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Serum binding of methyltetrahydrofolic acid

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The binding of folates to human plasma is complex [1–4]. However, the exact percentage of bound and free folate in human plasma *in vivo* is unknown. It might be important to determine the per cent of unbound plasma folate as opposed to total plasma folate, because only unbound plasma folate could readily pass into the tissues [5]. An example of a drug where it is helpful to measure the unbound as opposed to the total plasma drug level is diphenylhydantoin [6]. Therapeutic results are better correlated with the unbound diphenylhydantoin in plasma than with the total plasma diphenylhydantoin level [6].

The purpose of this study was to measure the percentage of unbound folate in fresh human serum and plasma under various conditions. This was accomplished by utilizing a rapid, ultrafiltration method that controls pH and protects labile substances from oxidation [5, 7]. To simulate plasma folate levels after the oral ingestion of folates, radiolabeled methyltetrahydrofate (MeTHF) was added to fasting plasma or serum. It is known that ingested folates are rapidly converted in the body to MeTHF, the principal folate of human plasma [8].

^{14}C-(±)-L-*N*-5-MeTHF (60 mCi/m-mole) and ^{3}H-folic acid (16 Ci/m-mole) were obtained from Amersham/Searle. ^{3}H-(+)-L-*N*-5-MeTHF (170 mCi/m-mole) was extracted and purified from fresh rat liver the day after the injection of 0·2 mCi ^{3}H-folic acid [9, 10]. Nonradioactive (±)-L-*N*-5-MeTHF was obtained from Sigma Co.

The methods employed to ultrafilter serum or balanced salt solutions have been described previously [5, 7]. Briefly, 2·5-ml samples of a balanced salt solution or serum containing radiolabeled MeTHF were placed in a 2·5-ml chamber. With constant stirring and the application of a constant pressure head of 5 pounds per square inch (p.s.i.) using a 95% N_2 and 5% CO_2 gas mixture, the balanced salt solution or serum (termed the parent solution) was ultrafiltered through a Millipore filter (PSED-01310) at 23°. The first 200 μl of ultrafiltrate was discarded and the second 200-μl aliquot retained for analysis. Recovery of ^{3}H- or ^{14}C-MeTHF in the second 200-μl aliquot of ultrafiltrate in the absence of protein was determined by adding ^{3}H- or ^{14}C-MeTHF to balanced salt solution to yield final concentrations of 2 ng/ml, 25 ng/ml and 20 μg/ml. The balanced salt solution also contained 5·0 mg sodium ascorbate and 0·2 mg thiourea per ml to protect the MeTHF from oxidation. Duplicate 100-μl aliquots of the second 200 μl of ultrafiltrate as well as the parent solution in the chamber (before and after the ultrafiltration) were assayed for radioactivity [5, 7].

To determine serum binding, fresh serum was obtained from two healthy overnight-fasted male donors. Immediately after centrifugation, 2 ml serum was added to 0·5 ml of a balanced salt solution. After mixing, an appropriate amount of ^{14}C- or ^{3}H-MeTHF (1–10 μl) was added, and this solution (the parent solution) was ultrafiltered. Two 100-μl aliquots of parent solution (before and after the filtration) and ultrafiltrate were assayed for radioactivity. The fraction of free radiolabeled MeTHF was calculated after correction for quench and nonspecific binding by the filter (*vide infra*) by dividing the dis/min in the filtrate by the average dis/min in the parent solution taken before and after the filtration. In another series of experiments, no radioactivity was added to the fasting serum, and the folate activity in the parent solution and ultrafiltrate was assayed by a competitive protein binding assay using β-lactoglobulin of milk [11].

Experiments were also performed to measure the ^{14}C-MeTHF binding to fresh human serum by ultrafiltration with the following variations: the temperature was decreased to 4° or increased to 43°; or sodium diphenylhydantoin or sodium salicylate or sodium probenecid was added to the parent solution to achieve concentrations of 0·2, 3·0 or 1·0 mM respectively. In order to measure ^{14}C-MeTHF binding to solutions other than fresh human serum, another series of experiments was performed with the following solutions substituted for fresh human serum: frozen human serum (1 year old), fresh human plasma, fresh rabbit plasma, and recrystallized albumin (4 times) at a concentration of 4 g/100 ml of balanced salt solution, which also contained 0·1 mg/ml of thiourea and 0·2 mg/ml of sodium ascorbate to protect the MeTHF from oxidation.

