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# INHIBITORY EFFECTS OF PROBENECID ON THE INDIVIDUAL TRANSPORT ROUTES WHICH MEDIATE THE INFLUX AND EFFLUX OF METHOTREXATE IN L1210 CELLS*

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**Abstract**—L1210 cells contain a single transport system which mediates the influx of methotrexate and at least three routes for drug efflux [G. B. Henderson and E. M. Zevely, *J. biol. Chem.* **259**, 1526 (1984)]; each of these processes is sensitive to probenecid. The influx carrier was inhibited reversibly and completely by probenecid with a $K_i$ of 0.25 mM, while efflux via the same system was relatively unaffected by this compound (50% inhibition above 2.0 mM). The two remaining efflux routes (which do not contribute to methotrexate influx) showed a much higher sensitivity to probenecid. Efflux via these components was reduced half-maximally at probenecid concentrations of 0.08 and 0.22 mM, respectively, and a complete block was achieved with excess amounts (2.0 mM) of the inhibitor. Intracellular levels of ATP, glucose metabolism, and the membrane potential were also reduced by probenecid, indicating that the mechanism for inhibiting methotrexate efflux may involve the ability of probenecid to act as a metabolic inhibitor. Probenecid may have a broad capacity for inhibiting anion transport processes since it also reduced sulfate influx and efflux via the general anion carrier system.

Probenecid (*p*-dipropylsulfamylbenzoic acid), an organic anion, inhibits both the renal transport [1] and cerebrospinal clearance [2] of methotrexate, and, when given in concert with methotrexate, enhances serum levels of the antifolate drug [3]. The therapeutic index of methotrexate is also improved in murine tumor cells by the concurrent administration of probenecid [4]. The mechanism for this enhancement was suggested to involve increased uptake of methotrexate due to a preferential inhibition of efflux [4, 5]. The proposed explanation for this asymmetry was that different routes are utilized for methotrexate influx and efflux and that the efflux components show a higher sensitivity to probenecid [5].

Multiple routes are utilized by L1210 cells for the transport of methotrexate across the plasma membrane [6–8]. Results obtained using specific transport inhibitors show that influx occurs only via a single high-affinity carrier system, while at least three routes participate in drug efflux [8]. The efflux routes include the methotrexate influx carrier, a second process that can be inhibited by bromosulfophthalein, and a bromosulfophthalein-insensitive component. The latter two efflux routes account for a majority of the efflux in energy-replete cells, while efflux via the influx carrier predominates in energetically deficient cells [8]. In the present study, evidence is presented which shows that probenecid inhibits each of the individual routes that contribute to methotrexate influx and efflux and that the two efflux routes that do not participate in methotrexate influx are the most sensitive to probenecid. These results provide an explanation for previous observations on the differential sensitivity of methotrexate influx and efflux to probenecid [5].

## MATERIALS AND METHODS

*Chemicals.* [3′,5′,9-$^3$H]Methotrexate (250 mCi/mmole) was obtained from Amersham, purified by thin-layer chromatography on cellulose sheets (Kodak 13255) with 0.1 M sodium-HEPES‡, pH 7.0, as the solvent, and diluted with unlabeled methotrexate to a specific activity of 150,000 dpm/nmole. Other compounds and their commercial sources were: [$^{35}$S]sulfate (Amersham); [$^{14}$C]tetraphenylphosphonium bromide (New England Nuclear); D-[U$^{14}$C]glucose (ICN); and methotrexate, probenecid, and bromosulfophthalein (Sigma).

*Cells.* L1210 mouse leukemia cells were grown as described previously [9], washed with HBBS, (20 mM sodium HEPES, 26 mM sodium-bicarbonate, 107 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, pH 7.4), and resuspended in the same buffer to a density of $3 \times 10^7$/ml.

