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[^{3}H]Methotrexate as a ligand for the folate receptor of *Dictyostelium discoideum*

(folate deaminase/chemotaxis/chemokinesis/developmental regulation/receptor coupling)

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ABSTRACT **Studies of the folate chemotactic receptor of vegetative *Dictyostelium discoideum* cells have been hampered by the presence of the degradative enzyme folate deaminase. The diaminopterin compounds aminopterin and methotrexate (MTX) are chemoattractants but are not attacked by the deaminase. [3′, 5′, 7, 9-^{3}H]methotrexate ([^{3}H]MTX) is a nondegraded radioligand for the folate receptor. Binding to the receptor is rapid, reaching steady state in less than one min, and reversible in less than 15 s by an excess of unlabeled MTX. A single class of binding sites is found with a K_d of 2×10^{-8} M, which correlates well with the concentration dependence of chemotaxis. Folate, aminopterin, and MTX all compete for [^{3}H]MTX binding, whereas pterin, *p*-aminobenzoate, and nucleotides do not. Analysis of the receptor during differentiation indicates a decrease in site number by a factor of 3 with no change in affinity during the first 7 hr. During this time, the directional response (chemotaxis) to MTX and folate is lost, but a nondirectional stimulation of motility rate (chemokinesis) is retained. The response to cyclic AMP displays reciprocal behavior, first appearing as a chemokinetic response and then as a chemotactic response.**

Vegetative *Dictyostelium discoideum* cells are chemotactic to folic acid and pterins (1). Because bacteria liberate these compounds, this chemotactic system has been considered to be a food-seeking device (2); however, growing evidence suggests that folate may have a role in early development (3, 4). Whatever its role, it should be of interest to compare the folate chemotactic system with the cyclic AMP (cAMP) system of aggregating cells. In particular, it seems likely that receptors for both chemoattractants share a common transducer mechanism by which the extracellular signal is coupled to the response of directional motility. A reflection of this general mechanism may be the finding that stimulation of cells in the appropriate stage of development with either folate or cAMP results in a transient increase in intracellular cyclic GMP (cGMP) levels (5, 6).

Whereas both the cAMP receptor (7, 8) and degradative enzyme (9) 3′:5′-cyclic-nucleotide phosphodiesterase (cNPDEase, EC 3.1.4.17) of differentiating cells have been well characterized, less is known about the analogous components of the folate system. In this vegetative chemotactic system, the degradative enzyme is a pterin deaminase (EC 3.5.4.11) (10,11) with a cellular distribution similar to that of the cNPDEase (7, 9–11). To perform radioligand binding studies with the folate receptor, it is necessary to either inhibit the deaminase or to use a ligand that is not attacked by the enzyme. We and others (11, 12) have not found a suitable inhibitor of the deaminase. However, we have found that both methotrexate (MTX) and aminopterin are not degraded by the folate deaminase but are chemoattractants of vegetative *D. discoideum* cells. Commercially available [^{3}H]MTX is a high-affinity ligand for the folate receptor that offers many advantages over [^{3}H]folate (12) in radioligand binding studies, the results of which are presented here.

