Biochemical Pharmacology, Vol. 26, pp. 1507–1511. Pergamon Press, 1977. Printed in Great Britain.

IDENTIFICATION OF DIHYDROFOLATE REDUCTASE IN RABBIT BRAIN

REYNOLD SPECTOR, PETER LEVY and HERBERT T. ABELSON

Departments of Medicine and Pediatrics, Harvard Medical School, Children's Hospital Medical Center, Peter Bent Brigham Hospital, and the Sidney Farber Cancer Institute, Boston, MA 02115, U.S.A.

(*Received* 15 *October* 1976; *accepted* 30 *December* 1976)

Abstract—After partial purification, rabbit brain extracts were assayed for dihydrofolate reductase by spectrophometric, radiochemical and methotrexate binding assays. By these assays, the specific activity of rabbit brain dihydrofolate reductase was about 15 per cent that of liver. Both rabbit liver and brain dihydrofolate reductase activities were abolished by methotrexate and had comparable K_m (3 μM), pH maxima (4.8), and cofactor requirements (NADPH). *In vivo*, a small percentage of [^{3}H]folic acid was reduced to [^{3}H]methyltetrahydrofolate when injected into the left lateral ventricle. Thus, both *in vitro* and *in vivo*, the central nervous system has the ability to reduce oxidized folates.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate: NADP oxidoreductase; EC 1.5.1.3) enzymatically reduces folate and dihydrofolate (DHF) to tetrahydrofolate (THF). Dihydrofolate reductase activity was not previously detected in rabbit or other mammalian brain extracts [1]. Therefore, in our studies of dihydrofolate reductase activity in rabbit choroid plexus, we used rabbit brain as a control tissue lacking dihydrofolate reductase. However, rabbit brain was an inappropriate control tissue for we consistently detected dihydrofolate reductase activity in partially purified rabbit brain preparations.

The purposes of this report are: (1) to demonstrate that dihydrofolate reductase exists in rabbit brain in significant amounts; (2) to characterize the dihydrofolate reductase activity in brain; and (3) to show that folic acid can be converted to tetrahydrofolate in the rabbit central nervous system *in vivo* as well as *in vitro*.

METHODS AND MATERIALS

[3′,5′,9-^{3}H]folic acid (56 Ci/m-mole) and [^{3}H]-methotrexate (6 Ci/m-mole) were obtained from Amersham-Searle. The [^{3}H]folic acid required purification on Sephadex G-25 biweekly [2] and the [^{3}H]methotrexate required purification on DEAE-Sephadex [3]. (±)-L-Tetrahydrofolate and (±)-L-*N*-5-methyltetrahydrofolate (MeTHF) were obtained from Sigma, purified on DEAE-Sephadex columns in 0.2 M mercaptoethanol and stored at −20° with 2 mg/ml sodium ascorbate [4]. NADPH and NADH were obtained from Sigma and methotrexate from Lederle Laboratories. All studies were performed on New Zealand white rabbits weighing about 1.5 kg.

Dihydrofolate reductase was obtained from fresh rabbit liver, choroid plexuses from six to eight rabbits (approximately 200 mg), whole blood, whole rabbit brain and about 0.4-g samples of rabbit brain above the lateral ventricle (including cortex, white matter and periventricular grey matter). Tissues were immediately homogenized in 7–10 vol. of cold isotonic saline in scintered glass tubes and the dihydrofolate reductase activity was purified through the second ammonium sulfate precipitation step as suggested by Perkins *et al.* [5]. The enzyme-containing pellet was taken up in either water or 0.02 M sodium citrate (pH = 4.8) [6] and stored at 2°. Dihydrofolate reductase was assayed within 2 days of enzyme preparation except as discussed below.

Dihydrofolate reductase activity in the partially purified preparations described above was determined on the pellets taken up in water by the spectrophotometric method of Mathews *et al.* [7], which measures the reduction of DHF to THF by NADPH, and on the pellets taken up in sodium citrate by the radiochemical method of Rothenberg [6, 8], which measures the reduction of [^{3}H]folic acid to [^{3}H]-THF. A third, albeit indirect, method employed for measuring dihydrofolate reductase activity in the various partially purified enzyme preparations described above was a methotrexate binding assay [9].