In order to be certain that no conversion or deterioration of the ^{14}C- or ^{3}H-MeTHF occurred during the ultrafiltration, both isotopes were submitted to paper chromatography [Whatman No. 1 paper; 0·1 M potassium phosphate buffer, pH 6, with 0·5% (v/v) mercaptoethanol (M.E.)] and/or thin-layer cellulose chromatography in two systems: (1) 3% NH_4Cl (w/v), pH 6·2, with 0·5% M.E.; and (2) 0·1 M potassium phosphate buffer, pH 6, with 0·5% M.E. [12]. In all three systems, the racemic ^{14}C-MeTHF received from the company (and dissolved in 2 ml distilled H_2O with 4 mg sodium ascorbate, pH 6) appeared as a single peak corresponding to carrier MeTHF. In six samples of ultrafiltrate, the ^{14}C appeared as a single peak corresponding to carrier MeTHF. In the two thin-layer systems, the naturally occurring stereoisomer, (+)^{3}H-MeTHF, appeared as two peaks with greater than 90 per cent appearing on a large peak cor-

Table 1. Per cent unbound MeTHF in serum

Conc added MeTHF*	% Unbound	Experimental condition†	% Unbound
7 ng/ml (±) ^{14}C-MeTHF	30 ± 3 (3)‡	Fresh human serum at 23°	31 ± 2 (9)
30 ng/ml (±) ^{14}C-MeTHF	31 ± 2 (9)	Fresh human serum at 4°	32 ± 2 (4)
30 ng/ml (±) ^{14}C-MeTHF		Fresh human serum at 43°	41 ± 2 (3)
20 μg/ml MeTHF	24 ± 4 (4)	Fresh human serum with 0·2 mM dilantin	26 ± 4 (3)
2 ng/ml (+) ^{3}H-MeTHF	36 ± 6 (5)	Fresh human serum with 1·0 mM probenecid	45 ± 4 (4)
		Fresh human serum with 3·0 mM salicylate	47 ± 4 (4)§
		Fresh human plasma	25 ± 4 (3)
		Frozen human serum	42 ± 2 (4)
		Human albumin, 40 mg/ml	40 ± 2 (3)
		Fresh rabbit plasma	57 ± 5 (3)§

* (±)^{14}C- or (+)^{3}H-MeTHF and/or carrier MeTHF was added to fresh human serum which was immediately filtered at 23° with 95% N_2 and 5% CO_2.

† 30 ng/ml (±)^{14}C-MeTHF was added to each sample of fresh human serum or other solution and immediately ultrafiltered at 23° with 95% N_2 and 5% CO_2, except as noted.

‡ The % unbound is the mean per cent of the added MeTHF unbound ± the standard error of the mean. The numbers in parentheses indicate the number of experiments at each point.

§ The means so indicated differ significantly from the % unbound (±)^{14}C-MeTHF in fresh human serum at 23° with $P < 0.05$ by Scheffe's method for multiple comparisons in the Gaussion analysis of variance [15].

responding to carrier MeTHF. None of the ^{3}H-MeTHF was volatile.

MeTHF did not pass quantitatively through the filter in balanced salt solution due to nonspecific binding by the cellulose base on which the filtering membrane rested [5, 7]. Thus, with 2 ng/ml of MeTHF in the balanced salt solution, only 67 ± 5 per cent (n = 5; S.E.M.) passed through in the second 200-μl aliquot; at 25 ng/ml, 88 ± 3 per cent (n = 6; S.E.M.) passed through in this aliquot. Therefore, the amount of MeTHF passing through the filter needed to be corrected for the concentration-dependent nonspecific binding.

The effect of diluting 2·0 ml serum or plasma with 0·5 ml buffer before the filtration was considered unimportant because the first 0·2 ml of ultrafiltrate was discarded and there was approximately a 0·1-ml dead space under the filter [5, 7]. Therefore, the concentration of protein in the parent solution during the collection period was approximately that of undiluted serum or plasma.

In Table 1, on the left, the percentages of unbound ^{14}C-(±)-MeTHF in serum as a function of the concentration of added ^{14}C-(±)-MeTHF are indicated. Also indicated is the percentage of unbound ^{3}H-(+)-MeTHF. Thus, increasing the concentration to 20 μg/ml of (±)MeTHF or employing the naturally occurring stereoisomer did not affect the binding. In Table 1, on the right, there are indicated the unbound percentages of MeTHF under various experimental conditions. Only 3 mM sodium salicylate and substitution of fresh rabbit plasma for fresh human serum significantly altered MeTHF binding under these experimental conditions at room temperature.

Determination of endogenous folate binding to human plasma using the competitive binding assay with β-lactoglobulin to measure the folate in the parent solution and in the second 200 μl aliquot of the ultrafiltrate [11] yielded different results. At 23° under 95% N_2 and 5% CO_2 at 5 p.s.i. and with no correction for nonspecific binding by the filter, 55 ± 1 per cent (n = 3; S.E.M.) of the folate activity determined by the competitive assay was unbound. The total serum folate level was 6·0 ± 0·1 ng/ml (n = 3; S.E.M.).

