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# PROTEIN BINDING AS A COMPONENT OF DRUG INTERACTION IN CELLULAR PHARMACOKINETIC STUDIES

## EFFECTS OF PROBENECID ON TRANSPORT AND ACCUMULATION OF METHOTREXATE IN EHRLICH ASCITES TUMOR CELLS *IN VITRO**

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**Abstract**—The organic acid probenecid has been shown to interfere with the active extrusion of methotrexate (MTX) from L1210 tumor cells *in vitro* leading to enhanced MTX accumulation and increased formation of MTX polyglutamate derivatives. In the presence of serum albumin (4 g/100 ml), to which probenecid is bound, the inhibition by probenecid of [$^3$H]MTX efflux from the Ehrlich ascites tumor cell was reduced markedly. While half-maximal inhibition of MTX efflux occurred with 0.12 mM probenecid in the absence of albumin, 1.45 mM probenecid was required in its presence. The presence of albumin also modified the probenecid-induced elevation of steady-state MTX levels in the tumor cell. Maximal elevation of cellular MTX levels occurred with 0.5 mM probenecid in the absence of albumin, and 3 mM probenecid in its presence. Serum albumin further reversed the effects of probenecid on MTX influx. While probenecid inhibited influx of 1 $\mu$M [$^3$H]MTX in the absence of albumin (half-maximal inhibition at ~1 mM probenecid), probenecid stimulated MTX influx in its presence (half-maximal effect at 0.5 to 1 mM). Equilibrium dialysis studies demonstrated that probenecid displaced MTX from albumin, increasing the effective free concentration of MTX in the incubation medium, and hence the rate of MTX influx. Therefore, probenecid may enhance the accumulation of MTX in the tumor cells by increasing the level of free (as opposed to albumin bound) MTX in the extracellular medium as well as by direct inhibition of MTX efflux. These observations may provide an additional explanation for probenecid enhancement of the therapeutic efficacy of MTX in tumor bearing mice and highlight the importance of assessing drug–protein interactions in an *in vitro* experimental model.

It is well established that drug association with serum protein may lower the "effective" circulating concentration of "free" drug and its uptake into tissue site(s); protein binding also provides a "depot" form of the drug which extends its half-life in the circulation [1]. Conversely, displacement of drug from serum protein would serve to elevate circulating drug concentrations and enhance tissue uptake of drug [2].

Studies of the cellular pharmacokinetics of drugs *in vitro* generally employ simple buffered-salt incubation mediums, which eliminate the potential contribution of serum proteins. While this experimental approach provides useful information regarding drug transport and metabolism, the absence of serum protein prevents effective extrapolation to the *in vivo* situation. This problem is magnified in experimental protocols which may involve two or more drugs which are protein bound; in such a case, drug interaction with serum protein may dramatically alter the cellular pharmacokinetics of chemotherapeutic agents.

A recent study by Sirotnak *et al.* [3] assessed the effects of the organic acid probenecid on the transport of the anticancer drug methotrexate (MTX) in L1210 leukemia cells harvested from tumor bearing mice. Probenecid was found to enhance the accumulation of MTX in these murine tumor cells by a preferential inhibition of MTX efflux. Subsequent studies determined that probenecid elevates MTX levels in L1210 leukemia cells *in vivo* and enhances the therapeutic effectiveness of the MTX against this cell line [4]. These effects *in vivo* are thought to reflect the inhibition by probenecid of MTX efflux observed *in vitro*.

We have investigated the interaction between probenecid and methotrexate utilizing Ehrlich ascites tumor cells *in vitro* and have confirmed the observations reported by Sirotnak *et al.* [3]. However, in the presence of serum albumin, the effects of probenecid on the cellular pharmacokinetics of methotrexate have proven to be markedly more complex than previously described; this results from the fact that both MTX and probenecid are bound to serum protein, which results in unexpected drug interactions. These studies demonstrate that the contribution of serum protein to drug binding may be a critical element in the extrapolation from *in vitro* drug studies to the whole animal.

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## MATERIALS AND METHODS

[3′,5′–7-$^3$H]Methotrexate was obtained from the Amersham Corp. (Arlington Heights, IL) and purified by high pressure liquid chromatography [5]. Unlabeled methotrexate was purified by DEAE cellulose chromatography [6]. Probenecid was obtained from the Sigma Chemical Co. (St. Louis, MO) and was dissolved in water and adjusted to pH 7.6 with sodium hydroxide. Bovine serum albumin (Cohn fraction V) was obtained from the Sigma Chemical Co. and dissolved in the incubation buffer on the day of the experiment.

