Biochemical Pharmacology, Vol. 29, pp. 3293–3298.
© Pergamon Press Ltd. 1980. Printed in Great Britain.

0006-2952/80/1215–3293 $02.00/0

ANALOG SPECIFIC ABERRANCIES IN ANTIFOLATE INHIBITION OF L1210 CELL DIHYDROFOLATE REDUCTASE

FRANCIS M. SIROTNAK,*† PAUL L. CHELLO,* DONNA M. MOCCIO,* JAMES R. PIPER,‡ JOHN A. MONTGOMERY‡ and JAMES C. PARHAM*

*Memorial Sloan–Kettering Cancer Center, New York, NY 10021, U.S.A. and ‡ Southern Research Institute, Birmingham, AL 35205, U.S.A.

(*Received* 14 *March* 1980; *accepted* 17 *June* 1980)

Abstract—During studies with L1210 cells and a variety of folate analogs, large discrepancies were revealed between data on membrane transport, on inhibition of dihydrofolate reductase in cell-free extracts, and on inhibition of growth in culture for 10-oxa-, 10-benzyl- and 10-phenethyl-aminopterin, and for 3-deaza, 10-methyl-aminopterin. While aminopterin, 10-methyl (methotrexate)-, 10-ethyl- and 10-propyl-aminopterin were tight binding inhibitors (K_i: $2–3 \times 10^{-12}$ M) of dihydrofolate reductase in cell-free extracts from L1210 cells, the other four analogs were only weak competitive inhibitors (K_i = $3–300 \times 10^{-8}$ M). Similar differences among analogs were observed for inhibition of dihydrofolate reductase in cell-free extracts from Sarcoma 180 and Ehrlich cells, but not for this enzyme in microbial cell-free extracts. There were only small differences in the transport of all of the analogs by L1210 cells. Inhibition of L1210 cell growth in culture by 10-oxa-, 10-benzyl- and 10-phenethyl-aminopterin and by 3-deaza, 10-methyl-aminopterin, in contrast to the other analogs, was several orders of magnitude greater than that predicted from the data on dihydrofolate reductase inhibition. The extent of binding of 10-oxa-, 10-benzyl- and 10-phenethyl-aminopterin, and of 3-deaza and 10-methyl-aminopterin to dihydrofolate reductase in intact L1210 cells, in contradistinction to that seen for the cell-free enzyme preparations, approached that observed for methotrexate; these estimates of drug–enzyme interaction *in situ* were more predictive of the extent of inhibition by these analogs of L1210 cell growth in culture.

Since the identification of dihydrofolate reductase as the primary target of 2,4-diamino-folate analogs [1–5], the characteristics of the inhibition of this enzyme by these analogs have been the subject of intense investigation in a number of laboratories [reviewed in Refs. 6 and 7]. It has been generally assumed in these studies that the extent of inhibition of this enzyme demonstrated for different analogs in cell-free systems is representative of a similar degree of drug-enzyme interaction *in situ*. Although this may appear to be a reasonably valid assumption for most derivatives, recent studies from our own laboratory have provided evidence which strongly suggests that this assumption may not apply in the case of all analogs. These studies were carried out with L1210 murine leukemia cells. In the case of four compounds, there were large discrepancies (three to five orders of magnitude) between the data on the inhibition of growth in culture and the data on the inhibition of dihydrofolate reductase from cell-free extract. Our results, which include detailed rate measurements of cellular membrane transport and estimates of intracellular binding to dihydrofolate reductase, show that these discrepancies can be attributed to the fact that the data obtained with some analogs in a standardized cell-free enzyme system provided a poor indication of the degree of inhibition of intracellular dihydrofolate reductase.

† Author to whom all correspondence should be addressed: Laboratory for Molecular Therapeutics, Memorial Sloan–Kettering Cancer Center, 1275 York Ave., New York, NY 10021.