The binding of folates to serum proteins is complex. Folate binding to albumin and to two other serum proteins (an α_2-macroglobulin and probably transferrin) has been documented [1–4]. At least 40 per cent of serum folate was shown to be bound [4]. However, these conclusions were based on gel filtration studies and, as mentioned by the author [1], might underestimate folate binding, since the folate–protein complex may dissociate as the complex passes through the column. This has been shown to occur with salicylate binding to human plasma [7]. Also, the folate in the serum and in the various fractions as they came off the gel column was measured by a microbiological assay [1–4]. However, the standardization, specificity and accuracy of the microbiological assay are uncertain [13]. Moreover, these studies were performed at 4° or 10° over approximately 20 hr at pH 7·0 [1–4]. In the present study, we avoided the nonphysiological conditions associated with gel filtration by employing a rapid ultrafiltration method [5, 7]. The pH was controlled at 7·4 and the binding of added radiolabeled MeTHF was measured within 2 hr on fresh serum at a low applied pressure under 95% N_2 and 5% CO_2 so that no oxidation of the labeled MeTHF occurred. In addition, the uncertainty associated with the microbiological assay for folates has been avoided by employing radiolabeled MeTHF and scintillation counting of the ultrafiltrate and parent solution [5, 7, 13].

The data herein reported confirm the conclusion that a significant amount of MeTHF is bound in human serum [1] (Table 1). Increasing the concentration to 20 μg/ml of (±)MeTHF did not alter the percentage bound, nor did (+)-MeTHF bind differently from (±)MeTHF (Table 1). Thus, as with the folate binding β-lactoglobulin of milk [13] and the folate binding protein of hog kidney [14], there was no demonstrable stereospecificity for MeTHF binding by fresh human serum. Moreover, most of the MeTHF binding could be accounted for by albumin (Table 1). Only rabbit

plasma and the addition of toxic doses of salicylate to human serum altered the binding of MeTHF (Table 1). It is possible, however, that, if a larger number of experiments had been performed, the binding of added (±)MeTHF to frozen serum or to serum at 43° or to serum to which probenecid was added might have differed significantly from that of controls. Similar effects of salicylate on the binding of other weak carboxylic acids has been reported [7].

This study, however, documents only the binding of added MeTHF to normal fasting serum and the binding of endogenous folate when measured by the competitive binding method with β-lactoglobulin [11]. The explanation for the different values obtained for folate binding by these two methods is not clear. Moreover, extrapolation of these results to folate-depleted patients may not be warranted [11, 14].

In conclusion, added MeTHF is 60–70 per cent bound to fresh serum of normal men after an overnight fast. Albumin could account for the major portion of the binding and the binding did not appear to be stereospecific. Drugs including diphenylhydantoin, probenecid and salicylate have only a minimal, if any, effect on added MeTHF binding at therapeutic dosages (Table 1). Moreover, increasing the concentration of MeTHF in serum by over a thousand times did not alter the percentage of MeTHF bound.

Departments of Medicine, Pharmacology, Radiology and Neurology,
Harvard Medical School,
The Children's Hospital Medical Center and
The Peter Bent Brigham Hospital,
Boston, Mass. 02115, U.S.A.

REYNOLD SPECTOR
A. V. LORENZO
DAVID E. DRUM

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Substrate and inhibitor kinetics of bovine phenylethanolamine-*N*-methyltransferase

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The methylation of norepinephrine, the final step in the synthesis of epinephrine, is catalyzed by phenylethanolamine-*N*-methyltransferase (PNMT). This enzyme was partially purified from monkey adrenal gland [1] and was found to have an exclusive specificity for both ring-substituted and unsubstituted phenylethanolamines. Subsequent studies [2] also pointed out that β-hydroxyphenylethylamines (phenylethanolamines) are much better substrates than the unsubstituted phenylethylamine derivatives (> 100:1), although the latter are methylated to a limited extent. Using PNMT isolated from the rabbit, Fuller *et al.* [3] showed that β-keto dopamine (arterenone) and 3,4-dichloro-β-amino-phenylethanolamine are also suitable substrates, suggesting that nucleophilic groups other than hydroxyl can interact with the enzyme. Recently, Laduron [4] reported the methylation of dopamine by PNMT. This paper will report on the substrate specificity and the inhibitor kinetics of PNMT using a purer enzyme preparation than other studies have utilized.

Bovine PNMT was purified by a modification of the method of Connett and Kirshner [5], as described by Ciaranello and Axelrod [6]. Briefly, protein from adrenal medullae that precipitates at an ammonium sulfate concentration of 30–60% was taken up in 50 mM potassium phosphate buffer, pH 7·2, and made 0·5 mM in dithiothreitol. After dialysis against the same buffer, the protein solution was adjusted to pH 5 and centrifuged. The pH of the supernatant was then brought up to 7, and the material was again dialyzed as before. Afterwards, the solution was applied to a G-100 Sephadex column. The peak, corresponding to a molecular weight of 40,000 was applied to a DEAE-Sephadex A-50 ion-exchange column. When the peak fractions from this column were pooled, concentrated and subjected to sodium dodecyl sulfate-acrylamide electrophoresis [6], a single staining band was observed. PNMT was assayed as previously described [1]. Each reaction tube contained 5 μmoles potassium phosphate buffer, pH 7·9, 25 μg protein, 2 nmoles ^{14}C-*S*-adenosylmethionine (52·3 mCi/