REFERENCES

1. R. Hammer, C. P. Berrie, N. J. M. Birdsall, A. S. V. Burgen and E. C. Hulme, *Nature, Lond* **283**, 90 (1980).
2. R. Hammer and A. Giachetti, *Life Sci.* **31**, 2991 (1982).
3. D. W. Gil and B. B. Wolfe, *J. Pharmac. exp. Ther.* **232**, 608 (1985).
4. M. McKinney, S. Stenstrom and E. Richelson, *Molec. Pharmac.* **27**, 223 (1985).
5. A. S. V. Burgen, C. R. Hiley and J. M. Young, *Br. J. Pharmac.* **50**, 145 (1974).
6. G. Galfre, S. Howe, C. Milstein, G. Butcher and J. Howard, *Nature, Lond.* **266**, 550 (1977).
7. S. de St. Groth and D. Scheidegger, *J. Immun. Meth.* **35**, 1 (1980).
8. E. Engvall and P. Perlman, *J. Immun.* **109**, 129 (1972).
9. G. Segal and N. Klinman, *J. Immun.* **116**, 1539 (1976).
10. O. Hurko, *Archs. Biochem. Biophys.* **190**, 434 (1978).
11. M. Meyer, M. Gainer and N. Nathanson, *Molec. Pharmac.* **21**, 280 (1982).
12. Y. Cheng and W. Prusoff, *Biochem. Pharmac.* **22**, 3099 (1973).
13. N. J. M. Birdsall, A. S. V. Burgen and E. C. Hulme, *Molec. Pharmac.* **14**, 723 (1978).
14. E. C. Hulme, N. J. M. Birdsall, A. S. V. Burgen and P. Mehta, *Molec. Pharmac.* **14**, 737 (1978).
15. A. Schreiber, P. Couraud, C. Andre and A. Strosberg, *Proc. natn. Acad. Sci. U.S.A.* **77**, 7385 (1980).
16. W. Cleveland and B. Erlanger, *Proc. natn. Acad. Sci. U.S.A.* **79**, 4810 (1982).
17. K. Sege and P. Peterson, *Proc. natn. Acad. Sci. U.S.A.* **75**, 2443 (1978).

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A re-evaluation of the competitive protein binding assay for methotrexate binding to dihydrofolate reductase

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Competitive ligand binding assays are widely used for the measurement of hormone [1], vitamin [2], and drug [3] levels. For small molecular weight ligands, activated charcoal is employed frequently to adsorb ligand that is not protein bound, leaving the protein-bound portion in the supernatant fraction after centrifugation [2]. Myers *et al.* [3] utilized charcoal adsorption and centrifugation steps in their assay for methotrexate (MTX), an important antineoplastic agent. In recent experiments designed to measure the "on" and "off" rates of methotrexate with its target enzyme, dihydrofolate reductase (DHFR), the binding protein used in drug assays, we noted that the stability of this complex was highly dependent on the concentration of free MTX and its cofactor NADPH, both of which are required for the formation of a stable ternary complex with the enzyme. At low NADPH concentration, the complex rapidly dissociated. The addition of charcoal to solutions containing ternary complex led to rapid dissociation of the complex through adsorption of free NADPH, a problem aggravated by the time required for centrifugation in order to separate charcoal from the aqueous solution. In this report we present a filtration-based method for separating protein-bound and free methotrexate; this method permits a more rapid and, therefore, more accurate quantitation of the drug–enzyme–NADPH complex for purposes of drug assay and biochemical study.

Materials and methods

Charcoal (acid-washed), albumin (fraction V), and dextran (mol. wt. 200,000–400,000) were purchased from the Sigma Chemical Co. (St. Louis, MO). Unlabeled MTX was obtained from the Drug Research and Development Branch of the National Cancer Institute (Bethesda, MD). [3′,5′,9(n)-^{3}H]Methotrexate (18 Ci/mmole) was purchased from Moravek Biochemicals (Brea, CA). NADP[^{3}H] (5 Ci/mmole) was a gift of Dr. James Phang of the National Cancer Institute. Acro-LC13 disposable filters (0.45 μm) were obtained from Gelman Scientific (Ann Arbor, MI). Human DHFR [4] was a gift of Dr. Bernard Kaufman of the National Cancer Institute (sp. act. 27.3 μmoles/min/mg at 37°). *Lactobacillus casei* DHFR was purchased from the New England Enzyme Center (Boston, MA) (sp. act. 0.64 μmole of tetrahydrofolate formed/min/mg at 37°).