*Preparation of NHS-methotrexate.* NHS-methotrexate [9, 10] was prepared by dissolving methotrexate (2.2 mg), 1-ethyl-3[3-dimethylaminopropyl]-carbodiimide (7.8 mg), and *N*-hydroxysuccinimide (5.8 mg) in 2.0 ml anhydrous

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‡ Abbreviations: HEPES, 4-2(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; NHS-methotrexate, *N*-hydroxysuccinimide ester of methotrexate; and HBBS, HEPES-bicarbonate-buffered saline.

dimethyl sulfoxide and allowing the mixture to stand for 60 min at 23°. The concentration of activated compound was determined from the millimolar extinction coefficient of methotrexate of 18.9 at 302 nm and pH 7.0.

*Transport measurements.* Influx of methotrexate was determined as described previously [10] in assay mixtures containing cells ($3 \times 10^7$), the desired additions, and [$^3$H]methotrexate (5.0 $\mu$M), in a final volume of 1.0 ml. After incubation with shaking for 2 min at 37°, the cells were centrifuged at 1000 *g* (5 min, 4°), washed with 4 ml of ice-cold 0.15 M NaCl, resuspended in 0.5 ml of water, and analyzed for radioactivity. Uptake at 4° served as the control.

Methotrexate efflux via combined or individual efflux routes was measured as described previously [8]. Cells were loaded with substrate by incubating for 15 min at 37° in HBBS containing 10 $\mu$M [$^3$H] methotrexate, washed at 4° to remove extracellular substrate, and resuspended in buffer to $3 \times 10^7$ cells/ml. An intracellular concentration of $80 \pm 10$ pmoles/mg protein was achieved under these conditions. Treatment with NHS-methotrexate was performed by exposing loaded cells to 10 $\mu$M NHS-methotrexate for 5 min at 23°. Excess reagent was removed by a single buffer wash at 4°. Inhibitors were then added to the desired concentration, and the cells were incubated for various times between 2 and 20 min at 37°, diluted with 4 ml of ice-cold 0.15 M NaCl, collected by centrifugation, and analyzed for residual radioactivity. Influx and efflux are reported in pmoles of methotrexate transported per min per mg protein. Cellular protein was measured by the biuret reaction [11] with bovine serum albumin as the standard.

The influx and efflux of [$^{35}$S]sulfate (0.5 mM) and the uptake of D-[U-$^{14}$C]glucose (1.0 mM) were measured using the same procedures described above for methotrexate.

*Other determinations.* ATP levels were measured in cells ($1.5 \times 10^7$) that had been combined with the desired additions (in 1.0 ml, final volume), incubated for 10 min at 37°, collected by centrifugation, and then dispersed in 0.5 ml of 0.3 M sulfuric acid. After 5 min at 23°, the samples were neutralized by the addition of 0.5 ml of 0.6 M NaOH and 4 ml of 20 mM potassium-phosphate, pH 7, and assayed by the procedure of Cheer *et al.* [12].

The membrane potential of L1210 cells was estimated as described previously [13] by the equilibrium distribution of [$^{14}$C]tetraphenylphosphonium$^+$ across the cell membrane. Assay mixtures contained $1.5 \times 10^7$ cells, the desired additions, and 2.0 $\mu$M [$^{14}$C]tetraphenylphosphonium bromide and were incubated for 30 min at 37° to achieve a steady state. Uptake in the presence of 5 $\mu$M each of valinomycin and nigericin served as a control.

## RESULTS

*Inhibition of methotrexate influx by probenecid.* The concentration dependence for inhibition of methotrexate influx by probenecid is shown in Fig. 1. A monophasic inhibition profile was observed in both the absence and presence of glucose; the concentration of probenecid required for half-maximal reduction in transport activity was 0.5 mM. A Dixon plot of these data (inset, Fig. 1) was linear, and the calculated $K_i$ value for probenecid was 0.25 mM. In parallel samples (not shown) in which the cells had been incubated (5 min, 37°) with various concentrations of the inhibitor (with or without glucose) and then washed to remove the reagent, influx activity was restored by greater than 90%.