*Cells and incubation conditions.* Ehrlich ascites tumor cells were grown in male $CF_1$ mice (Sprague–Dawley, Madison, WI) and passed weekly by intraperitoneal inoculation of 0.2 ml of undiluted ascitic fluid. The cells were harvested after 7–10 days and washed twice with 0.85% NaCl solution (0°). All experiments were performed at 37° in specially designed flasks in a modified Krebs–Henseleit buffer [7]. The pH of the incubation medium was maintained at 7.4 by passing warm and humidified 95% $O_2$/5% $CO_2$ over the cell suspension [6].

*Analysis of intracellular radiolabel.* Experiments were initiated by addition of [$^3$H]MTX to the cell suspension. Transport fluxes were terminated by injection of the cell suspension into 10 vol. of 0° 0.85% NaCl solution. The cell fraction was separated by centrifugation (500 *g*, 2 min) and washed twice with the same solution. The cell pellet was aspirated into the tip of a Pasteur pipet and then extruded onto a polyethylene tare. After overnight drying at 70°, pellets were weighed directly on a Cahn model 4700 electrobalance, placed in scintillation vials, and digested with 0.2 ml of 1 N KOH for 1 hr at 70°. After neutralization with 0.2 ml of 1 N HCl, 3 ml of Ready Solv scintillation solution (Beckman, Irving, CA) was added and radioactivity was determined.

*Equilibrium dialysis.* Equilibrium dialysis was performed overnight at 37° utilizing dialysis tubing with a molecular weight cutoff of 12,000 (Arthur H. Thomas Co., Philadelphia, PA) which had been boiled in 1 mM EDTA and rinsed with deionized water immediately before initiation of the experiment. The dialysis tubing was affixed over the blunted end of a plastic pipette tip, supported within a 1.5 ml plastic microfuge tube, and placed in a 7 ml plastic counting vial which was capped to prevent evaporation. The volume of solution within the dialysis bag was 100 μl and contained either buffer alone or buffer with bovine serum albumin added. The volume of solution surrounding the dialysis bag was 700 μl and initially contained [$^3$H]MTX with or without probenecid. After 24 hr, 50 μl of inner solution was sampled and 100 μl of the outer solution was sampled for $^3$H. Bound drug was calculated as the difference between total $^3$H within the bag, and free drug outside the bag after attainment of equilibrium.

There was no evidence of bacterial contamination in these equilibrium dialysis studies. To test for leakage of protein across the dialysis membrane, as well as bacterial protein, the solution surrounding the dialysis bag was tested for protein precipitation using concentrated trichloroacetic acid. There was no indication of protein in this solution.

## RESULTS

It has been established that various mammalian tumor cell lines have the capacity to eliminate the antifolate drug methotrexate by an energy-dependent transport mechanism [8] which limits the cellular accumulation of methotrexate. Sirotnak *et al.* [3] have demonstrated that MTX efflux from L1210 leukemia cells is markedly inhibited in the presence of low concentrations of probenecid. This finding has potential relevance in enhancing the therapeutic effectiveness of methotrexate. Our initial studies were designed to evaluate the effects of probenecid on MTX efflux from the Ehrlich ascites tumor cell in both the presence and absence of albumin, as probenecid is known to be extensively bound to serum proteins [9].

Figure 1 is a log plot showing the effect of 0.5 mM probenecid on efflux of MTX from the Ehrlich ascites tumor cell. Cells which had achieved steady-state levels of intracellular MTX after incubation with 5 μm [$^3$H]MTX for 30 min were washed and resuspended in incubation buffer alone or in buffer containing 4 g/100 ml bovine serum albumin (BSA). As reported by Sirotnak *et al.* for the L1210 leukemia cell [3], probenecid markedly reduced efflux of cellular $^3$H (compare lines A and B). Efflux of [$^3$H]-MTX from the Ehrlich ascites cells was inhibited by 57.89 ± 13.49% by 0.5 mM probenecid, while this concentration of probenecid inhibited MTX efflux from the L1210 cell by approximately 70%. When albumin was present in the washout buffer, the rate of MTX efflux was enhanced by 17.8 ± 1.3% in three experiments (compare lines A and C). When both albumin and 0.5 mM probenecid were present, the capacity of probenecid to inhibit MTX efflux was reduced by ~52% (lines C and D) when compared to efflux in the absence of BSA (lines A and B).