MTX binding assay. Ternary complex was formed with tritiated MTX, enzyme, and 10^{-4} M or 10^{-8} M NADPH. After ternary complex formation reached an equilibrium (10 min), the unbound drug was removed by adsorption to a mixture of albumin–dextran-coated charcoal. All assays were performed at 23° and were carried out by the sequential addition of the following: (1) 0.1 to 1.0 nCi [^{3}H]MTX in 150 μl of water; (2) 200 μl of aqueous solutions containing various concentrations of unlabeled MTX in water; and (3) 50 μl of 0.5 M potassium phosphate buffer, pH 6.2 or 7.2, containing 0.0027 units of DHFR and 5×10^{-8} or 5×10^{-13} moles of NADPH. Following addition of all assay components, tubes were vortexed and allowed to equilibrate for 10 min. To each tube was then added 50 μl of a charcoal suspension prepared as follows: Norit A-activated untreated charcoal, 10 g; (2) bovine serum albumin, fraction V, 2.5 g; and (3) high molecular weight dextran, 0.1 g in a total volume of 20 ml. The pH of the charcoal suspension was adjusted to either 6.2 with 1 N HCl or pH 7.2 with 1 N NaOH immediately prior to use. After addition of the charcoal, the tubes were processed in one of two ways: (1) *Conventional method:* Tubes were vortexed, allowed to stand for up to 10 min, and then centrifuged at 1200 *g* for 30 min. A 200-μl aliquot of supernatant fraction was added to 10 ml of scintillant (Ready-Solv, Beckman) and counted in a Searle liquid scintillation counter. (2) *Filtration method.* Tubes were vortexed and then transferred to a 3-ml syringe fitted with a Gelman Acro-LC13 filter; at exactly 60 sec after addition of charcoal, the sample was filtered into a counting vial, thus separating the charcoal containing non-enzyme-bound [^{3}H]MTX from that associated with the ternary complex.

Charcoal adsorption of NADPH and MTX. To demonstrate that NADPH is adsorbed by charcoal, [^{3}H]-NADPH was added to unlabeled NADPH to obtain final concentrations of 10^{-9} M to 10^{-4} M. A 0.45-ml aliquot of various concentrations of labeled NADPH solutions was adjusted to pH 6.2 or pH 7.2. Charcoal slurry (50 μl) adjusted to the appropriate pH was added to the NADPH solutions and vortexed, and the NADPH was allowed to remain in contact with the charcoal for various amounts of time prior to separation of charcoal from the aqueous solution with an Acro-LC13 filter. In a similar fashion, solutions of [^{3}H]MTX with concentrations up to 10^{-4} M were incubated with charcoal for various amounts of time prior to separation with an Acro-LC13 filter.

Results

Since the stability of the ternary complex is dependent on the presence of a reservoir of unbound NADPH and MTX, we initially tested the ability of charcoal to adsorb NADPH and MTX. We observed a rapid adsorption of NADPH and MTX by albumin–dextran-coated charcoal. In less than 10 sec after the addition of charcoal, the concentration of NADPH and MTX in solutions with initial concentrations of up to 10^{-4} M was reduced to less than 10^{-11} M. To reduce the duration of exposure of the complex to charcoal, we separated the charcoal from the aqueous phase by use of an Acro-LC13 filter. The filter retained 99% of [^{3}H]MTX bound to charcoal while allowing recovery of 99% of complex.

We next determined the stability of ternary complex in the presence of charcoal for up to 20 min as compared to the stability of the ternary complex as measured by the filtration method, which allows for a briefer (1-min) period of charcoal exposure. Tubes containing ternary complex at pH 7.2 were prepared as described in Materials and Methods and allowed to reach equilibrium for 30 min. Charcoal was added to one tube at time 0, and aliquots were taken from the solution at various intervals up to 20 min and immediately filtered through an Acro-LC13 filter to ascertain the concentration of [^{3}H]MTX remaining in the ternary complex. A second tube contained the same components as above except the charcoal. In this tube at various times up to 20 min, aliquots were taken, charcoal was added, and the mixture filtered after exactly 60 sec. Figure 1 illustrates the inherent stability of the ternary complex over time in the absence of charcoal and its rapid deterioration after the addition of charcoal ($T_{\frac{1}{2}}$ = 9 min).