The product of the enzymatic reduction of [^{3}H]-folic acid by NADPH [6, 8] was identified in two chromatographic systems [4, 10]. Partially purified dihydrofolate reductase was incubated for 20 min at 37°. with 2.5 or 5 pmoles [^{3}H]folic acid and 0.1 mg NADPH in 1 ml of 0.02 M citrate buffer (pH = 4.8) containing 0.01% mercaptoethanol [6]. At the end of the incubation, 10 μl mercaptoethanol and, in the case of samples chromatographed on columns, 1.0 mg sodium ascorbate as well as 50 μg carrier THF were added to the incubation mixture which was then heated for 30 min at 75°. After cooling in ice and centrifugation at 1740 *g* for 5 min, 10 μl of the supernatant was chromatographed on thin-layer cellulose plates and developed in 3% ammonium chloride (pH = 6.2) containing 0.5% mercaptoethanol [10, 11]. In some cases, 0.90 ml of the supernatant was added to 2.1 ml of 0.1 M sodium phosphate buffer (pH = 6.0) containing about 1.0 mg purified carrier THF and chromatographed on a 18 × 0.55 cm DEAE-Sephadex column equilibrated with 0.1 M sodium phosphate (pH = 6.0) containing 0.2 M mercaptoethanol [4]. The column was eluted with phosphate buffer (pH = 6.0) of increasing ionic strength containing 0.2 M mercaptoethanol [4]. Both these chromatographic techniques separate folic acid, DHF,

THF, MeTHF and *p*-aminobenzoylglutamate, a known breakdown product of THF [4, 10].

To measure the metabolism of [^{3}H]folate in the central nervous system, a hole was drilled in the skull after the induction of anesthesia with sodium pentothal, and 2.0 μCi [^{3}H]folic acid in 0.1 ml of artificial cerebrospinal fluid (CSF) was injected stereotactically into the left lateral ventricle [2]. The hole in the skull was sealed with bone wax [2]. After 2 hr, the rabbit was reanesthetized and 1 ml of cisternal CSF was withdrawn. The nature of the radioactivity in the CSF was determined on the thin-layer and column chromatographic systems described above except that, in some cases, carrier MeTHF was substituted for THF [4, 10].

The ability of rabbit brain slices to concentrate and metabolize [^{3}H]folic acid *in vitro* was also measured. Rabbit brain above the left lateral ventricle (approximately 0.7 g) was sliced into 0.25-mm pieces on a McIlwain tissue chopper and washed twice for 2 min in chilled artificial CSF [12]. About 30 mg tissue was added to 3 ml of artificial CSF and preincubated at 37° for 5 min under 95% O_2 in a metabolic shaker [11, 12]. After the preincubation, [^{3}H]folic acid was added (20 nM) and the incubation continued for 30 min. At the end of the incubation, the brain slices were filtered and washed with two 5-ml rinses of iced artificial CSF [11, 12]. The ^{3}H concentration of the slices and medium was determined [11, 12]. In one experiment, the nature of the ^{3}H within the tissue at the end of the incubation was determined by homogenizing the filtered brain slices in 1 ml of 0.01 M sodium phosphate buffer (pH = 6.5) containing 10 μl mercaptoethanol and 0.5 mg of carrier MeTHF, heating at 75° for 30 min, centrifuging and chromatographing the supernatant on the thin-layer system described above [4, 11].

RESULTS

The dihydrofolate reductase specific activities in partially purified rabbit brain, choroid plexus and liver as determined spectrophotometrically and radiochemically are shown in Table 1. Also shown in Table 1 are the pmoles methotrexate bound/mg of brain and liver protein [9]. Although indirect, methotrexate binding is an independent measure of dihydrofolate reductase [9]. As shown in Table 1, there was an excellent correlation between the results of the functional and binding assays. By all three assays, rabbit brain extracts contained about 15 per cent as much dihydrofolate reductase activity as liver/mg of protein.

Definitive evidence that rabbit brain and liver extracts can convert [^{3}H]folic acid to [^{3}H]THF was forthcoming when [^{3}H]folic acid was incubated with NADPH and the partially purified dihydrofolate reductase preparations. After a 20-min incubation, the product co-chromatographed with carrier THF in both the thin-layer and column chromatographic systems (Fig. 1) when 1 mg/ml of sodium ascorbate was added to the incubation medium at the start of the incubation [4, 10]. When sodium ascorbate was excluded, the product co-chromatographed (in the thin-layer system) predominantly with aminobenzoylglutamate, a known breakdown product of THF [8, 10].