Figure 2 presents a dose–response relationship for the inhibition of MTX efflux by probenecid in the presence and absence of albumin. It is important to note that the range of probenecid concentrations required for inhibition of MTX efflux in the presence of albumin was approximately 10-fold greater than needed in the absence of serum protein. In the absence of albumin, half-maximal inhibition of efflux occurred at 0.12 mM probenecid (Fig. 2A), while in the presence of albumin 1.45 mM probenecid was required to achieve the same degree of inhibition of efflux (Fig. 2B). This observation suggests that binding of probenecid to albumin lowers its effective free concentration in the incubation medium, and the consequent inhibition of MTX efflux. These findings further suggest that the presence of albumin would reduce or eliminate enhancement of cellular accumulation of MTX by low concentrations of probenecid [3].

The next series of experiments was designed to assess the effects of probenecid on net MTX accumulation in the absence and presence of albumin. Figure 3 demonstrates the effects of simultaneous addition of 0.1 or 1 mM probenecid with [$^3$H]MTX on the net cellular accumulation of methotrexate in the absence

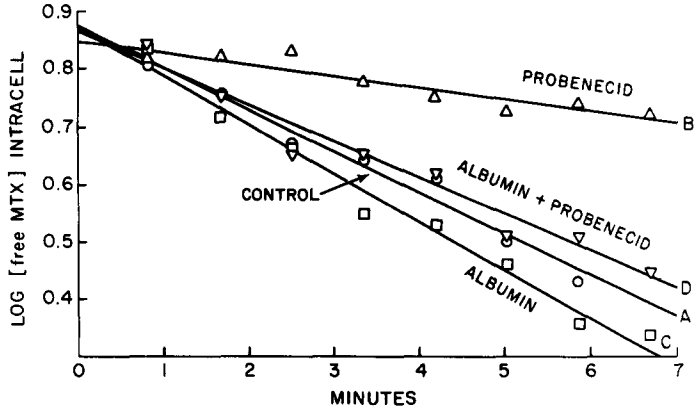


Fig. 1. Effect of probenecid on methotrexate efflux in the presence and absence of albumin. Cells were loaded with methotrexate by incubation with 5 $\mu$M [$^3$H]methotrexate for 30 min. Uptake of [$^3$H]-methotrexate was terminated by transfer of cell suspension to saline at 4°; cells were washed with 4° saline. Efflux of [$^3$H]MTX was initiated by resuspension of the cell pellet in 37° buffer (A), or buffer containing 0.5 mM probenecid alone (B), 4 g/100 ml albumin alone (C), or albumin + probenecid (D). Intracellular [$^3$H]MTX was monitored at 50-sec intervals. Free methotrexate was calculated by subtracting the nonexchangeable fraction (i.e. drug bound to dihydrofolate reductase) from total intracellular $^3$H. Lines were drawn by least squares linear regression analysis.

(panel A) or presence (panel B) of albumin. Addition of probenecid enhanced net methotrexate accumulation in the absence of albumin (Fig. 3A), as was expected from the inhibition by probenecid of MTX efflux. There was also an apparent inhibition of initial methotrexate uptake. These observations are consistent with those of Sirotnak *et al.* [3]. Albumin alone reduced net MTX accumulation (compare control levels in Fig. 3A and 3B), indicating that binding of MTX to albumin reduces the concentration of free drug available for uptake into the cell. In the presence of albumin, probenecid enhanced net MTX accumulation, utilizing a probenecid concentration as low as 0.1 mM (Fig. 3B). This was a surprising observation in view of the relatively small degree of inhibition of MTX efflux by 0.1 mM probenecid in the presence of albumin (Figs. 1 and 2B). Albumin also eliminated the inhibition of initial MTX uptake by probenecid observed in Fig. 3A; this again indicates that the effective probenecid concentration had been reduced by its binding to albumin.

