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MOUSE BRAIN CONTAINS A HIGH MOLECULAR WEIGHT NONFUNCTIONAL PROTEIN WITH ANTIGENIC HOMOLOGY TO DIHYDROFOLATE REDUCTASE [1]

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Summary: The cytosol of brain tissue from mature BDF_1 mice contains very little dihydrofolate reductase activity but it does contain a high molecular weight nonfunctional protein which cross-reacts in a radioimmunoassay for the active enzyme. Liver cytosol contains less of this high molecular weight cross-reacting protein and more of the functional dihydrofolate reductase. The cytosol from kidney contains very little of the high molecular weight cross-reacting protein, 95% of the immunoreactive proteins being the functional form of dihydrofolate reductase. The modification of dihydrofolate reductase into a nonfunctional form may be an intrinsic property of some cells and this finding could explain the variability in measuring the activity of this enzyme in brain tissue of mature animals.

Dihydrofolate reductase (DHFR, EC 1.5.1.3) activity has been detected in the brain of a number of mammalian species (1-4) but the level of activity appears to be lower than observed in other tissues such as liver (5) or kidney (6). Makulu et at. (7) were unable to measure DHFR activity in newborn and adult rat brain and Duch and coworkers (8) could not detect activity in brain tissue from rats older than 3 weeks of age. Although these differing reports of DHFR in brain tissue reflect in part differences in methodology for measuring the activity of this enzyme, another possibility is that some intracellular process modifies the structure of the enzyme into some inactive form.

Radioimmunoassay (RIA) provides a method for identifying and quantifying proteins, such as enzymes, even if they lack function. This method has recently been used to detect a high

Abbreviations: MTX, methotrexate; PMSF, phenylmethylsulfonyl fluoride; DHFR; dihydrofolate reductase; RIA, radioimmunoassay; CRP, cross-reacting protein.

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molecular weight non-functional but immunoreactive form of DHFR in L1210 leukemia cells (9). This cross-reacting protein (CRP) also had the same isoelectric points as DHFR indicating that both proteins must be structurally homologous. In this report we present the results of a study to assay the immunoreactive forms of DHFR in normal adult mouse brain, liver, and kidney, using this RIA.

Materials and Methods

[^{3}H]Methotrexate (MTX) (specific activity, >5Ci/mmole), and sodium [^{125}I] iodide were purchased from Amersham/Searle Corp., Arlington Heights, Ill. NADPH and phenylmethylsulfonyl fluoride (PMSF) were purchased from the Sigma Chemical Co., St. Louis, Mo. MTX was obtained through the courtesy of Lederle Laboratories, Inc., Pearl River, N.Y., and was purified by the method of Gallelli and Yokoyama (10). Sephadex G-75 was obtained from Pharmacia Fine Chemicals, Piscataway, N.J. Trasylol was obtained from the Mobay Chemical Corp., New York, N.Y. Rabbit antichicken gamma globulin was purchased from Miles Laboratories, Inc., Elkhart, Ind. All other chemicals were of reagent grade. BDF_1 mice (C57BL X DBA/2 F_1) were purchased from the Jackson Laboratories, Bar Harbor, Me.

Preparation of cytosol from mouse tissue

Five BDF_1 mice, each weighing approximately 20 gm, were sacrificed by decapitation and the liver, kidney, and brain of each animal was immediately removed, rinsed with ice cold 0.15 M NaCl, dried by filter paper and weighed. Each tissue was homogenized in 3 volumes of 0.06 M sodium citrate, pH 7.2, containing Trasylol (1000 KIU/liter) and PMSF (3.5 mg/liter). The homogenization was carried out at 4°C for 2 min using a motorized teflon homogenizer. The cytosol, obtained by centrifuging the particulate components at 100,000 x g for 1 hour at 4°C, was stored at -35°C.