The characteristics of the dihydrofolate reductase activity in the partially purified brain and liver enzyme preparations were similar as measured by the method of Rothenberg [6, 8]. Both brain and liver preparations required NADPH as a cofactor; when NADH was substituted for NADPH on an equal weight basis, there was no detectable dihydrofolate reductase activity in brain or liver. Whole blood in brain (about 1–2 per cent of brain weight) could not account for the dihydrofolate reductase activity in brain, since whole blood had less than 5 per cent of the dihydrofolate reductase activity of brain/mg of protein. Also, the addition of extracts of fresh whole rabbit blood (from 1 and 10 μl of whole blood) to brain extracts containing about 1 mg protein did not alter the dihydrofolate reductase activity/mg of brain protein. Methotrexate (1.0 μM) abolished totally the dihydrofolate reductase activity in both brain and liver. The pH dependence of the partially purified brain and liver dihydrofolate reductase preparations was similar. Both brain and liver had maximum activity at pH 4.8 [6, 8]. At pH 4.0 and 7.0, brain had 42 and 18 per cent respectively, of the activity at pH 4.8, whereas liver had 45 and 15 per cent respectively. The K_m values for brain and liver were about 3 μM, a value similar to those previously reported [8].

Table 1. Dihydrofolate reductase activity in liver, brain and choroid plexus extracts by three different methods*

	Rothenberg method†		Spectrophotometric method‡		Methotrexate binding method§	
	(pmoles/min × mg)	% Liver activity	(units/mg)	% Liver activity	(pmoles/mg)	% Liver binding
Liver	0.40 ± 0.13 (6)		4.5 ± 1.8 (4)		2.0 ± 0.3 (5)	
Brain	0.07 ± 0.02 (6)	18	0.4 ± 0.1 (3)	10	0.3 ± 0.1 (5)	16
Choroid plexus	0.04 (2)	10	0.6 ± 0.2 (3)	14		

* Values are expressed as means ± standard error of the mean. The numbers in parentheses indicate the number of experiments at each point.

† The method of Rothenberg [6, 8] was employed with 5 pmoles [^{3}H]folic acid, 1 mg sodium ascorbate, and about 0.2 mg of partially purified liver dihydrofolate reductase or 1.0 mg of brain or choroid plexus dihydrofolate reductase. The pmoles/min/mg of protein of [^{3}H]folic acid reduced to [^{3}H]THF shown are the initial rates.

‡ The spectrophotometric method was followed [7]. One unit of activity equals a decline of 0.01 absorbance units/min and equals 1.05 nmoles DHF reduced/min [7].

§ The ability of the partially purified dihydrofolate reductase to bind [^{3}H]methotrexate/mg of protein was determined [9].

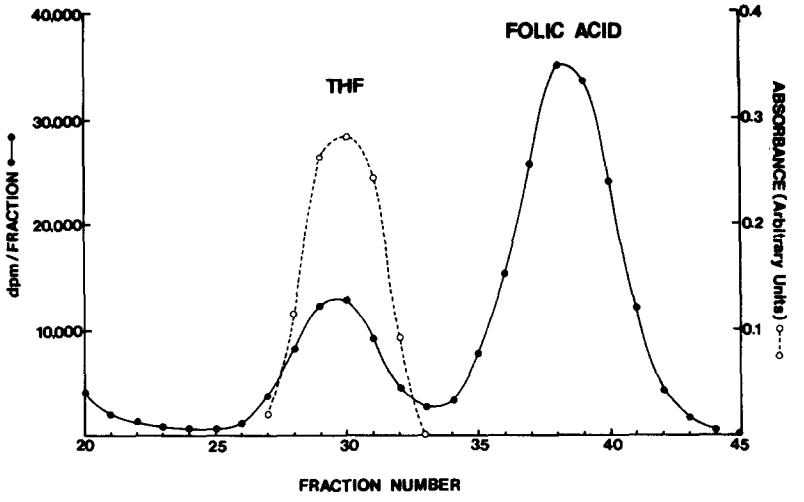

Fig. 1. Partially purified brain dihydrofolate reductase (1.2 mg) and 2.5 pmoles [^{3}H]folic acid were added to 1 ml of citrate buffer containing 0.01% mercaptoethanol, 0.1 mg NADPH and 1 mg sodium ascorbate. The mixture was incubated for 20 min at 37° [6, 8]. At the end of the incubation, the protein was denatured and the mixture centrifuged. Then 1 mg tetrahydrofolate was added to the supernatant which was, in turn, applied to a DEAE-Sephadex column as described in the text [4]. The column was eluted with phosphate buffer of increasing ionic strength containing 0.2 M mercaptoethanol and 2-ml fractions were collected [4]. Shown in Fig. 1 are the disintegrations/min/fraction and the absorbance/fraction in arbitrary units vs the fraction number.