MATERIALS AND METHODS

All chemicals were purchased from Sigma with the exception of [3′, 5′, 7, 9-^{3}H]methotrexate ([^{3}H]MTX; 14–18 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) and [^{3}H]folate (45–50 Ci/mmol), which were purchased from Amersham. All batches of tritiated and unlabeled ligands were tested for purity by high-performance liquid chromatography (HPLC). The wild-type strain of *D. discoideum*, NC-4, was grown on *Escherichia coli* B/r in suspension (13), harvested at a density of $4–6 \times 10^6$ cells per ml, and washed free of bacteria. Cells were then either allowed to differentiate (at 10^7 cells per ml) in suspension (14) in 17 mM sodium/potassium phosphate (pH 6.4) or were suspended in the same buffer for assay. [^{3}H]MTX binding was assayed by either a rapid filtration method (8) or a centrifugation assay (15). For both methods, cells were suspended at the indicated density (see Figs. 1–5) in 17 mM sodium/potassium phosphate (pH 6.4) at 4°C. [^{3}H]MTX was added at 50–70 nM, and a 0.5-ml aliquot periodically was taken for assay. Filtration was on 10-place Hoeffer manifolds with 0.8-μm polycarbonate filters (Bio-Rad), followed by a 1.0-ml wash with cold buffer. For the centrifugation assay, cells were incubated with [^{3}H]MTX for 1.5 min and the 0.5-ml aliquots were layered above 1.0 ml of 12% (wt/vol) polyethylene glycol 6000 in a 1.5-ml polypropylene tube (Bio-Rad) and centrifuged for 30 s in a Beckman Microfuge B at 4°C. The filters or the resuspended cell pellets were measured for radioactivity in 10 ml of 3a70 scintillation fluid (Research Products, Elk Grove, IL). Nonspecific binding was determined in the presence of 50 μM MTX. Chemotaxis assays were performed and photographed as described (14). The gradient of MTX formed in the 2% (wt/vol) agar well plates was determined by placing 1 μM MTX (containing 200,000 dpm of [^{3}H]MTX) in the center well of the plates. After 3 hr of diffusion at room temperature, several 3-mm strips of agar were cut from three plates, cut into 1-mm slices with a gel slicer, and measured for radioactivity. The logarithm of MTX concentration is a linear function of distance from the center well (from 1 to 11 mm).

RESULTS

Studies of folate deaminase (to be published elsewhere) indicated that both methotrexate and aminopterin (2, 4,-diaminopterin compounds) are competitive inhibitors of the enzyme and that neither compound is a substrate. This suggested that [^{3}H]MTX might be a useful radioligand for the folate receptor.

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Abbreviations: MTX, methotrexate; HPLC, high-performance liquid chromatography; cNPDEase, cyclic nucleotide phosphodiesterase; cAMP, cyclic AMP; cGMP, cyclic GMP.

Thus, we tested the potency of MTX and aminopterin in comparison with that of folate in chemotaxis assays (14). At all initial concentrations (0.1–100 μM) of the three compounds tested, MTX and aminopterin were found to be equipotent with folate as chemoattractants of vegetative *D. discoideum* cells (see Fig. 5 for MTX assay). The response to a gradient of folate or MTX was also tested in the presence of a constant higher concentration of the other compound. Folate and MTX were each able to inhibit the chemotactic response of cells to a gradient of either compound (not shown), suggesting that chemotaxis to both folate and MTX is mediated by the same receptor. Based on these data, binding studies with [^{3}H]MTX were undertaken to characterize the folate receptor and to confirm that folate and MTX were, in fact, ligands for the same receptor.

Kinetics of Binding. Fig. 1 shows that [^{3}H]MTX bound to intact, vegetative NC-4 cells rapidly with the maximal level being reached by 1 min. This level of binding remained relatively constant for 30 min, indicating the expected lack of degradation of the [^{3}H]MTX by cells. At 30 min, unlabeled MTX was added to the incubation mixture to a final concentration of 50 μM to induce the dissociation of reversibly bound [^{3}H]MTX. Within 15 s about 90% of the bound [^{3}H]MTX was dissociated (Fig. 1), indicating that this amount of the labeled ligand was reversibly bound to cell surface receptors. The remaining 10% of the bound MTX represented nonspecific binding, primarily to the filters. A centrifugation assay was developed, and kinetic data identical to that in Fig. 1 were obtained with this assay as well. Binding to the same batch of cells was tested with the filtration and the centrifugation assays and found to give the same levels of specific binding (not shown).

Equilibrium Binding Properties. The concentration dependence of [^{3}H]MTX binding was investigated with both assays, and data from a centrifugation experiment is shown because this method gives lower nonspecific binding values (<0.1% of input cpm). In this experiment (shown as a Scatchard plot in Fig. 2), a single class of binding sites was evident with an apparent dissociation constant, K_d, of 2×10^{-8} M (MTX). The number of binding sites per cell was 20,000. The number of sites per cell varied with the growth state of the NC-4 cells, being as high as 40,000 in early subculture passage (1–3) cells and as low as 5000 in later passage cells, even though all assays were performed on vegetative cells harvested at the same density. There was also some variability in the apparent K_d derived from Scatchard analysis of binding data with different batches of cells, the range being from 2×10^{-8} M (Fig. 2) to 1×10^{-7}M (MTX). These concentrations correlated well with the concentrations of MTX (or folate) to which the cells were chemotactically responsive. We directly measured the MTX concentration gradient in the agar of the plates used for the chemotaxis assay at the position of chemotaxing cells. A response was detectable at 5 nM, and an optimal response was seen in the range of 10–100 nM local MTX. When the local concentration was about 100 nM, the difference in concentration across a cell's diameter (ca. 10 μm) was only 0.5 nM, or a gradient of 0.5% of the absolute local concentration.

