–1074
30 Lefkowitz, R.J., Stadel, J.M. and Caron, M.G. (1983) Annu. Rev. Biochem. 52, 159–186
31 Snyderman, R., Pike, M.C., Edge, S. and Lane, B. (1984) J. Cell Biol. 98, 444–448
32 Van Haastert, P.J.M. and De Wit, R.J.W. (1984) J. Biol. Chem. 259, 13321–13328
33 Snyderman, R. (1983) Am. J. Med. 75, 10–18
34 Neufeld, G., Steiner, G., Korner, M. and Schramm, M. (1983) Proc. Natl. Acad. Sci. USA 80, 6441–6445
35 Devreotes, P.N., Derstine, P.L. and Steck, T.L (1979) J. Cell Biol. 80, 291–299
36 Devreotes, P.N. (1982) in The development of *Dictyostelium discoideum* (Loomis, W.F., ed.), pp. 117–168, Academic Press, New York and London
37 Van Haastert, P.J.M. (1983) J. Biol. Chem. 258, 9643–9648
38 Mastropaolo, D., Camerman, A. and Camerman, N. (1980) Science 210, 334–336