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IRREVERSIBLE INHIBITORS OF METHOTREXATE TRANSPORT IN L1210 CELLS

CHARACTERISTICS OF INHIBITION BY AN *N*-HYDROXYSUCCINIMIDE ESTER OF METHOTREXATE

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Methotrexate, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and *N*-hydroxysuccinimide react to form an activated ester of methotrexate which is a potent irreversible inhibitor of methotrexate transport in L1210 cells. In cells treated with the reagent at 37°C, inhibition was rapid ($t_{1/2} < 1$ min), optimal at pH 6.8, half-maximal at an inhibitor concentration of 20 nM, and complete at high levels of the reagent. Specificity was indicated by the fact that excess methotrexate added during the pretreatment step protected the transport system against inactivation. Irreversible inhibition was also observed in cells exposed to the reagent at 4°C. Inactivation in this case was qualitatively similar to the corresponding process at 37°C; it appeared rapidly, was half-maximal at 20 nM, and could be prevented by the addition of high concentrations of the substrate. The extent of the inhibition, however, reached a maximum of only 75%, even in samples containing excess or multiple additions of reagent. The latter findings suggest that at 4°C the transport protein exists in two forms, one (75% of the total) containing binding sites which are accessible to the active ester, and the other (25% of the total) with inaccessible sites. The identity of these sites is suggested to be transport proteins which have outward and inward orientations, respectively.

Introduction

L1210 mouse leukemia cells contain a single, high-affinity transport system for the internalization of methotrexate and other folate compounds [1–5]. A principal component of this system is a membrane-associated binding protein which has been identified and quantitated by low-temperature binding studies [6]. An unusual property of this protein is that its substrate binding site can accommodate a variety of organic and inorganic anions, in addition to folate compounds [2,6–10]. The basis for this broad binding specificity has been suggested to reflect its ability to mediate the exchange of extracellular folate compounds for intracellular anions of diverse structure [2,8–12]. The high affinity of this carrier protein for large organic anions has also been employed to develop irreversible inhibitors of the transport system that are directed against the substrate binding site. Effective compounds include 8-azidoadenosine 5′-monophosphate [7], 4,4′-diisothiocyanostilbene-2,2′-disulfonate [9] and carbodiimide-activated folate compounds [13]. In the present study, evidence is presented which shows that methotrexate

Abbreviations: EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

transport in L1210 cells can also be irreversibly inhibited by an *N*-hydroxysuccinimide ester of methotrexate, and that this inactivation occurs much more rapidly and at much lower concentrations than had been observed previously with other inhibitors.

Materials and Methods

Chemicals. [3′,5′,9-^{3}H]Methotrexate (0.25 Ci/mmol) was obtained from Amersham, purified by thin-layer chromatography [11], and diluted with unlabeled methotrexate to a specific activity of 150000 dpm/nmol. Other chemicals were obtained as follows: methotrexate, *N*-hydroxysuccinimide, and EDC, Sigma; Hepes, CalBiochem. Me_2SO was purchased from J.T. Baker and stored in the presence of activated molecular sieve (4 Å, 8–12 mesh) (Sigma) to remove residual water.

Cells. L1210 mouse leukemia cells were grown in RPMI 1640 medium containing 4% fetal bovine serum (Flow Laboratories) and 100 units each of penicillin and streptomycin (Irvine Scientific). Culture flasks (2 liter), which contained medium (1 liter) and an inoculum of 10^8 cells, were capped and then incubated (with gentle shaking) for 48 h at 37°C. Cells were harvested by centrifugation at $1000 \times g$ (5 min, 4°C), washed with the desired buffer and suspended to a density of $3 \cdot 10^7$/ml. The buffers employed were: Hepes-buffered saline (20 mM Hepes, 140 mM NaCl, 10 mM KCl and 2 mM $MgCl_2$, pH 7.4 with NaOH) and Mg-Hepes-sucrose (20 mM Hepes and 225 mM sucrose, adjusted to pH 7.4 (unless otherwise indicated) with MgO). The usefulness and validity of employing non-physiological buffer systems to analyze methotrexate transport in L1210 cells has been discussed elsewhere [8,10,14].

Preparation of methotrexate esters. Carbodiimide-activated methotrexate were prepared as described previously [13] by dissolving 4 μmol methotrexate (free acid) and 40 μmol EDC in 2.0 ml anhydrous Me_2SO and incubating the mixture for 1 h at 23°C. Esters of methotrexate were prepared by the same procedure, except that 40 μmol of either *N*-hydroxysuccinimide, *p*-nitrophenol, dinitrophenol or *p*-nitroaniline were also included in the reaction mixture. Concentrations of carbodiimide-activated methotrexate and the corresponding *N*-hydroxysuccinimide ester were determined from the extinction coefficient of unreacted methotrexate of 18.9 at 302 nm and pH 7.