The effects on methotrexate accumulation of the addition of various concentrations of probenecid is presented in Fig. 4. While probenecid elevated net methotrexate accumulation in both the presence and absence of albumin, the relationship between methotrexate accumulation and the concentration of probenecid added was markedly different when serum protein was present. Maximal enhancement of methotrexate accumulation was observed utilizing 0.5 mM probenecid in the absence of albumin (Fig. 4A); with increasing concentrations of probenecid, the inhibition of MTX influx ultimately overwhelmed the inhibition of efflux, so that at 5 mM probenecid net MTX accumulation was reduced as compared to the control. In the presence of albumin (Fig. 4B), concentrations of probenecid between 0.5 mM and 5 mM elevated MTX accumulation.

Additional studies examined the effects of probenecid addition to cells which had accumulated steady-state levels of [$^3$H]MTX in the presence and absence of albumin. The addition of probenecid was found to elevate intracellular MTX levels in the

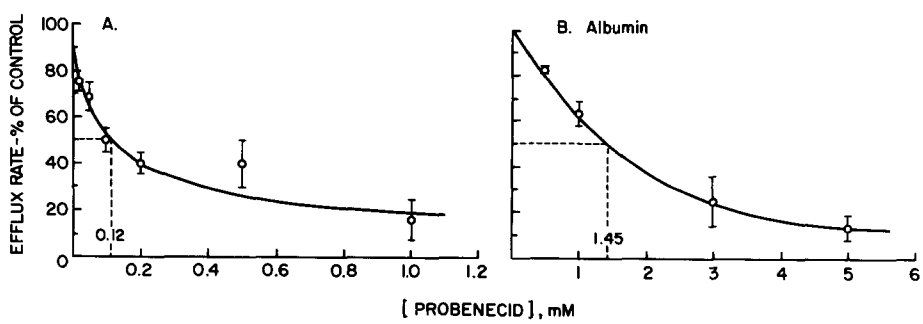


Fig. 2. Inhibition of [$^3$H]MTX efflux by various concentrations of probenecid; effect of albumin. Cells were loaded with 5 $\mu$M [$^3$H]methotrexate in the absence of albumin, and efflux of $^3$H was assessed in the presence of various concentrations of probenecid (as described in the legend to Fig. 1). Panel A: Efflux in the absence of albumin. Panel B: Efflux in the presence of 4 g/100 ml albumin. Each point represents the mean ± S.E. for three to five experiments.

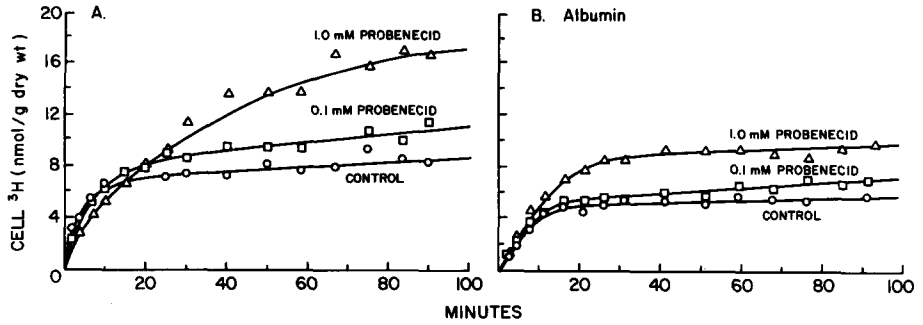


Fig. 3. Effect of 0.1 or 1.0 mM probenecid on uptake and net accumulation of [³H]MTX in the presence and absence of albumin. Cells were incubated with 5 μM [³H]MTX and either 0.1 mM or 1 mM probenecid in a buffer without albumin (panel A) or containing 4 g/100 ml albumin (panel B). Cellular accumulation of ³H was monitored for 90 min.

absence as well as the presence of albumin; however, the pattern of stimulation by probenecid was markedly different in the two conditions. Figure 5 shows the percent increase in intracellular ³H achieved by the addition of increasing concentrations of probenecid in the absence or presence of albumin. The upper panel represents simultaneous exposure of cells to probenecid and [³H]MTX. The lower panel represents addition of probenecid to cells with steady-state levels of cellular ³H. The profile of effects was markedly different when serum protein was present. It is clear that maximal enhancement of net MTX accumulation was achieved with approximately 0.5 mM probenecid in the absence of BSA, while a probenecid concentration of ~3 mM was required to achieve maximal stimulation of net MTX accumulation in the presence of albumin. This finding is consistent with protein binding of probenecid reducing the effective free concentration of the organic acid. However, the enhancement of net MTX accumulation in the presence of albumin by concentrations of probenecid which only modestly inhibit MTX efflux suggests another component of drug interaction, i.e. dissociation of MTX from the serum protein in the presence of probenecid, which stimulates methotrexate transport into the cell.