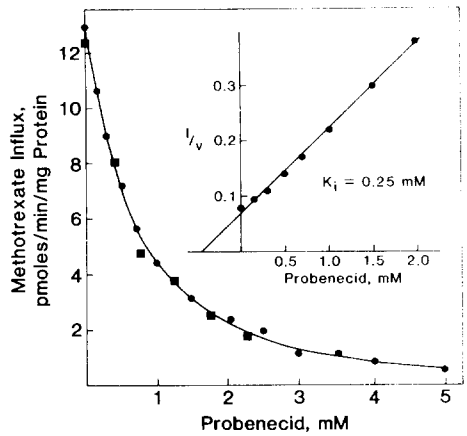


Fig. 1. Concentration dependence for the inhibition of methotrexate influx by probenecid. Key: (●) buffer alone; (■), buffer plus 5.0 mM glucose. Inset: Dixon plot of the data; *v*, methotrexate influx.

*Effect of probenecid on total efflux.* When total efflux was examined in the presence of increasing concentrations of probenecid (Fig. 2), inhibition was observed which was half-maximal at a probenecid concentration of 0.11 mM. A complete loss in activity, however, was not achieved even at relatively high concentrations of the inhibitor (up to 2.0 mM). A similar profile was obtained when glucose was added to the cells (Fig. 2), although a higher initial efflux was observed in the control, and inhibition was half-maximal at a slightly lower probenecid concentration (0.09 mM). The probenecid-insensitive

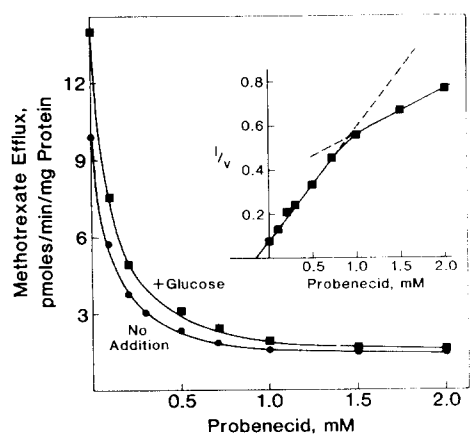


Fig. 2. Efflux of methotrexate as a function of probenecid concentration in the absence (●) and presence (■) of glucose. Glucose concentration, 5.0 mM. Inset: Dixon plot of the data; *v*, efflux of methotrexate.

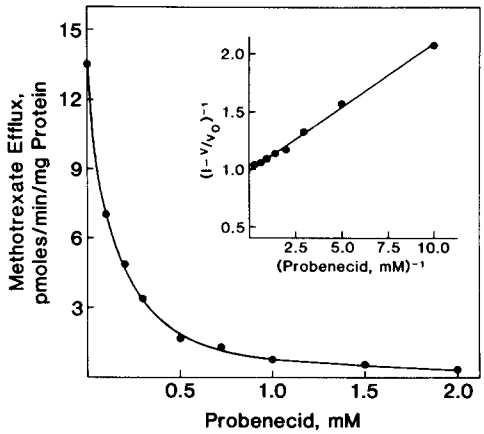


Fig. 3. Efflux of methotrexate as a function of probenecid concentration in cells treated with NHS-methotrexate. Suspending medium; HBBS plus 5.0 mM glucose. Inset, replot of the data by the method of Preston *et al.* [14]; $v$, observed rate; $v_o$, uninhibited rate.