Treatment of cells with the N-hydroxysuccinimide ester of methotrexate. Aliquots (0.01 ml) of the reagent (diluted to the desired concentration with Me_2SO containing 2 mM each of EDC and *N*-hydroxysuccinimide) were added to washed cell suspensions (0.99 ml) and incubated under the desired conditions of time and temperature. The cells were then recovered by centrifugation at $1000 \times g$ (5 min, 4°C) and washed with 4 ml of ice-cold Mg-Hepes-sucrose buffer (pH 7.4). Treatment with carbodiimide-activated methotrexate or with other reagents was accomplished by a similar procedure, except that the EDC and *N*-hydroxysuccinimide were omitted from the diluting solvent.

Transport measurements. Control or treated cells (in duplicate) were suspended in Mg-Hepes-sucrose buffer (pH 7.4), containing 2.0 μM [^{3}H]methotrexate and incubated (with shaking) for 5 min at 37°C. The cells were then chilled to 4°C, diluted with 7 ml ice-cold 0.15 M NaCl, recovered by centrifugation at $1000 \times g$ (5 min, 4°C), resuspended in 0.5 ml of water, and analyzed for radioactivity. Uptake at 4°C served as the control, and the results are reported in pmol of methotrexate transported per min per mg protein. Protein was measured by the biuret reaction [15] using bovine serum albumin as the standard.

Measurement of [^{3}H]methotrexate uptake at 4°C. The time-dependence for [^{3}H]methotrexate uptake at 4°C was measured in triplicate as described previously [6]. Control samples contained excess (200 μM) unlabeled methotrexate, which was added prior to the [^{3}H]methotrexate. Results are expressed in pmol per mg protein.

Results

Evaluation of active esters of methotrexate as irreversible inhibitors of methotrexate transport

Previous studies have shown that methotrexate and other folate compounds react with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) to form activated compounds which specifically and irreversibly inhibit methotrexate transport in L1210 cells [13]. The reactive species have been

tentatively identified as α,γ-anhydrides [13,16], and inactivation was shown to occur at reagent concentrations near the K_t for half-maximal transport of the non-activated compounds. Due to the potential uses of covalent-labeling agents in studying various aspects of the transport process, the possibility was pursued that perhaps other activated derivatives of methotrexate could be synthesized and that a specific agent might be found which exhibited increased reactivity towards this system. A screening procedure was subsequently devised in which various nucleophiles (with the potential of forming an activated bond) were allowed to react with EDC-activated methotrexate and the resulting mixtures were tested for the ability to irreversibly inhibit methotrexate transport (see Materials and Methods for the pretreatment conditions). It was observed (Table I) that transport was unaffected in cells exposed to each of the nucleophiles alone and that reagent mixtures containing EDC-activated methotrexate and either *p*-nitrophenol, 2,4-dinitrophenol or *p*-nitroaniline were no more effective than EDC-activated methotrexate tested alone (Table I). The inhibition observed with each of the latter mixtures was approx. 40%. In contrast, when *N*-hydroxysuccinimide was employed as the nucleophile, an active ester of methotrexate was formed which, under the same conditions, inhibited methotrexate transport by 95%. Examination of the conditions for synthesis of this ester (Fig. 1) revealed that inactivation was optimal in a reaction mixture containing methotrexate and *N*-hydroxysuccinimide in a molar ratio of 1 : 10 (and incubated for 1 h at 23°C), and that half-maximal conversion of EDC-activated methotrexate to the *N*-hydroxysuccinimide ester occurred when this ratio was reduced to 1.0 : 0.7.

TABLE I

IRREVERSIBLE INHIBITION OF METHOTREXATE TRANSPORT BY VARIOUS AGENTS

Activated methotrexate (4 μmol), added compounds (40 μmol) or combinations of these agents in the same amounts were dissolved in 2.0 ml Me_2SO, incubated for 20 min at 23°C, diluted 20-fold in Me_2SO, and then added in 10 μl aliquots to 0.99 ml cells (in Mg-Hepes-sucrose buffer, pH 7.4). After 5 min at 37°C, the cells were collected by centrifugation, washed with buffer, and analyzed for methotrexate transport activity. The rate of transport in untreated control samples was 12.0 pmol/min per mg protein.