A series of experiments was designed to determine how probenecid alters influx of MTX in the presence and absence of albumin. Figure 6 shows that, in the presence of serum albumin alone, MTX influx was reduced, presumably because the concentration of methotrexate was reduced as a result of its binding to serum protein (compare lines A and B). However, probenecid in the presence of albumin *stimulated* influx of methotrexate, to exceed that in the presence of albumin alone (compare lines B and C). This was in marked contrast to inhibition of MTX influx by probenecid in the absence of albumin (compare lines A and D).

To define this phenomenon in greater detail, the effects of varied probenecid concentrations on influx of MTX were assessed. In the absence of albumin (Fig. 7A), increasing the concentration of probenecid resulted in a corresponding inhibition of MTX influx. However, when the effects of probenecid on MTX influx were assessed in the presence of albumin, only *stimulation* of MTX influx was observed (Fig. 7B). Maximal stimulation of MTX influx was observed at 0.5 to 1 mM probenecid; at higher concentrations of probenecid, the stimulation of influx (though still significant) was reduced. The dramatic difference in the effects of probenecid on MTX influx in the presence and absence of BSA is summarized in Fig. 8. Increasing concentrations of probenecid resulted in a proportionate inhibition of MTX influx in the absence of serum protein; however, in the presence of serum protein, probenecid stimulated influx in a dose-dependent man-

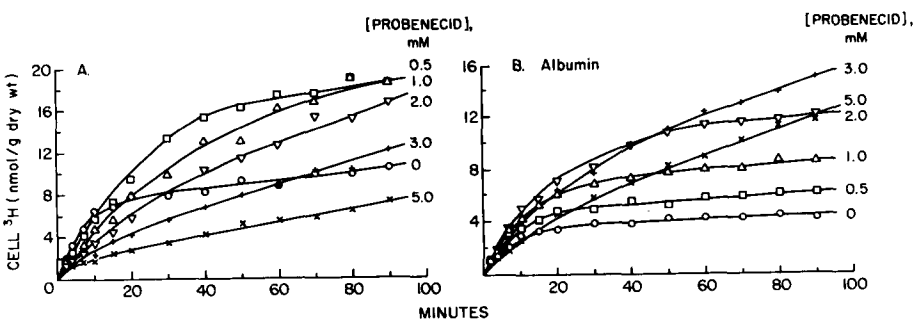


Fig. 4. Time course of cellular accumulation of [³H]MTX with increasing concentrations of probenecid; effect of albumin. Experimental conditions are described in the legend to Fig. 3. A series of probenecid concentrations (0.5 to 5 mM probenecid) were utilized.

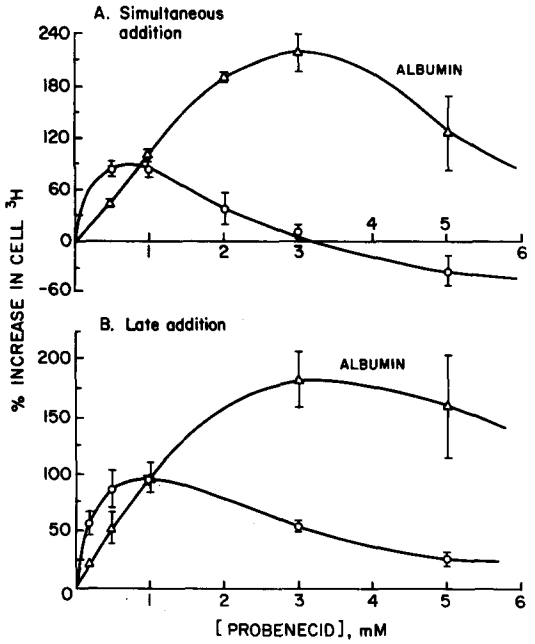


Fig. 5. Summary of the relationship between probenecid concentration and net [³H]MTX accumulation in the presence and absence of albumen. The percentage increase in net cellular ³H accumulation induced by various concentrations of probenecid is presented in the presence and absence of 4 g/100 ml albumin. Panel A presents the results after simultaneous exposure of the cells to probenecid and 5 μM [³H]MTX. Panel B presents results from addition of probenecid to cells with steady-state levels of [³H]MTX (initial [MTX] was 5 μM). Each point represents the mean ± S.E. for three to six experiments.