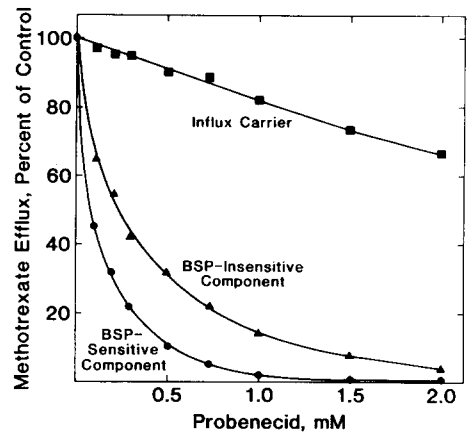


Fig. 4. Sensitivity of individual efflux routes to inhibition by probenecid. Suspending medium; HBBS plus 5.0 mM glucose. Initial efflux: methotrexate influx carrier, 2.0 pmoles/min/mg protein; bromosulfophthalein-sensitive route, 8.1 pmoles/min/mg protein; and bromosulfophthalein-insensitive route, 3.7 pmoles/min/mg protein. BSP, bromosulfophthalein.

portion of efflux that was observed in both the absence and presence of glucose proceeded at approximately 2 pmoles/min/mg protein. The biphasic nature of the inhibition profile was illustrated further by the non-linearity of a Dixon plot of these data (inset, Fig. 2).

*Effect of probenecid on individual efflux components*. Specific inhibitors can be employed to identify and quantitate three efflux routes for methotrexate in L1210 cells [8]. One of these routes is the methotrexate influx carrier, which can be inhibited irreversibly by an *N*-hydroxysuccinimde ester of methotrexate [8, 9]. The effect of NHS-methotrexate treatment on the ability of probenecid to inhibit methotrexate efflux is shown in Fig. 3. The inhibition profile obtained in these treated cells was monophasic and a nearly complete loss in efflux activity was observed at high levels of the inhibitor. A replot of the data (inset, Fig. 3) showed that inhibition occurred half-maximally at a probenecid concentration of 0.11 mM and that the data points produced a straight line which extrapolated to 100% inhibition (y-intercept = 1.0). Active ester treatment thus eliminated the portion of total efflux which was insensitive to probenecid (see Fig. 2).

The contribution to total efflux by the methotrexate influx carrier can be determined directly from the difference in efflux between untreated cells and cells treated with NHS-methotrexate [8]. Efflux via this component in HBBS containing glucose is 2.0 to 2.5 pmoles/min/mg protein or approximately 20% of total efflux. The addition of increasing amounts of probenecid inhibited this efflux component (Fig. 4), although the extent of inhibition was low and did not reach 50% at the highest concentration of probenecid tested (2.0 mM).

The remaining two efflux components for methotrexate can be separated by their sensitivity to bromosulfophthalein [8]. Quantitation of the bromosulfophthalein-sensitive component is obtained by comparing efflux in cells treated with NHS-methotrexate in the absence and presence of bromosulfophthalein, while the bromosulfophthalein-insensitive route corresponds directly to the efflux activity remaining after exposure to both NHS-methotrexate and bromosulfophthalein. The concentration dependence for the inhibition of these two efflux components by probenecid is shown in Fig. 4. The most potent response to probenecid was observed for the bromosulfophthalein-sensitive route, which was inhibited half-maximally at a probenecid concentration of 0.08 mM. Moreover, a

Table 1. Effect of probenecid on ATP levels and the membrane potential of L1210 cells*

| Additions | ATP (nmoles/mg protein) | Membrane potential (m$V$) |
|---|---|---|
| None | 11.6 | −69 |
| Probenecid | 7.0 | −73 |
| Glucose | 17.6 | −68 |
| Glucose + probenecid | 14.0 | −51 |
| Valinomycin + nigericin | 0.3 | 0 |

* ATP levels and the membrane potential were measured as described in Methods. Concentrations of additions were: probenecid, 1.0 mM; glucose, 5.0 mM; valinomycin, 5.0 μM; and nigericin, 5.0 μM.