Pretreatment additions	Inhibition (%)
EDC-activated methotrexate	42
p-Nitrophenol	2
2,4-Dinitrophenol	1
p-Nitroaniline	4
N-Hydroxysuccinimide	0
EDC-activated methotrexate + *p*-nitrophenol	42
EDC-activated methotrexate + 2,4-dinitrophenol	41
EDC-activated methotrexate + *p*-nitroaniline	40
EDC-activated methotrexate + *N*-hydroxysuccinimide	95

Inhibition by ester treatment at 37°C

When L1210 cells (suspended in Mg-Hepes-sucrose buffer, pH 7.4) were pretreated with varying amounts of the *N*-hydroxysuccinimide ester for 5 min at 37°C, inhibition of methotrexate trans-

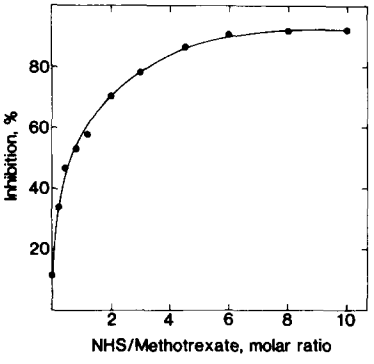

Fig. 1. Effect of *N*-hydroxysuccinimide (NHS) concentration on the synthesis of active ester. Reaction mixtures were prepared by combining methotrexate (4 μmol), EDC (40 μmol) and the indicated amounts of *N*-hydroxysuccinimide in 2.0 ml Me_2SO and incubating the solutions for 1 h at 23°C. Samples were then diluted 20-fold into Me_2SO (containing 2 mM EDC) and added in 10-μl amounts to 0.99 ml of cells. After 5 min at 37°C, the cells were recovered by centrifugation, washed with buffer, and analyzed for methotrexate transport activity. The uninhibited transport rate for methotrexate (12.9 pmol/min per mg protein) was determined in cells (0.99 ml) that had been exposed only to Me_2SO (10 μl) for 5 min at 37°C.

port was found to be half-maximal at an inhibitor concentration of 32 nM and to exceed 95% at a higher level (500 nM) of the reagent (Fig. 2). Moreover, the reaction appeared to be specific for this transport system, since substantial protection against inactivation was afforded by the inclusion of methotrexate (200 μM) during the pretreatment step (Fig. 2). Exposure of cells over the same range of reagent concentrations (i.e., 10–500 nM) but for either a shorter (1 min) or a longer (10 min) time interval, in a different buffer (Hepes-buffered saline), or at 25°C had no effect on the inhibition profile (data not shown). Inhibition was complete (over 99%) when the concentration of ester was increased to 10 μM, and this inhibition was not reversed in cells that had been exposed subsequently (5 min, 37°C) to mercaptoethanol (20 mM). When the pH was varied (Fig. 3), the concentration of methotrexate ester required for half-maximal inhibition reached a minimal value of 20 nM in cells treated with the reagent at pH 6.8. The effectiveness of the reagent remained relatively high at pH values as low as 6.2, but it diminished rapidly as the pH was raised above 7.4. A similar variation with pH was observed for the K_t value for half-maximal transport of methotrexate (Fig. 3).

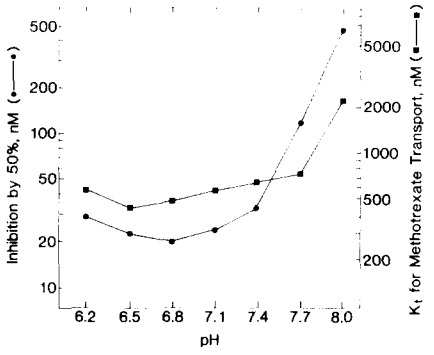

Fig. 3. Comparison of the pH dependence for inhibition of methotrexate transport by active ester treatment and the K_t for half-maximal transport. Half-maximal inhibition of transport by the *N*-hydroxysuccinimide ester of methotrexate (at 37°C) was determined in buffer of the indicated pH from a plot of inhibition as a function of reagent concentration (see Fig. 2). K_t values for methotrexate transport in untreated cells were determined as described in Materials and Methods. Buffer composition: 20 mM Hepes plus 225 mM sucrose, adjusted to the indicated pH with MgO.