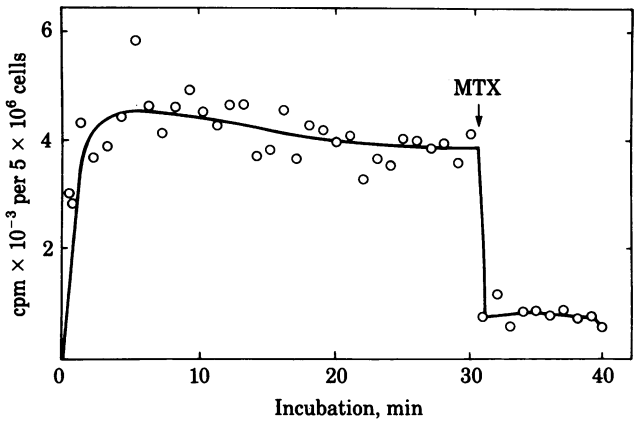

FIG. 1. Time course of [^{3}H]MTX binding to vegetative NC-4 cells. To a suspension of 10^7 cells per ml was added 50 nM [^{3}H]MTX. Aliquots (0.5 ml) were filtered at the indicated times. At 30 min (arrow), unlabled MTX was added to a final concentration of 50 μM, and sampling was continued. The data points are averages of seven experiments. The level of binding between 0 and 30 min is 4127 ± 580 cpm, and the nonspecific (irreversible) binding level (after 30 min) is 842 ± 171 cpm.

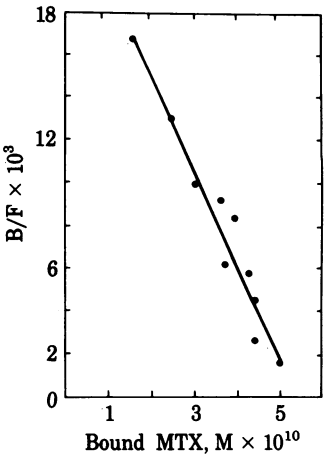

FIG. 2. Scatchard plot of MTX binding data. Cells (1.7×10^7/ml) were incubated with [^{3}H]MTX and various concentrations of MTX, and the bound and free forms were separated by centrifugation. Points were obtained in duplicate. The experiment was performed eight times. Due to variability with different batches of cells, a single experiment is shown. Maximum [^{3}H]MTX binding in this experiment was 2190 cpm, and nonspecific binding was 180 cpm. B and F, bound and free ligand, respectively.

Specificity of Binding. The ability of a variety of compounds to compete for the binding of [^{3}H]MTX was tested. Fig. 3 shows that aminopterin was 1/5th as effective as MTX in competing for the binding of [^{3}H]MTX. Folate also competed for binding but at a 10-fold higher concentration than aminopterin, probably due to rapid degradation by the folate deaminase, which was present on the cell surface of the washed cells used for the binding assays. Pan *et al.* (1, 2) reported that pterin is a chemoattractant and that the pterin moiety of folate could account for the chemotactic potency of folate. We have found pterin to be a chemoattractant but with about 1/10th the potency of folate. Pterin did not compete for [^{3}H]MTX binding at concentrations as high as 0.5 mM (Fig. 3). Due to insolubility, we could not assay higher pterin concentrations, but it is unlikely that pterin is more than 1% as effective as folate at competing for [^{3}H]MTX binding. In addition to the compounds shown in Fig. 3, Table 1 shows that *p*-aminobenzoate, another component of the folate structure, did not compete for [^{3}H]MTX binding, nor did pterin-6-carboxylate, a photooxidative degradation product of folate (16). cAMP showed no inhibition of MTX binding, whereas cGMP showed only weak inhibition. The triphosphates ATP and GTP showed neither inhibition nor enhancement of binding. The data in Fig. 3 indicate that MTX and folate bind to the same receptor and, thus, [^{3}H]MTX is, in fact, a probe for the folate receptor of *D. discoideum*.