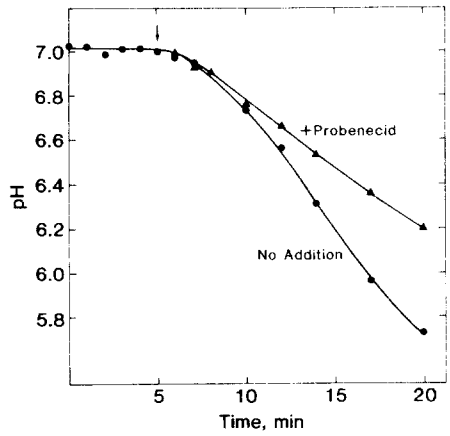


Fig. 5. Effect of probenecid on acid production from glucose. Suspending medium: 1 mM sodium HEPES, 160 mM NaCl, 2 mM $MgCl_2$, pH 7.0. Arrow indicates time of glucose addition. Probenecid concentration, 1.0 mM; glucose concentration, 5.0 mM.

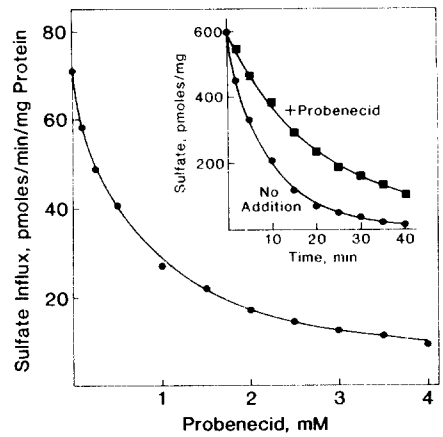


Fig. 6. Concentration dependence for the inhibition of sulfate influx by probenecid. Suspending medium: HBBS. Inset: efflux of sulfate in the absence and presence of 2.0 mM probenecid.

complete inhibition of this efflux route could be achieved at high levels of the inhibitor. A similar profile was observed for the bromosulfophthalein-insensitive route, although a somewhat higher concentration of probenecid (0.22 mM) was required for 50% inhibition.

*Effect of probenecid on energy metabolism in L1210 cells.* Since a reduction in the energetic state of L1210 cells can substantially reduce methotrexate efflux via both the bromosulfophthalein-sensitive and bromosulfophthalein-insensitive components [8], the possibility was considered that probenecid may affect efflux by acting as a metabolic inhibitor. Several parameters that reflect cellular metabolism were therefore measured in the absence and presence of probenecid. It was observed that probenecid (at 1.0 mM) reduced ATP levels by 40% in control cells, although this reduction was less pronounced (20%) in cells with added glucose (Table 1). At a lower concentration of probenecid (0.2 mM), ATP levels decreased by only 17 and 3%, respectively, in the absence and presence of glucose. A reduction in the membrane potential was also noted (Table 1), although this occurred only in the presence of glucose. When the metabolism of glucose was examined, probenecid was found to reduce both acid production from glucose (Fig. 5) and the uptake of [$^{14}$C]glucose (Table 2).

*Inhibition of sulfate transport by probenecid.* Sulfate transport via the general anion carrier system of L1210 cells [15] was also sensitive to probenecid (Fig. 6). Inhibition of influx appeared to be monophasic and it was half-maximum at a probenecid concentration of 0.65 mM. Calculations using the Dixon equation and an observed $K_i$ for sulfate of 4.0 mM yielded a $K_i$ for probenecid of 0.60 mM. Probenecid also inhibited sulfate efflux, with a reduction of 65% being observed in the presence of 2.0 mM probenecid (inset, Fig. 6).

## DISCUSSION

Methotrexate influx in L1210 cells is susceptible to competitive inhibition by a variety of organic and inorganic anions [8, 10, 13, 15–17]. The basis for this inhibition appears to be that the transport system mediates uptake via an anion-exchange mechanism [8, 10, 15–17] and that anions of diverse structures are utilized *in vivo* as exchange substrates for methotrexate [10]. Consequently, the binding site on the transport protein has a broad anion-binding capacity, although specificity has been maintained by exhibiting a large difference in affinity for various anions. Binding preference is given to folate compounds, although other divalent anions with aromatic substituents are also bound with a high degree of efficiency [10]. Monovalent anions generally bind to the