Inhibition by ester treatment at 4°C

The *N*-hydroxysuccinimide ester also inactivated methotrexate transport in cells treated with the reagent at 4°C (Fig. 4). Inhibition (at pH 6.8) was half-maximal at a reagent concentration

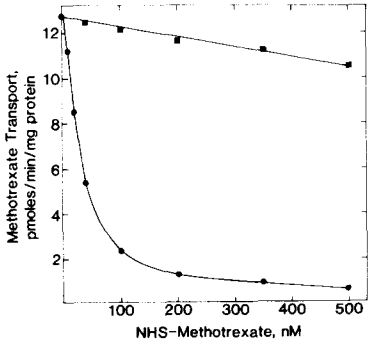

Fig. 2. Inhibition of methotrexate transport as a function of *N*-hydroxysuccinimide ester concentration in cells treated with reagent at 37°C. ● Cells treated with reagent only; ■, cells treated with reagent plus 200 μM methotrexate. Pretreatment time, 5 min; pretreatment buffer, Mg-Hepes-sucrose, pH 7.4. NHS-methotrexate, *N*-hydroxysuccinimide ester of methotrexate.

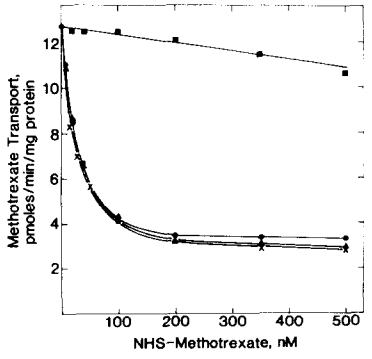

Fig. 4. Inhibition of methotrexate transport as a function of *N*-hydroxysuccinimide ester concentration in cells treated with reagent at 4°C. ●, Cells treated (pH 6.8) with reagent only for 5 min; ▲, cells treated (pH 6.8) with reagent only for 30 min; ×, cells treated (pH 7.4) with reagent only for 5 min; ■, cells treated (pH 6.8) with reagent plus 200 μM methotrexate for 5 min. All treatments at 4°C. Pretreatment buffer, Mg-Hepes-sucrose. NHS-methotrexate, *N*-hydroxysuccinimide ester of methotrexate.

(20 nM) which was comparable to that achieved at 37°C (see Fig. 3), and protection against inactivation was obtained by the inclusion of methotrexate during the treatment step. Unlike 37°C, however, the observed inhibition at 4°C was incomplete, reaching a maximum of only 75% at high levels of the inhibitor. Inactivation also occurred rapidly at 4°C, since no additional inhibition was observed when the time of exposure to the active ester was increased from 5 min to 30 min (Fig. 4). Increasing the reagent concentration to 10 μM or exposing the cells to three consecutive additions of reagent (each at 0.5 μM and followed by a 5 min incubation at 4°C) also failed to enhance the extent of inhibition (data not shown). When the pretreatment pH was increased to 7.4 (Fig. 4), values similar to those at pH 6.8 were observed, both for the extent of inhibition (78%) and for the concentration of reagent required for half-maximal inhibition (20 nM). Substitution of the standard buffer (Mg-Hepes-sucrose, pH 7.4) with Hepes-buffered saline of the same pH (data not shown) also had little effect on the inhibition profile, with the extent of inhibition reaching a maximum of 70% and half-maximal inhibition occurring at 20 nM.

Further analysis of the inhibitor-insensitive transport component

Additional experiments were devised in an effort to determine why a portion of methotrexate transport had been insensitive to reagent treatment at 4°C. One possibility was that the cells had clumped at low temperatures (creating inaccessible areas on the cell surface), but no evidence for this phenomenon was observed upon examination of the cells microscopically. A second possibility was that the ester was less reactive at 4°C and that, due to reagent instability, decomposition occurred before sufficient time had elapsed for reaction to have occurred with all of the binding sites. This explanation could also be ruled out (Fig. 5), since reagent that had been diluted into buffer and incubated up to 30 min at 4°C showed only a small loss in ability to inhibit the transport system. In contrast, reagent diluted similarly but incubated at 37°C (see Fig. 5) was much less stable, requiring only 8 min of incubation to decrease in potency by 50%. Finally, ester treatment did not