Because the addition of charcoal caused an immediate decrease in free NADPH and MTX concentration and a rapid destabilization of the ternary complex, we compared the equilibrium constant (K_D) for MTX binding to DHFR, using either the centrifugation or the Acro-LC13 filtration method to separate bound from free MTX. Table 1 details the K_D values obtained by Scatchard analysis [5] of binding curves generated using either *L. casei* or purified human DHFR. The greatest difference in K_D using the two processing methods was apparent when using the purified human DHFR at pH 7.2. Under these conditions the change in methodology indicated a 70-fold difference in the apparent K_D (3.8×10^{-9} for the centrifugation method vs 5.8×10^{-11} for the filter method). Comparisons at pH 6.2 using either of the two enzyme sources produced less dramatic differences but, nonetheless, disclosed lower K_D values (greater affinity of MTX for DHFR) when the filtration method was used to separate bound from free MTX.

To further illustrate the utility of the filtration method versus centrifugation, we compared the sensitivity of MTX measurements using these two methods. Figure 2 illustrates the MTX binding curves generated in the process of measuring MTX levels using the above-mentioned methods for separating bound from free ligand. The filtration method had a 5-fold lower limit of sensitivity (1×10^{-9} M) as compared to the sensitivity of the conventional centrifugation method (5×10^{-9} M) and had a steeper slope over the critical MTX concentration range of 10^{-7} to 10^{-9} M.

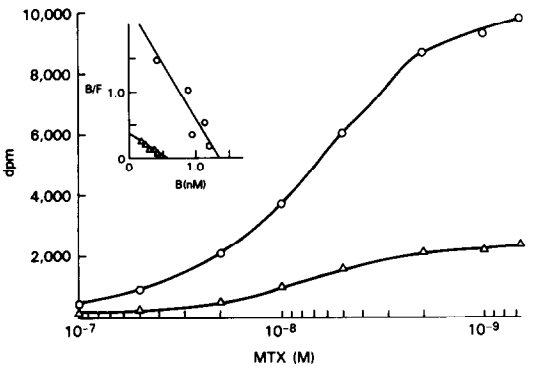

Fig. 2. Competitive binding curves for quantitating standard MTX concentrations using either the centrifugation method or the filtration method. Concentrations of unlabeled MTX standards were quantitated by competition with 1 nCi [^{3}H]MTX for binding 2.7×10^{-3} units of DHFR in the presence of NADPH (10^{-4} M). Following equilibrium, the unbound ligand was separated from enzyme-bound ligand by either the centrifugation method (△——△) or the filtration method (○——○). The graph depicts the amount of [^{3}H]MTX (dpm) bound to DHFR as a function of the concentration of standard amounts of unlabeled MTX. The inset represents a Scatchard analysis of the two curves.

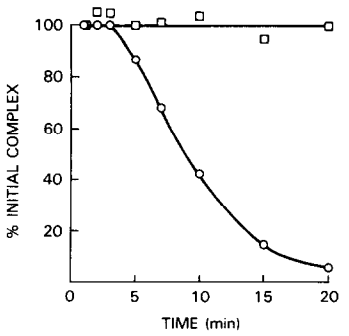

Fig. 1. Stability of ternary complex exposed to charcoal. The stability of NADPH–DHFR–MTX ternary complex was measured over time in the absence of charcoal (□——□) and in the continuous presence of charcoal (○——○). Following ternary complex formation, unbound ligand was separated from protein-bound ligand with charcoal and the isolated ternary complex made 100 mM in NADPH. The complex/NADPH solution was then either resuspended in charcoal (○——○) or not resuspended in charcoal (□——□), and the residual ternary complex measured over time.

Discussion

The above data show that the stability of ternary complex formed by MTX–DHFR–NADPH is dependent on the length of exposure to activated charcoal. Since charcoal is used frequently to separate unbound ligand from that bound to protein for kinetic experiments and serum measurement of MTX levels, we have investigated the effect of charcoal on various factors that affect the stability of the ternary complex. Kamen and co-workers [6] reported that the presence of 10^{-4} M NADPH results in a 100-fold enhanced affinity of MTX for DHFR when compared to its affinity at NADPH levels of less than 10^{-7} M. Clearly, the degree of MTX binding to DHFR will also depend on the concentration of MTX. As Fig. 1 illustrates, the concentrations of unbound MTX and NADPH were essentially unmeasurable following exposure to charcoal for as little as 10 sec. It is likely that the destabilization of the ternary complex exposed to charcoal was the result of diminishing concentrations of free NADPH and free MTX.