ner up to 1 mM probenecid. As the concentration of probenecid was increased (so that more free probenecid was available), the stimulation of influx was balanced by inhibition of influx, so that at 5 mM probenecid there was a small net effect on MTX influx.

To directly establish that probenecid displaces methotrexate from serum albumin, thereby stimulating MTX influx, equilibrium dialysis studies were performed to assess the association of methotrexate with albumin in the presence and absence of probenecid. Utilizing 1 μM [³H]MTX, the percentage of MTX bound was found to reflect the albumin concentration in the medium (e.g. at 1 g/100 ml albumin, 40% of MTX was bound) (Fig. 9A). Over a concentration range of 1 to 100 μM [³H]MTX, the percentage of MTX bound to serum albumin averaged 60–70% when the albumin was 4 g/100 ml. In the presence of probenecid, MTX was effectively displaced from albumin binding sites. Figure 9B shows that 0.1 mM probenecid reduced the percentage of albumin-bound [³H]MTX to 50%, while at 1 mM probenecid only 6% of the [³H]MTX was bound to serum protein.

## DISCUSSION

The importance of serum protein binding in reducing the effective concentration of circulating drugs has long been recognized. Acidic drugs including the salicylates, sulphonamides, coumarins, anticoagulants and phenylbutazone have been shown to bind strongly to serum albumin [10]. The possibility that competition for protein binding sites may elevate circulating drug concentrations has been investigated with such paired groupings as phenylbutazone and warfarin [11], and diazapam and valproate [12].

Various anticancer agents, including methotrexate, bind to serum albumin [13]. Approximately 50–70% of MTX, at a concentration of $10^{-6}$ M, is bound to serum protein, primarily albumin [14–16]. A number of drugs may compete with methotrexate for binding sites to serum protein, including adriamycin, bleomycin, cyclophosphamide and salicylate [17]. Studies in mice have shown that toxicity of methotrexate is inversely correlated with the degree of plasma protein binding [18]; in studies using leukemic cells in tissue culture, uptake of MTX has been reduced by the addition of protein binders [19]. These findings indicate that serum protein plays an important role in altering the cellular pharmacokinetics of methotrexate.

Probenecid has been shown to prolong and

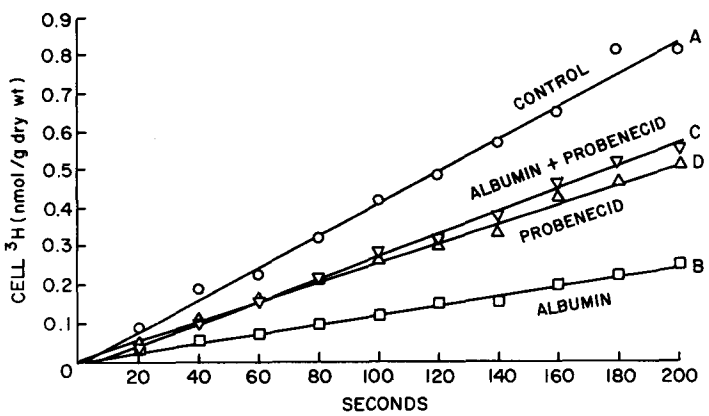


Fig. 6. Effect of probenecid on [³H]MTX influx in the presence and absence of albumin. The unidirectional uptake of 1 μM [³H]MTX was assessed in incubation buffer alone (A), in the presence of 4 g/100 ml albumin (B), in the presence of albumin + probenecid (C) or in the presence of 0.5 mM probenecid (D). Lines were drawn by least squares linear regression analysis.