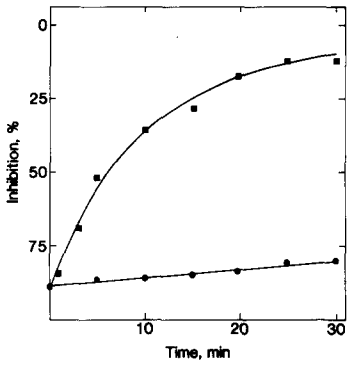

Fig. 5. Stability of the *N*-hydroxysuccinimide ester of methotrexate as a function of time and temperature in Mg-Hepes-sucrose buffer. Samples containing the active ester (1 mM) were diluted 50-fold into Mg-Hepes-sucrose buffer (pH 6.8) at either 4°C (●) or 37°C (■) and then incubated for the indicated times prior to the addition of 10-μl aliquots to 0.99 ml of cells. After 5 min at 37°C, the cells were centrifuged, washed and then examined for methotrexate transport activity.

cause the appearance of unusual uptake kinetics for methotrexate since: (A) transport by the treated cells, although reduced in rate relative to the untreated control, remained linear with time for at least 10 min (Fig. 6); and (B) the V_{max} was decreased by 4-fold, but no effect was observed on the K_t (0.7 μM) for half-maximal influx (data not shown).

Another possibility was that a fraction (25%) of the carrier proteins were directed toward the inner membrane surface and thus had binding sites which

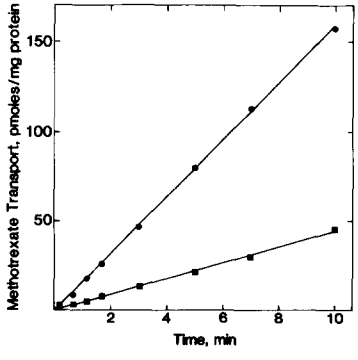

Fig. 6. Time-dependent uptake of [^{3}H]methotrexate in untreated cells (●) and in cells treated with *N*-hydroxysuccinimide ester (500 nM) for 5 min at 4°C (■). Pretreatment buffer, Mg-Hepes-sucrose, pH 6.8.

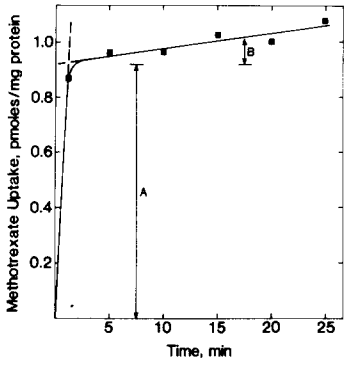

Fig. 7. Time-dependent uptake of [^{3}H]methotrexate at 4°C. Measurements were performed as described in Materials and Methods. Suspending buffer, Mg-Hepes-sucrose, pH 6.8; [^{3}H]methotrexate concentration, 0.5 μM.

were inaccessible to the reagent. To examine the feasibility of this latter model, the time-dependence for uptake of [^{3}H]methodtrexate was measured under the same conditions that had been employed to pretreat cells with the *N*-hydroxysuccinimide ester. It was observed (Fig. 7) that uptake (at 4°C) could be separated into a rapid component (component A), which has been identified as substrate binding to exposed carrier proteins [6], and a much slower second component (B), which appears to reflect either the rate of reorientation of binding sites from the cell interior or the rate at which a complete transport cycle can occur via exposed sites.

Discussion

Irreversible inhibition of methotrexate transport occurs in L1210 cells that have been treated with an *N*-hydroxysuccinimide ester of methotrexate. The inhibitor can be readily synthesized in a reaction mixture containing methotrexate and excess amounts of a carboxyl-activating agent (EDC) and *N*-hydroxysuccinimide. The active component in this reagent mixture would appear to be either an α- or γ-*N*-hydroxysuccinimide monoester of methotrexate, although further studies will be required for structural verification. The corresponding α,γ-diester may also form under the reaction conditions, but it is probably not the primary reactive species, since the reagent is half-maximally effective when the molar ratio of *N*-hydroxysuccinimide to methotrexate in the reaction mixture is less than 1 : 1 (Fig. 1). A similar *N*-hydroxysuccinimide ester has been employed in a previous study [17] to link methotrexate covalently to immunoglobulins, and an *N*-hydroxysuccinimide ester of biotin has been shown, along with other active esters of biotin, to inhibit biotin transport irreversibly in *Saccharomyces cerevisiae* [18]. The covalent bond formed with the yeast transport protein differs, however, from that of the present system, since only the former can be reversed by reducing agents [19].