Shown in Fig. 2 are Hofsteé transformations of the velocity (y) of the formation of THF and the concentration (S) of folate in the medium. Both the partially purified brain and liver dihydrofolate reductase preparations used in Fig. 2 were from a single rabbit. The partially purified dihydrofolate reductase activity in brain was not stable when stored in a refrigerator at 2°. After 9 and 19 days, the dihydrofolate reductase specific activity was 41 and 17 per cent, respectively, of the specific activity on day 1.

After the injection of 2.0 μCi [^{3}H]folic acid into the left lateral ventricle of two rabbits, an average of 9 per cent of the injected [^{3}H]folic acid (in an assumed volume of CSF of 3 ml) was recovered in 2 hr [2]. These results confirmed a previous study which showed that [^{3}H]folic acid is rapidly cleared from CSF [2]. The ^{3}H radioactivity recovered in the CSF co-chromatographed with carrier folic acid (49 per cent; N = 2) and MeTHF (51 per cent) in the thin-layer chromatographic system [10, 11]. No ^{3}H

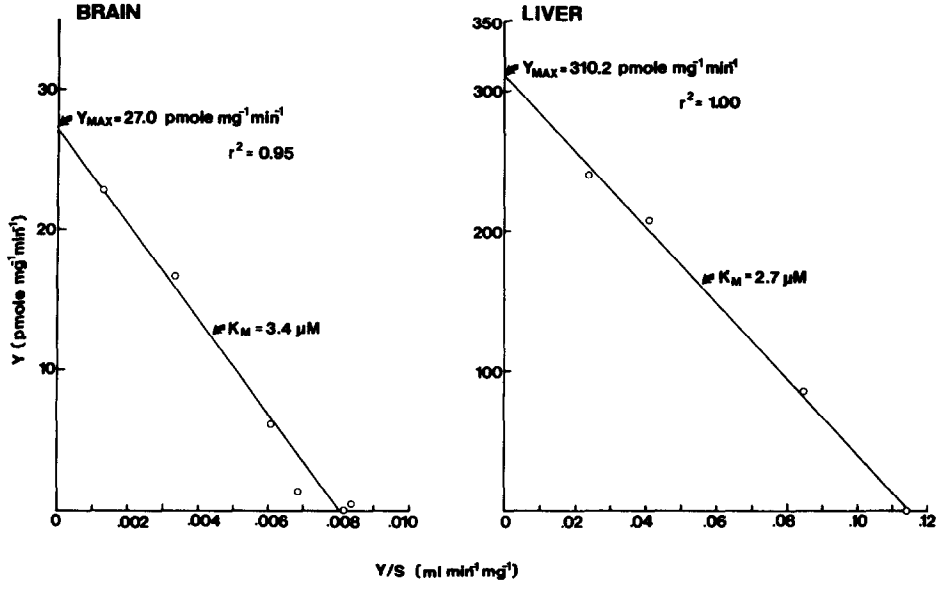

Fig. 2. [^{3}H]folic acid (2.5 pmoles) and various amounts of carrier folate were incubated for 15 min with 1.18 mg (brain) or 0.14 mg (liver) of partially purified dihydrofolate reductase obtained from one rabbit as in Fig. 1 [6, 8]. Plotted in Fig. 2 are the Hofsteé transformations of the velocity and concentration data for brain and liver respectively [11]. The respective K_m and Y_{max} are shown [6, 11]. The coefficient of determination (r^2) in each case is shown. In both cases, less than 25 per cent of the [^{3}H]folic acid was converted to THF when no carrier was added [6].