Developmental Regulation of Binding and Chemotaxis. It has been observed that aggregation-competent cells respond chemotactically to cAMP but not to folate (2). Using the chemotaxis assay and [^{3}H]MTX binding as a folate receptor assay, we

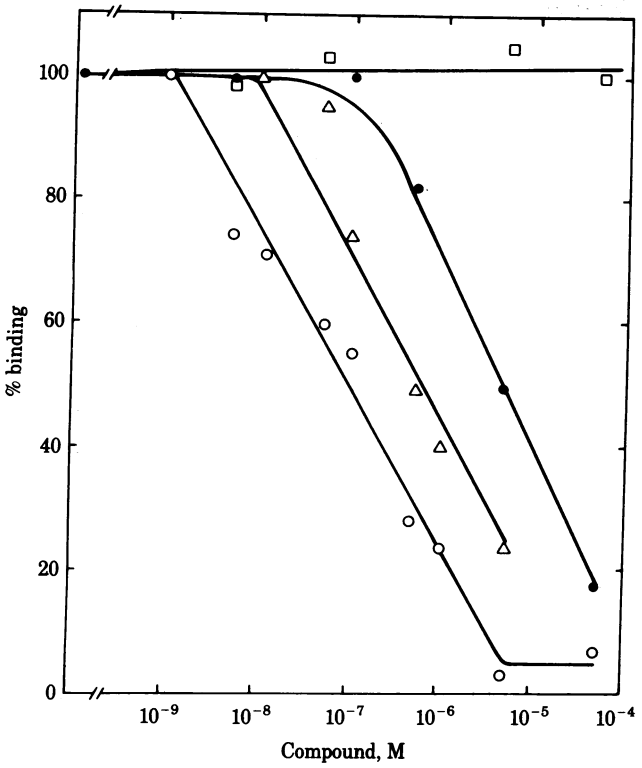

FIG. 3. Specificity of [^{3}H]MTX binding. Cells (2×10^7/ml) were incubated with 70 nM [^{3}H]MTX and the indicated compounds at various concentrations. Bound and free forms were separated by centrifugation. ○, MTX; △, aminopterin; ●, folate; and □, pterin. 100% binding was 8270 cpm, and nonspecific binding was 280 cpm.

examined the time course of the transition from folate chemotactic competence in the vegetative state to cAMP chemotactic competence in differentiated cells. Fig. 4*A* shows a series of binding curves obtained as a function of MTX concentration for cells allowed to differentiate in suspension for 0 (vegetative), 3.5, 7 (aggregation competent), and 9 hr. The K_d derived from these data indicate that the receptor affinity was unchanged (from $K_d = 3 \times 10^{-8}$ M) during differentiation, but that the apparent number of binding sites decreased to about 1/3rd, from 39,000 to 12,000 per cell during the first 9 hr of development (Fig. 4*B*).

Cells from these binding experiments were tested also for their motility response to MTX and cAMP. Gradients of MTX and cAMP were established in agar well plates with an initial concentration of 10 μM because the gradient formed by this concentration will yield a chemotactic response, yet the concentration is high enough relative to receptor K_d that chemokinesis can be detected as well. As expected, 0-hr (vegetative) cells responded with directional movement to MTX but not at all to cAMP (Fig. 5). By 3 hr of differentiation, the cells still displayed a chemotactic response to MTX, but it appeared somewhat less well oriented than at 0 hr. At 3 hr, cAMP caused a slight stimulation of random or radial motility (chemokinesis). In 7-hr cells, the cAMP response was much greater and became directional, whereas the MTX response, though still large in magnitude, became radial or chemokinetic rather than oriented. By 9 hr of differentiation, the radial MTX response was somewhat attenuated, whereas the cAMP response remained directional. Cells spotted on control agar showed little or no increase in random motility during differentiation (not shown) and appeared similar to the cAMP response at 0 hr (Fig. 5). Under our developmental conditions, cells acquired the maximal number of cAMP receptors at 7 hr (not shown). Thus, although the number of MTX receptors on the cells' plasma membranes decreased by 7 hr, a significant number remained. Interestingly, these appeared to become "uncoupled" from oriented movement (chemotaxis) yet remained able to stimulate random motility rate (chemokinesis).