Inactivation of methotrexate transport by the *N*-hydroxysuccinimide ester of methotrexate occurs at an unusually rapid rate. Even at the lowest levels of reagent tested (5–50 nM), inhibition reached a maximum value after exposure of the cells to the reagent either for 1 min at 37°C (cf. Fig. 2) or for 5 min at 4°C (Fig. 4). The reagent was specific for the methotrexate transport system, since protection against inactivation could be obtained at both temperatures (cf. Figs. 2 and 4) by the addition of methotrexate during the pretreatment step. The unlabeled substrate, however, had to be added at relatively high levels (200 μM) to afford maximum protection. The concentration of reagent required for half-maximal inhibition was also unusually low, reaching a minimum value of 20 nM at the pH optimum of 6.8 (cf. Figs. 3 and 4). Inactivation thus occurred at inhibitor concentrations which were much lower than the range of K_t or K_i values (500–5000 nM) of the transport system for either methotrexate (cf. Fig. 3), various derivatives of methotrexate [20], or other folate compounds [2,3,10,20]. Moreover, it was observed that the sensitivity of the transport system to inactivation at either 37°C or at 4°C was independent of buffer composition, even though a 6-fold difference in binding affinity is obtained for methotrexate in the Mg-Hepes-sucrose buffer, compared to that in Hepes-buffered saline [14]. These results indicate that the concentration of ester required for half-inactivation of the transport process does not reflect a binding constant for the active compound, but, instead, corresponds only to the reagent concentration at which inactivation can be drawn to half-completion. Thus, even though only a fraction of the exposed sites may contain bound inhibitor at a given time, reaction

with these filled sites is immediate, and, given an adequate incubation period (i.e., 1–5 min) and a stable reagent (Fig. 5), modification continues until all of the exposed sites have reacted or the reagent has been consumed. The inactivation process thus becomes nearly quantitative, with a half-maximal loss in activity occurring with an amount of reagent (20 pmol) which is close to the level of added binding sites (5–10 pmol). These latter values are similar, in spite of the fact that a reduction in effectiveness by the reagent could have resulted from a variety of factors including: (A) a less-than-quantitative synthesis of the reactive species or its partial hydrolysis during the pretreatment step; (B) the likelihood that the reaction mixture contains both α- and γ-esters of methotrexate and that one of these esters may not bind to or react with the transport protein; and (C) the possibility that a portion of the reagent molecules are inactive due to racemization of the glutamate side-chain during the activation process[21].

The unusually high sensitivity of the transport process to inhibition by the *N*-hydroxysuccinimide ester indicates that this agent, if prepared using [^{3}H]methotrexate, should act as an effective affinity-labeling agent for the L1210 transport protein. The use of a reactive compound of this type could provide a means for following the binding protein during its solubilization from the membrane and subsequent purification. This would be a particularly useful technique for the L1210 protein, since it is present in relatively small amounts per cell [6]. An attempt has been made to label this carrier protein using EDC-activated [^{3}H]methotrexate, but the reagent was found to exhibits insufficient selectivity [22]. At concentrations required to half-saturate the binding site, reaction occurred to a substantial degree with cellular components other than the methotrexate carrier. The latter results contrast with a parallel study in which carbodiimide-activated [^{3}H]folate was used to label specifically the folate transport protein of *Lactobacillus casei* [16]. Following purification to homogeneity, covalent modification of the bacterial protein was shown to have occurred via an amide bound between one of the carboxyl groups of folate and an ϵ-amino group of a lysine residue on the protein.

The ability of the *N*-hydroxysuccinimide ester of methotrexate to react with the transport system at low temperatures may have also provided information on the membrane orientation of carrier proteins. It was observed that, in cells treated with the reagent at 4°C, a portion (25%) of the transport sites is refractory to reagent treatment (Fig. 4). This residual component could not be inactivated by multiple additions or high concentrations of the reagent, and its presence could not be explained by cell clumping, instability of the reagent (Fig. 5), or the appearance of abnormal transport kinetics (Fig. 6). It was concluded that the methotrexate transport protein can exist in two forms and that these forms can be distinguished by their differential rates of reaction with the active ester. The proposed explanation for these results is that, by lowering the temperature to 4°C, 25% of the transport proteins become fixed in an inward orientation and thus have binding sites which are inaccessible to the reagent. This would not be the case, however, at 37°C, a temperature at which reorientation would occur rapidly and all sites would have a finite time of exposure to the external medium. In support of this hypothesis, uptake studies (Fig. 7) showed that at 4°C substrate binding to exposed carrier proteins (component A) occurs rapidly, while the rate of substrate transport across the membrane or the appearance of new substrate-binding sites at the cell surface (component B) is very slow. The presence of binding sites on both sides of the membrane might also be expected in a system that appears to mediate transport by an anion-exchange mechanism.

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