appeared as THF. To confirm the thin-layer chromatographic results, about 0.9 ml of the withdrawn CSF from one rabbit was chromatographed on a DEAE-Sephadex column after pretreatment of the withdrawn CSF as described above and the addition of approximately 1 mg of carrier THF. Fifty-eight per cent of the ^3H remained associated with folic acid (vs 59 per cent in the thin-layer system) and 42 per cent chromatographed as a single peak just before the carrier THF, consistent with the position of MeTHF [4]. Thus, 2 hr after the injection of 2.0 μCi [^{3}H]folic acid into the left lateral ventricle, 4.6 per cent could be recovered as MeTHF and 4.4 per cent as [^{3}H]folic acid in the CSF. These experiments do now allow interpretation of where the [^{3}H]folic acid was reduced, i.e. in brain and/or choroid plexus and/or meninges and/or outside the central nervous system with subsequent transport back into the CSF. However, the latter possibility is unlikely since we have previously shown that tracer amounts of [^{14}C]methyltetrahydrofolate do not achieve higher levels in CSF than plasma even after a 3-hr intravenous infusion [2]. In the present experiments, plasma contained 300 dis/min/ml (N = 2) at the end of the study, whereas CSF contained a concentration of [^{3}H]MeTHF more than two orders of magnitude higher.

As previously reported by two groups of investigators, we found that rabbit brain slices did not accumulate or metabolize [^{3}H]folic acid when incubated in 20 nM [^{3}H]folic acid for 30 min [1, 13].

DISCUSSION

Partially purified New Zealand white rabbit brain homogenates appear to have about 15 per cent as much dihydrofolate reductase activity/mg of protein as comparably prepared and assayed rabbit liver extracts (Table 1). The characteristics of the rabbit brain dihydrofolate reductase activity are similar to those of liver as shown by the absolute requirement of the enzyme from both liver and brain for NADPH (over NADH), the similar pH maxima, the comparable K_m (Fig. 2) and the abolishment of enzyme activity of both preparations with 1.0 μM methotrexate.

The tritiated product of the reaction between brain and liver dihydrofolate reductase, NADPH and [^{3}H]folic acid as described by Rothenberg [6, 8] co-chromatographed with THF in two systems when 1 mg sodium ascorbate was added to the reaction mixture and measures were taken to prevent oxidation during the chromatography [4, 10] (Fig. 1). However, when the sodium ascorbate was excluded from the reaction mixture, the tritiated reaction product chromatographed predominantly with aminobenzoylglutamate, a known breakdown product of THF. Thus, we have confirmed that the assay as described by Rothenberg [6, 8] is a measure of the enzymatic activity of dihydrofolate reductase. However, the product does not remain [^{3}H]THF as thought by Rothenberg [6, 8], unless sodium ascorbate is added to the reaction mixture.

The explanation of our detection of dihydrofolate reductase activity in rabbit brain contrary to the finding of other investigators is not clear [1]. Possibly, our method of preparing the extracts or the fact that the brain enzyme is unstable even at 2° could explain the difference. Alternatively, our strain of rabbits might be different. The latter explanation, however, is unlikely since we have also found dihydrofolate reductase activity in mouse brain.*

We have confirmed that [^{3}H]folic acid, when injected in tracer doses into the left lateral ventricle, is rapidly cleared from the CSF into blood [2]. However, of the small amount of ^3H activity remaining in the CSF (9 per cent), about 50 per cent had been reduced and converted to MeTHF. The site of reduction of [^{3}H]folic acid in the central nervous system is unclear. Possibilities include brain, choroid plexus, arachnoid tissue and, although unlikely, the CSF compartment. Choroid plexus, unlike brain as discussed below, contains a very vigorous uptake system for [^{3}H]folic acid [11]. Like brain, choroid plexus contains dihydrofolate reductase activity (Table 1). Previously, Spector and Lorenzo [2, 11] have suggested that the choroid plexus may be the locus of [^{3}H]folic acid transport from CSF to blood. Thus, it is possible that the choroid plexus and not the brain is the place wherein [^{3}H]folic acid is reduced to [^{3}H]THF when [^{3}H]folic acid is injected into the cerebrospinal fluid.

The possibility that [^{3}H]MeTHF entered CSF from plasma after injection of [^{3}H]folic acid intraventricularly is unlikely. Although radiolabeled MeTHF enters CSF from plasma, the levels in CSF were still less than in plasma even after 2.5 hr [2]. Thus, the very low levels of ^3H in plasma at the end of the experiment (300 dis/min/ml) in the rabbits who received intraventricular [^{3}H]folic acid could not explain the high concentration of [^{3}H]MeTHF in CSF (35,000 dis./min/ml).