Gel Filtration Chromatography

Sephadex G-75 was equilibrated with 0.05 M Tris-HCL, pH 7.4, and the column (1.5 x 75 cm) was packed at a flow rate of 13 ml/hr at 4°C. The cytosol (2.5ml) from liver, kidney, and brain was concentrated to approximately 0.75 ml by evaporation from a dialysis bag at 4°C and each preparation chromatographed through the Sephadex column, collecting 0.8 ml fractions. Each fraction was assayed for immunoreactive protein by an RIA using ^{125}I-labeled purified DHFR and a monospecific chicken antiserum to the enzyme (9), and for active DHFR by the binding of [^{3}H]MTX, as previously described (9).

Results

The cytosol of brain, liver, and kidney from these mice contained immunoreactive proteins which could be separated by filtration through Sephadex G-75. The brain cytosol (Fig. 1) contained only the high molecular weight CRP which eluted in the void volume of the column. The fractions which correspond to the elution position of active DHFR neither bound any significant [^{3}H]MTX nor contained any immunoreactive protein.

In contrast to the brain, the high molecular weight CRP constituted only 5% of the immunoreactive proteins in the kidney cytosol (Fig. 2). The major immunoreactive component (95%) eluted in the fractions which also contained the active form of DHFR, which by titration with [^{3}H]MTX, was equivalent to the concentration measured by RIA.

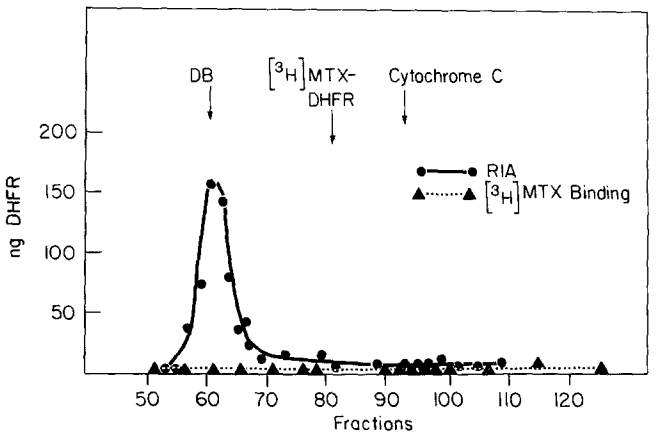

Figure 1. The chromatogram of immunoreative proteins measured by RIA, and functional DHFR measured by the binding of [^{3}H]MTX, in the cytosol of brain from normal BDF$_1$ mice after filtration through a Sephadex G-75 column. Immunoreactive protein (●—●); binding of [^{3}H]MTX (▲·····▲); D.B. is blue dextran.

The concentration of the high molecular weight CRP in the liver cytosol was approximately 1/3 of the total immunoreactive protein (Fig. 3), the remaining 2/3 corresponding to the active DHFR.

Discussion

Since DHFR is an enzyme which is required to maintain the pool of tetrahydrofolate in cells which are replicating and synthesizing thymidine by the de novo pathway, the highest level of activity of this enzyme is found in rapidly dividing cells (11). The low level of DHFR activity in brain, therefore, is quite consistent with the fact that this tissue in mature animals

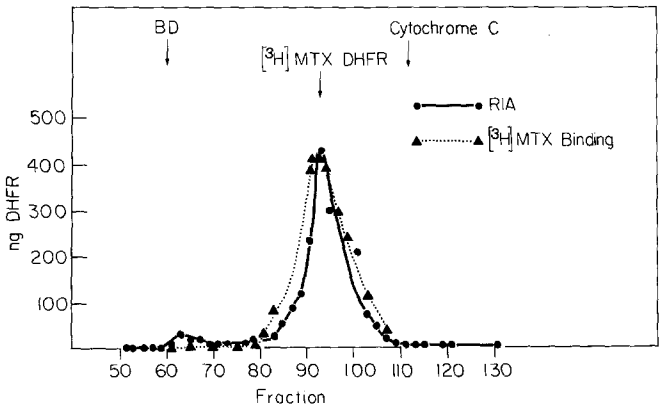

Figure 2. The chromatogram of immunoreactive proteins measured by RIA, and funtional DHFR measured by the binding of [^{3}H]MTX, in the cytosol of kidney from normal BDF$_1$ mice after filtration through a Sephadex G-75 column. Immunoreactive protein (●—●); binding of [^{3}H]MTX (▲·····▲); D.B. is blue dextran.