Table 2. Effect of probenecid on glucose uptake by L1210 cells*

| Addition | Time (min) | Glucose uptake (nmoles/mg protein) | Inhibition (%) |
|---|---|---|---|
| None | 1 | 1.8 | 0 |
| Probenecid | 1 | 1.8 | 0 |
| None | 15 | 15.5 | 0 |
| Probenecid | 15 | 9.5 | 39 |

* Glucose uptake was determined (at 37°) as described in Methods. Probenecid concentration, 1.0 mM.

transport protein with an affinity that is 3 to 4 orders of magnitude lower than that of folate compounds, although the presence of an aromatic substituent, as is the case for probenecid ($K_i = 0.25$ mM), can substantially improve binding. Probenecid, however, lacks the structural requirements to allow this anion to serve as an effective exchange substrate for methotrexate [10].

Probenecid inhibited each of the three routes that mediate the efflux of methotrexate in L1210 cells (cf. Figs. 2–4). These routes differed, however, in their sensitivity to probenecid, as shown by the biphasic nature of the inhibition profile for total efflux (Fig. 2), and by variable responses to the inhibitor when the individual efflux components were measured separately (Fig. 4). Efflux via the methotrexate influx carrier was the least sensitive to probenecid and appeared to constitute the unresponsive portion of total efflux (see Fig. 2). Inhibition of this route was less than 50% at the highest level (2.0 mM) of probenecid tested (Fig. 4). Far greater inhibition was observed for efflux via the bromosulfophthalein-sensitive and -insensitive components. Efflux via these routes decreased by 50% at 0.08 and 0.22 mM respectively. Previous results documenting a preferential inhibition by probenecid of methotrexate efflux relative to influx can thus be explained by the combined observations that: (a) the influx of methotrexate proceeds via a single transport system while efflux proceeds via at least three distinct routes [8]; (b) efflux routes that do not contribute to influx account for a majority of total efflux (*ca.* 80% in the presence of glucose and bicarbonate) [8]; and (c) both of the latter efflux routes were more sensitive to probenecid than cell entry of methotrexate via the influx carrier (cf. Figs. 1 and 4). The result of this combination of factors is that the uptake of methotrexate at the steady-state will be enhanced by probenecid [5].

The mechanisms involved in the inhibition of methotrexate efflux by probenecid remain unclear, although it is likely that at least some of the inhibition is the result of a direct interaction of probenecid with binding sites on individual transport proteins. Bound probenecid might then inhibit efflux by acting as a poor substrate, which would reduce the rate of membrane cycling of the binding site. Alternatively, probenecid could be a non-substrate and consequently block transport by trapping binding sites at the external membrane surface. A second factor in the interpretation of these inhibitory effects is that probenecid acts as a weak metabolic inhibitor (cf. Fig. 5 and Tables 1 and 2). Since the energetic state of the cell affects efflux via both the bromosulfophthalein-sensitive and -insensitive efflux routes [8], inhibition of the latter efflux routes by probenecid might then be a consequence of changes in energy metabolism. Alterations in cellular energetics, however, appear to be insufficient to account for all of the observed inhibition since substantial changes in ATP levels, membrane potential, and glucose utilization occurred only at concentrations of probenecid (1.0 mM) that are 10-fold higher than needed to inhibit efflux (0.1 mM).

An emerging feature of probenecid is that it exhibits a relatively broad capacity for inhibiting anion transport systems. In L1210 cells, this property extends to each of the three processes that mediate the influx and/or efflux of methotrexate (Figs. 1 and 4) and, also, to sulfate influx and efflux via the general anion carrier system (Fig. 6). These effects were generally seen below millimolar concentrations of the inhibitor, indicating that there was a moderate degree of specificity for each of the processes involved. In other cell lines, inhibition of anion transport by probenecid has been reported for chloride [18], urate [19], citrate [20], and $\alpha$-ketoglutarate [20].

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