Using a filter technique designed to minimize exposure of ternary complex to activated charcoal and yet ensure complete removal of unbound labeled ligand, we demonstrated a markedly increased accuracy in determining the

Table 1. Dissociation constants of human and *L. casei* DHFR using the centrifugation versus the filtration methods

	Human DHFR (purified) K_D (nM)	*L. casei* DHFR K_D (nM)
pH 7.2		
Centrifugation method	3.8	2.1
Filtration method	0.058	0.57
pH 6.2		
Centrifugation method	0.5	1.04
Filtration method	0.094	0.54

All experiments were performed at 10^{-4} M NADPH. Dissociation constants were measured by Scatchard analysis. Conditions for complex formation and processing for both methods are detailed in the text.

actual amount of MTX bound to DHFR at a given ligand concentration. Exposure to charcoal for 1 min allowed complete adsorption of all unbound ligand while maintaining the maximum concentration of ternary complex, as the complex was relatively stable for 3 min following charcoal exposure (Fig. 2), We applied this new technique to a study of MTX binding constants (K_D) to DHFR purified from both a human and a bacterial source and compared these results to those obtained in parallel experiments utilizing centrifugation to separate bound from free drug. As Table 1 shows, the method used for measuring ternary complex formation produced constants of dissociation that differed by up to 70-fold, depending on the assay conditions and enzyme source. The lower affinity figure likely reflected the binding constant of MTX to DHFR at low concentrations of NADPH.

The filtration method not only improved the accuracy of determination of binding constants but also enhanced the sensitivity by at least 5-fold. A gain in sensitivity also occurred in the MTX concentration range of 10^{-7} to 10^{-9} M due to the greater steepness of the binding curve (Fig. 2). This range is important in that drug concentrations above 10^{-8} M are cytotoxic for bone marrow myeloid precursor and dividing cells of the gastrointestinal epithelium [7].

In summary, the use of rapid separation techniques that minimize the exposure of the DHFR–NADPH–MTX complex to activated charcoal allows a more accurate measurement of this complex. This technique facilitates the study of kinetic interactions of DHFR with MTX and the measurement of MTX concentrations.

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REFERENCES

1. U. Westphal, *Steroid-Protein Interactions*. Springer, New York (1971).
2. S. Waxman and C. Schreiber, *Blood* **42**, 281 (1973).
3. C. E. Myers, M. E. Lippman, H. M. Eliot and B. A. Chabner, *Proc. natn. Acad. Sci. U.S.A.* **72**, 3683 (1975).
4. B. T. Kaufman and V. F. Kemener, *Archs Biochem. Biophys.* **179**, 420 (1977).
5. G. Scatchard, *Ann. N.Y. Acad. Sci.* **51**, 660 (1949).
6. B. A. Kamen, W. Whyth-Bauer and J. R. Bertino, *Biochem. Pharmac.* **32**, 837 (1983).
7. B. A. Chabner and R. C. Young, *J. clin. Invest.* **52**, 1804 (1973).

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Genetic differences in inhibition of 2-aminofluorene *N*-acetyltransferase activity between C57BL/6J and A/J mice

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Competitive inhibition of one or more of the metabolic steps in the activation of chemical carcinogens is a potential chemopreventive mechanism. The first step in the metabolic activation of AF* to reactive electrophiles, and a potential point for control, is N-acetylation to AAF by liver NAT [1]. Differences in AF NAT activity are under genetic control in C57BL/6J and A/J mice [2], and differences in human arylamine N-acetylating capacity have been correlated with differences in susceptibility to urinary bladder cancer [3–5]. In the present study, we wanted to identify relatively non-toxic, preferably competitive inhibitors of liver AF NAT, and to determine if there was a differential susceptibility to the effect of these inhibitors between C57BL/6J and A/J mice. This paper presents evidence that the beta-carboline derivatives have no effect on NAT, and that PABA competitively inhibits, and folic acid and MTX noncompetitively inhibit NAT. In addition, there was a statistically significant difference in K_i values for folic acid and MTX between C57BL/6J and A/J NAT.

* Abbreviations: AF, 2-aminofluorene; AAF, 2-acetylaminofluorene; NAT, *N*-acetyltransferase; PABA, *p*-aminobenzoic acid; MTX, methotrexate; NF, 2-nitrofluorene; DTT, dithiothreitol; K_i, inhibition constant; TCA, trichloroacetic acid; TFA, trifluoracetic acid; DMSO, dimethyl sulfoxide; and HPLC, high performance liquid chromatography.

Materials and methods

Chemicals. AF was purchased from K & K Laboratories, Plainview, NY; AAF from the Aldrich Chemical Co., Milwaukee, WI; acetyl CoA from P-L Biochemicals, Inc., Milwaukee, WI; folic acid from the Nutritional Biochemical Corp., Cleveland, OH; and PABA, MTX, harmine, har-