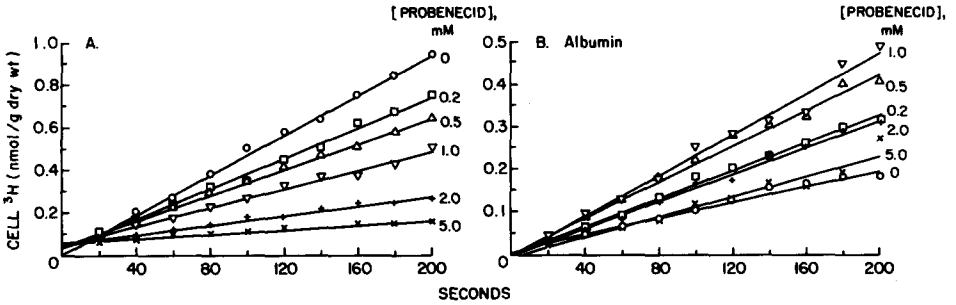


Fig. 7. Effects of increasing concentrations of probenecid on [$^3$H]MTX influx in the presence and absence of albumin. Influx of 1 $\mu$M [$^3$H]methotrexate was assessed in the presence of increasing concentrations of probenecid (0.2 to 5 mM). Panel A: Incubation buffer. Panel B: Incubation buffer containing 4 g/100 ml albumin. Lines were drawn by least squares linear regression analysis.

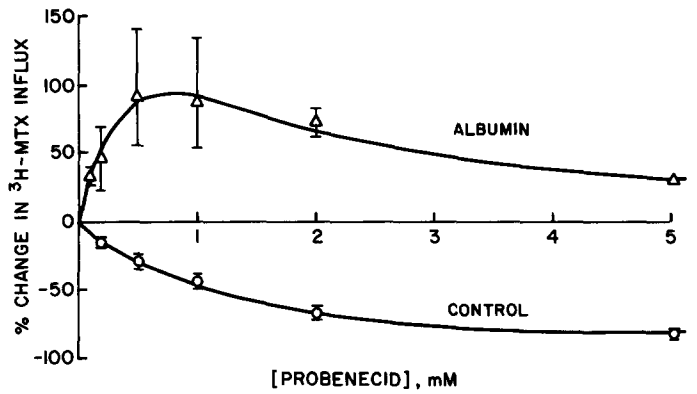


Fig. 8. Summary of the relationship between probenecid concentration and [$^3$H]MTX influx in the presence and absence of albumin. Each point represents the mean ± S.E. of three to four experiments.

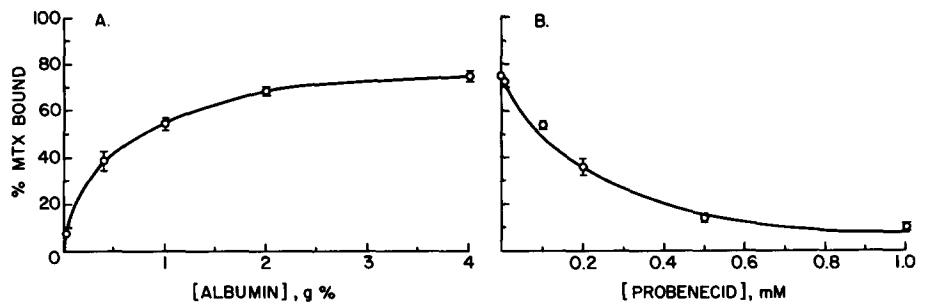


Fig. 9. Equilibrium dialysis binding of [$^3$H]MTX to bovine serum albumin. Panel A: Percent of [$^3$H]-MTX (1 $\mu$M) bound is plotted as a function of increasing concentrations of albumin in the dialysis bag. Panel B: Effect of probenecid on binding of 1 $\mu$M [$^3$H]MTX to 4 g/100 ml albumin. Each point represents the mean ± S.E. for four experiments.

enhance the levels of serum methotrexate [20, 21], presumably by interfering with renal and hepatic excretory pathways [22–24]. Probenecid interferes with efflux of methotrexate from tumor cells *in vitro* [3, 25] and elevates tumor MTX levels in mice *in vivo* [4]. However, an understanding of the mechanism of interaction of probenecid with methotrexate *in vivo* is incomplete without an examination of how serum proteins alter the interaction among MTX, probenecid and cells.