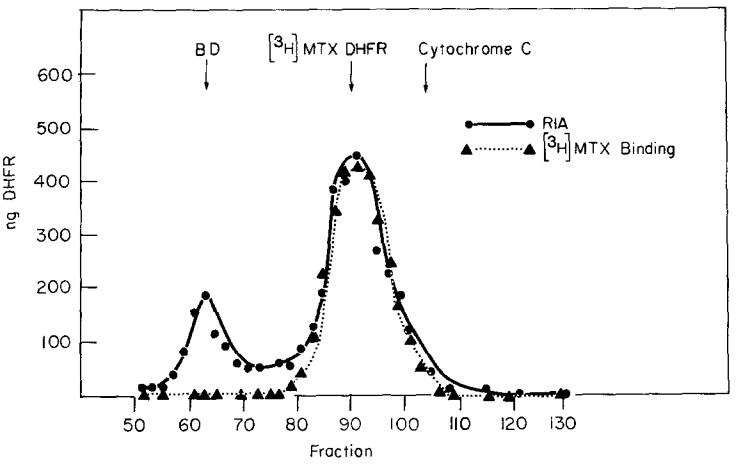

Figure 3. The chromatogram of immunoreactive proteins measured by RIA, and functional DHFR measured by the binding of [^{3}H]MTX, in the cytosol of liver from normal BDF$_1$ mice after filtration through a Sephadex G-75 column. Immunoreactive protein (●——●); binding of [^{3}H]MTX (▲·····▲); D.B. is blue dextran.

contains few replicating cells. Similarly, the high level of DHFR activity in kidney tissue is quite consistent with the fact this tissue contains a substantial fraction of rapidly dividing tubular epithelium. The finding that the high molecular weight CRP in brain and kidney is reciprocally related to the level of the DHFR suggests that the process by which the active enzyme is modified into this nonfunctional derivative is in some way related to the rate of cell replication. A similar protein has been found in L1210 murine leukemia cells (9) and its concentration in the cytosol varied inversely with the concentration of active DHFR, being highest in early log (day 2) phase and then falling rapidly in late log and stationary growth phases (9). The disparate concentration of this high molecular weight CRP and active DHFR in these normal mouse tissues also indicates that the CRP is not an artifact resulting from an alteration of DHFR during the preparation of the cytosol because the kidney contained almost no CRP and yet had the highest concentration of active enzyme.

We have not as yet isolated the CRP from brain to determine the conditions necessary to derive active DHFR and thereby show that the process of inactivation is reversible. However, preliminary studies of the CRP isolated from the cytosol of L1210 cells indicates that it can be dissociated by treatment with 8M urea and 40 mM dithiothreital into smaller immunoreactive forms[4] indicating that disulfide bridging is the basis of the polymeric structure. These smaller

[4] Presented at the 8th International Symposium : Pteridines and Folic Acid Derivatives, Montreal, Canada, June, 1986.

immunoreactive molecules did not have catalytic activity but similar denaturing and reduction also inactivated functional DHFR indicating that the catalytic site of the enzyme is sensitive to such treatment. The mechanism by which an intracellular metabolic process may reduce these disulfide bonds is likely to be more selective, leaving the catalytic site on the molecule intact.

Even though we have not as yet shown that active enzyme may be derived from CRP, the importance of this in brain tissue merits some discussion. The post-translational modification of DHFR into an inactive form may serve a biological function of conserving the enzyme if the genetic expression of this protein diminishes as the cells reach full maturation, as, for example, brain tissue. Since approximately 8% of brain folate is dihydrofolate (13) generated probably by the non-enzymic oxidation of tetrahydrofolate (2), a low but constant level of DHFR will be needed in this tissue to maintain the pool of reduced folate. In addition, DHFR may play a role in the regulation of tetrahydrobiopterin levels in brain tissue since its immediate precursor, 7,8,-dihydrobioterin, is a substrate for this enzyme (14). The slow release of active DHFR from this inactive reservoir could subserve these functions in the mature brain.

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