DISCUSSION

These data indicate that [^{3}H]MTX is a useful radioligand for the folate receptor of *D. discoideum*. Unlike folate and pterin derivatives (10, 11), MTX is not degraded by the folate (or pterin) deaminase and, thus, is stable during long-term exposure to cells. In early experiments using [^{3}H]folate as a ligand, we found that, at the low concentrations used in binding assays, folate is extremely unstable to light and oxygen. MTX has proven to be much more stable under assay conditions, such that ascorbate

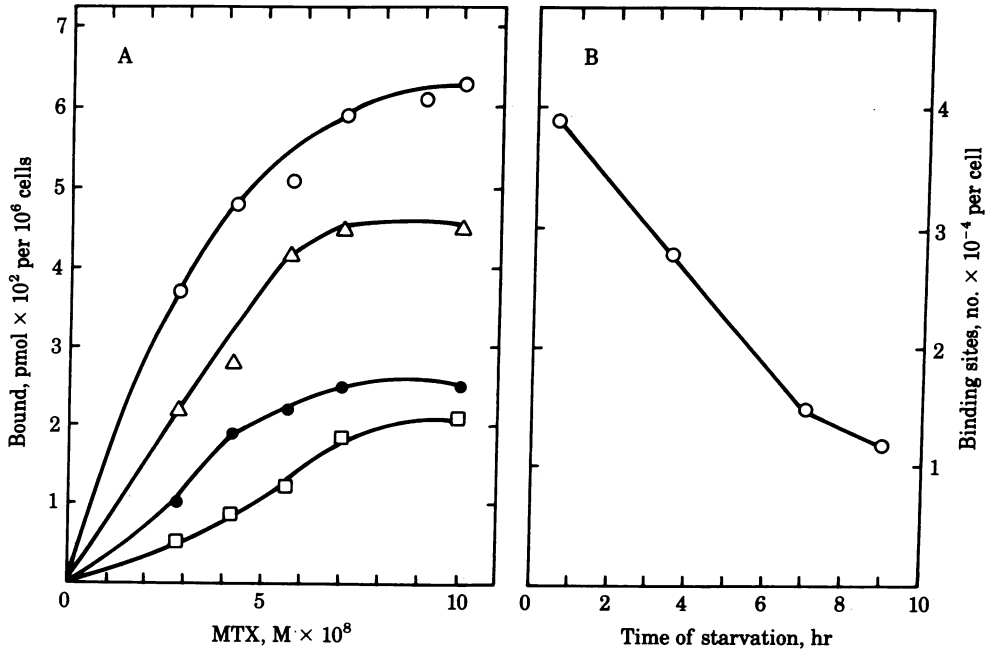

FIG. 4. Developmental time course of [^{3}H]MTX binding. NC-4 cells were differentiated for the indicated times and tested for binding as a function of [^{3}H]MTX concentration at 1.7×10^7 cells per ml. (*A*) Binding data for cells differentiated for 0 hr (○), 3.5 hr (△), 7 hr (●), and 9 hr (□). (*B*) Specific binding at saturation, expressed as sites per cell as determined from *A*.