The reason for and the function of dihydrofolate reductase in brain are not clear. First, reduced folates (MeTHF) readily enter CSF and brain from blood via a saturable transport system [2]. Second, as noted by us and other investigators [1, 13], brain slices *in vitro* do not accumulate (and, then, metabolize) [^{3}H]folic acid. It is possible that, in the brain, intracellular reduced folates become oxidized; the role of brain dihydrofolate reductase might be to reduce intracellular oxidized folates as opposed to oxidized folates outside the cell. The choroid plexus or some other mechanism might reduce oxidized folates in the CSF-extracellular space of brain compartment. Of the total folates in rat brain, 8 per cent are dihydrofolates [14]. Alternatively, brain dihydrofolate reductase, like liver dihydrofolate reductase, might reduce 7,8-dihydrobiopterin to tetrahydrobiopterin [15]. Tetrahydrobiopterin is a cofactor for brain tyrosine and tryptophan hydroxylases [16].

Whatever the role of dihydrofolate reductase in mammalian brain, its presence and sensitivity to methotrexate might explain the heretofore puzzling toxicity of methotrexate in the central nervous system [17, 18]. Low dose methotrexate does not have serious central nervous system toxicity except when injected intrathecally, thus bypassing the blood–brain and blood–CSF barrier, or when administered in con-

* H. T. Abelson, unpublished observations.

junction with X-ray therapy to the brain [17, 18]. We speculate that methotrexate may bind to brain dihydrofolate reductase, thus inhibiting the reduction of oxidized folates or some other unknown substrate to a critical degree.

In summary, rabbit brain appears to contain significant dihydrofolate reductase activity. Further studies will be required to detect its presence in other mammalian species (particularly humans) and ascertain its function(s) in the brain.

Acknowledgements—The authors wish to thank Caroline Gorka for her expert technical assistance. These studies were supported by grants from the National Foundation, the National Institutes of Health (NS-12274 and CA-18662). H. T. A. is the recipient of Research Career Development Award CA-00075.

REFERENCES

1. D. R. Makulu, E. F. Smith and J. R. Bertino, *J. Neurochem.* **21,** 241 (1973).
2. R. Spector and A. V. Lorenzo, *Am. J. Physiol.* **229,** 777 (1975).
3. J. M. Kinkade, W. R. Vogler and P. G. Dayton, *Biochem. Med.* **10,** 337 (1974).
4. P. F. Nixon and J. R. Bertino, in *Vitamins and Coenzymes* (Eds. D. B. McCormick and L. D. Wright), p. 661. Academic Press, New York (1971).
5. J. P. Perkins, B. L. Hillcoat and J. R. Bertino, *J. biol. Chem.* **242,** 4771 (1967).
6. S. P. Rothenberg, *Analyt. Biochem.* **16,** 176 (1966).
7. C. K. Mathews, K. G. Scrimgeour and F. M. Huennekens, in *Methods in Enzymology*, (Eds. S. P. Colowick and N. D. Kaplan), Vol. VI, p. 364. Academic Press, New York (1963).
8. S. P. Rothenberg, *Analyt. Biochem.* **13,** 530 (1965).
9. B. A. Kamen, P. L. Takach, R. Vatev and J. D. Caston, *Analyt. Biochem.* **70,** 54 (1976).
10. J. P. Brown, G. E. Davidson and J. M. Scott, *J. Chromat.* **79,** 195 (1973).
11. R. Spector and A. V. Lorenzo, *Science, N.Y.* **187,** 540 (1975).
12. H. McIlwain and R. Radman, *Practical Neurochemistry*, p. 109. Churchill, London (1962).
13. M. G. N. Bourne, S. K. Sharma and R. G. Spector, *Biochem. Pharmac.* **25,** 1917 (1976).
14. T. Brody, Y. S. Shin and E. L. R. Stockstad, *J. Neurochem.* **27,** 409 (1976).
15. S. Kaufman, in *Vitamins and Coenzymes* (Eds. D. B. McCormick and L. D. Wright), p. 614. Academic Press, New York (1971).
16. A. J. Turner, F. Ponzio and S. Algeri, *Brain Res.* **70,** 553 (1974).
17. W. A. Bleyer, J. C. Drake and B. A. Chabner, *New Engl. J. Med.* **289,** 770 (1973).
18. A. T. Meadows and A. E. Evans, *Cancer, N.Y.* **37,** 1079 (1976).