The present studies demonstrate the critical importance of providing serum protein binders for *in vitro* studies of drug pharmacokinetics—in particular where drug interaction may result in alterations in drug binding. In the absence of albumin, a concentration of probenecid as low as 0.1 mM elevated

intracellular MTX levels in Ehrlich ascites tumor cells by inhibition of MTX efflux, with minimal effects on MTX influx. In the presence of albumin, this concentration of probenecid minimally affected MTX efflux, since probenecid is effectively bound to the albumin [9]. However, *net* MTX accumulation *was* enhanced, presumably by effecting the dissociation of MTX from albumin binding sites and thereby elevating the free MTX concentration in the incubation medium. While intracellular MTX levels were elevated in either case, the concentration profile for the effects of probenecid was markedly different, as was the mechanism by which probenecid was achieving its ultimate effects. In the absence of albumin, maximal enhancement of cellular MTX levels occurred with 0.5 mM probenecid; in the presence of albumin, 3 mM probenecid was required to achieve this effect. This profile of activity was similar when probenecid was added simultaneously with the MTX or after the cells had achieved a steady state.

An explanation for the differing profiles of activity for probenecid in the absence and presence of albumin is the binding of probenecid by serum albumin [9], which lowers the effective concentration of probenecid available for inhibition of methotrexate efflux. However, another consequence of this binding is the apparent dissociation of methotrexate from protein binding sites. This dissociation of MTX from protein by probenecid and concomitant elevation of the extracellular methotrexate concentration is dramatically demonstrated by the stimulation of methotrexate influx in the presence of serum albumin. A levelling off in the concentration-dependent stimulation of MTX influx by probenecid occurs when significant levels of free probenecid become available to inhibit influx of methotrexate.

Maximal cellular accumulation of methotrexate in the presence of albumin occurred with 3 mM probenecid. At this concentration of probenecid, methotrexate influx was stimulated by approximately 50% (Fig. 8) while methotrexate efflux was inhibited by approximately 75% (Fig. 2). Clearly, it is the combination of probenecid effects on influx and efflux which results in maximal enhancement of net drug accumulation. At a lower concentration of probenecid (e.g. 1 mM), the stimulation of influx (~80%) would presumably play a greater role than inhibition of efflux (~35%).

Equilibrium dialysis studies clearly demonstrate that probenecid displaced methotrexate from albumin. However, it is at present unclear whether this is a direct competitive effect or simple steric interaction with the serum protein; that is, occupation of contiguous sites on the albumin by probenecid may alter the binding of methotrexate to the albumin molecule. Preliminary studies suggest this may be the case as the degree of displacement of [$^3$H]MTX from albumin by a given concentration of probenecid was not related to the concentration of methotrexate used. A recent paper by Paxton [26] demonstrates that probenecid displaces MTX from human plasma proteins, and we have confirmed this effect in our laboratory (unpublished results).

The relevance of our studies to the clinical situations remains to be defined. The concentrations of both methotrexate and probenecid used are consistent with clinical protocols. The peak plasma concentrations of probenecid achieved in man range from 0.1 to 0.5 mM [27, 28] while methotrexate concentrations may range between 0.1 and 100 $\mu$M within the first 24 hr after a single intravenous injection [20, 21]. However, methotrexate has a volume of distribution of about 90 liters at steady state [29] while the volume of distribution of drug albumin complexes is thought to be 5.5 liters [30]. To achieve significant elevation of the free circulating concentration of serum protein bound-drug, it is thought that drug must initially be greater than 90% protein bound [30]. The degree of methotrexate binding to human serum protein has been reported to be as low as 40% [26] and as high as 87% [31]. Therefore, it is difficult to predict the relative effects of probenecid in enhancing free circulating methotrexate concentrations by displacement of drug from serum protein versus elevation of circulating methotrexate by the inhibition of renal and hepatic excretion of methotrexate.

In summary, the inclusion of a protein binder in these *in vitro* studies serves to further elucidate the multiple interactions of probenecid with the cellular pharmacokinetics of methotrexate. In addition to inhibition of MTX efflux with low concentrations of "free" probenecid, and inhibition of MTX influx with higher probenecid concentrations [3], probenecid may serve to elevate the levels of MTX in the tumor cell by causing the dissociation of MTX from serum-protein binding sites.

In more general terms, these studies demonstrate that studies of drug–cell interaction in an *in vitro* experiment model are altered by the presence of serum proteins, which would likewise modify drug–cell interaction in the whole animal.

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