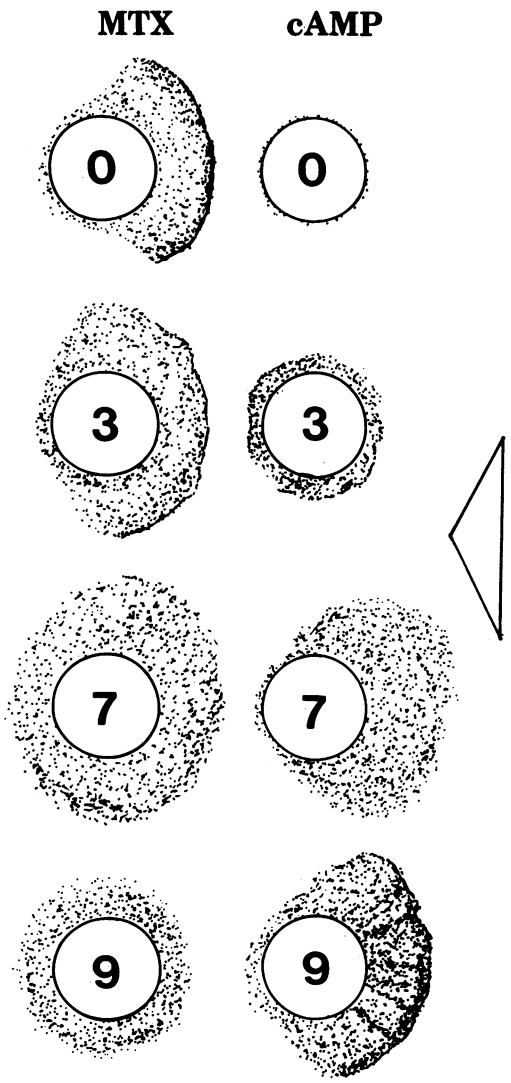

FIG. 5. Response of cells to cAMP and MTX during differentiation. Aliquots of cells from the experiment in Fig. 4 were concentrated to 3×10^8 cells per ml and spotted on 2% buffered agar plates poured in 10 × 35 mm Falcon plastic Petri dishes. At the center of each plate, a 5-mm-diameter well was filled with either MTX or cAMP at 10 μM. Each circle represents the initial boundary of a drop of cells and the number of hours of differentiation before spotting the cells. The arrowhead indicates the direction of the gradient in the agar. After 3 hr, all cell drops were photographed with a Polaroid camera on an Olympus stereo dissecting microscope, and the figure was drawn from representative photographs. (The agar on which the cells rest makes it difficult to obtain high-contrast photos for reproduction.) Identical results were obtained in two independent experiments.

is not required to prevent oxidation, and assays do not have to be performed in the dark. In addition, [^{3}H]folate gives higher and more variable nonspecific binding. Thus, MTX appears to be the ligand of choice for further characterization of the folate receptor.

The binding of [^{3}H]MTX to vegetative cells is rapid and reversible (Fig. 1). Like the binding of cAMP to its receptor, binding is complete in less than 1 min and [^{3}H]MTX dissociates in less than 15 s in the presence of a large excess of unlabeled ligand (7, 8). Binding to the folate receptor on both vegetative and differentiated cells reaches steady state and does not appear to oscillate. In eight experiments with different batches of cells, the apparent K_d ranged from 2×10^{-8} M to 1×10^{-7} M. Although receptor number is quite variable with the batch of cells used, there is no apparent correlation between receptor number and the observed affinity. The reason for this variation is not known. In general, axenic cells (A3) have many fewer receptors per cell compared with the NC-4 cells grown on bacteria used in this study. Because it is very difficult to remove all bacteria from harvested cells, we checked the *E. coli* B/r cells for specific [^{3}H]MTX binding at 1000 times the density at which they contaminate harvested cells and found none. Thus, the specific binding of MTX is a property of the NC-4 cells. The range of values for the K_d of MTX binding is similar to the concentrations of MTX, aminopterin, and folate that are found to stimulate chemotaxis.

The specificity studies of [^{3}H]MTX binding (Fig. 3 and Table 1) show that MTX, aminopterin, and folate all interact with the same receptor. In addition, a constant high concentration of MTX inhibits the directional response of cells to a gradient of folate, providing further evidence for a common receptor for both molecules. The apparent lower affinity of folate for the receptor relative to MTX and aminopterin (Fig. 3) may be a reflection of its degradation by the membrane-bound folate deaminase. This enzyme also deaminates pterin (11) with a K_m comparable to that for folate (unpublished data), thus, the lack of inhibition of MTX binding by pterin indicates that none of the observed MTX binding is associated with the membrane-bound deaminase. We and others (1, 2) have observed that pterin is a chemoattractant of vegetative cells. Thus, it was somewhat surprising that pterin was not a competitor of MTX binding. This observation along with the data of Pan *et al.* (1) on the chemotactic potency of pterin analogs suggests that yet another receptor may exist that specifically recognizes the pterin structure, in particular the 2-amino, 4-oxy region of the ring. Such a receptor might be expected to bind the pterin moiety of folate. However, Wurster and Butz (12), using [^{3}H]folate as a receptor probe, found that pterin did not compete for binding. The K_d reported for [^{3}H]folate binding was 2×10^{-7} M (12), up to 10-fold higher than that found for [^{3}H]MTX (Fig. 2), in agreement with the lower potency of folate as a competitor of [^{3}H]MTX binding (Fig. 3).

The fact that both MTX and aminopterin are potent chemoattractants in the absence of degradation implies that degradation of the attractant is not necessary for detection of the gradient by cells in the assay we employ (Fig. 5). In this assay (14), a static gradient is established; thus, it is probable that cells sense the gradient by comparing concentrations at their "front" and "back." If so, they are extremely sensitive, being able to detect a difference in concentration of about 0.5% across their

Table 1. Effect of folate-related compounds and nucleotides on [^{3}H]MTX binding

Unlabeled compound	[^{3}H]MTX bound, %
None	100
MTX	7
Aminopterin	23
Folate	49
p-Aminobenzoate	105
Pterin	111
Pterin-6-carboxylate	103
cAMP	100
cGMP	79
ATP	101
GTP	106

Specific binding of [^{3}H]MTX (70 nM) was determined by the centrifugation assay with each compound at a concentration of 5 μM. Binding is expressed as percentage of the specific binding in the absence of competitors.

diameter. This compares with the ability of leukocytes to detect a spatial gradient of about 1% (17). In the normal situation, degradation of bacterially secreted folate and related compounds (2) may serve to increase the sensitivity of the chemotactic system. However, our findings do rule out the obligatory participation of signal degradation as part of the mechanism by which a gradient is sensed.

The number of folate receptors decreases during the first 7–9 hr of differentiation, with no change in apparent receptor affinity (Fig. 4). During this period, the response to folate or MTX changes from a directional, chemotactic response to a random or radial chemokinetic response in which motility rate is greatly stimulated compared to controls (Fig. 5). This change from a tactic to a kinetic response is not simply due to a decrease in receptor number because in many experiments cells with as few as 5000 receptors per cell in the vegetative state were seen to respond chemotactically to MTX and folate (not shown). Thus, this shift in the motility response to folate or MTX may represent a change in the coupling of the receptor to the contractile apparatus of the cell. During the same period of development in which this shift in coupling of the folate receptor occurs, the cAMP receptor appears on the cell surface (7, 8) and becomes coupled to the chemotactic response. It is interesting to note that, in time course experiments such as that in Fig. 5, when a detectable response to cAMP first appears at about 3–4 hr of differentiation, it is not a chemotactic but a chemokinetic response. Varnum and Soll (18) have noted the rapid shift from a chemotactic response to cAMP to a chemotactic response to folate after refeeding differentiated cells to "erase" the differentiated state (19). They observed that after erasure, the cAMP response is one of chemokinesis (18). Thus, it appears that both the folate and the cAMP receptors can exist on the plasma membrane in two states, one coupled to a directional response and one in which motility rate, but not direction, is affected. Wurster and Butz (12) have noted that by 6 hr of differentiation, cells fail to respond to folate with alterations in their light-scattering properties in suspension. Thus, they suggested that the receptor is completely disconnected from signal processing. Our data and that of Varnum and Soll (18) indicate that the folate receptor on differentiating cells remains coupled to the motility apparatus in such a way that rate of movement is stimulated, but the vectorial nature of the movement is lost. Furthermore, it is tempting to speculate (*i*) that both the folate and the cAMP receptors utilize the same coupling apparatus and (*ii*) that as the cAMP receptor appears in the membrane as differentiation proceeds, it displaces the folate receptor from these hypothetical sites that transduce receptor occupancy into directional movement. A significant number of folate receptors remain in the membrane, and these, perhaps because of a lower affinity for the transducer sites or a change in their distribution on the cell surface, can only stimulate motility rate. It should be informative to compare the intracellular responses to stimulation of the folate and cAMP receptors when they are each coupled to chemotaxis and to chemokinesis.

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