EXPERIMENTAL

L1210 leukemia, Sarcoma 180, and Ehrlich cells were obtained as intraperitoneal ascites suspensions from $BD2F_1$ mice [8] or in culture [9] and were processed in a manner described previously for dihydrofolate reductase isolation [8] and transport experiments [8]. The L1210 cell-line in culture employed during these experiments was derived from the parental L1210V line carried in mice. Antifolate-resistant variants of the L1210V line [10] and *Diplococcus pneumoniae* [11], which overproduce dihydrofolate reductase, were also employed as sources of this enzyme for some of the inhibition experiments to be described that measure the effect of drug on the reduction of dihydrofolate [3]. Methods for isolation and growth of L1210 cells in culture have been given earlier [9]. The medium employed was RPMI and 1640 (Grand Island Biologicals, Grand Island, NY) supplemented with 10% fetal calf serum (Microbiological Associates, Bethesda, MD). The suspending medium during transport experiments, and the conditions employed during measurements of influx and efflux of methotrexate, have been given [8, 12]. During these transport experiments, measurements of intracellular drug that was not radioactively labeled were carried out by titration inhibition of *D. pneumoniae* dihydrofolate reductase [8, 13]. Measurement of intracellular [^{3}H]methotrexate was done by scintillation counting. Methods for extracting drug have been described [8, 12, 13]. [3′, 5′, 9′-^{3}H]Methotrexate (Morvek Biochemicals, City of Industry, CA) was purified by

paper chromatography [14] to a final purity of > 98 per cent. Samples of unlabeled methotrexate were provided by the Drug Procurement and Synthesis Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, and were determined spectrophotometrically [15] to be > 96 per cent pure. Syntheses of 3-deaza-methotrexate [16], 10-oxa-aminopterin [17, 18], 10-benzyl-aminopterin [18, 19], and 10-phenethyl-aminopterin [18] have been described. The preparation of 10-propyl-aminopterin was carried out in a manner similar to that reported for the other analogs [18] and will be reported in a future paper. The samples of each of these analogs employed were > 98 per cent pure. Individual experimental details are provided in the legend of each figure and table.

RESULTS AND DISCUSSION

For the general category of 2,4-diamino folate analogs, the extent of inhibition of cell growth in culture by any particular analog can usually be predicted [8, 20, 21] from data on membrane transport and inhibition of the target enzyme, dihydrofolate reductase. During some recent studies in our laboratory of a large number of these analogs, major discrepancies in these relationships were revealed. This is illustrated in Table 1, where data are compared for a number of analogs modified at the 10 position and for a related analog, 3-deaza-methotrexate. The values of the influx V_{max} and of the efflux rate constants derived for these analogs are virtually the same, and the values of the influx K_m are distributed over a relatively narrow range. In the case of aminopterin itself, 10-deaza-aminopterin, and the 10-methyl, 10-ethyl and 10-propyl derivatives, the K_i values derived for dihydrofolate reductase inhibition were essentially identical. For these analogs, the differences in the corresponding values for IC_{50} appear to reflect the small differences in the values for influx K_m. For the other four analogs shown, i.e. the 10-oxa, 10-benzyl, and 10-phenethyl derivatives of aminopterin and the 3-deaza derivative of methotrexate, the values for IC_{50} were much smaller than would be expected based on the extremely large values derived for the K_i of dihydrofolate reductase inhibition. Values of K_i for these analogs were 10^4 to 10^6-fold higher than those derived

Table 1. Folate analogs and membrane transport, growth, and dihydrofolate reductase inhibition in L1210 cells

	Dihydrofolate reductase inhibition*	Membrane transport†			Growth inhibition‡
		Influx		Efflux	
	K_i (nM)	K_m (μM)	V_{max} [nmoles · min^{-1} · (g dry wt)$^{-1}$]	K (min^{-1})	IC_{50} (nM)
Aminopterin	0.0032	1.2	2.90	0.24	0.8
10-Deaza-aminopterin	0.0029	1.9	3.24	0.21	0.9
10-Oxa-aminopterin	280.0	3.4	2.84	0.24	1.6
10-CH_3-aminopterin	0.0043	3.3	3.13	0.23	5.4
10-C_2H_5-aminopterin	0.0039	3.8	3.01	0.25	1.7
10-C_3H_7-aminopterin	0.0034	3.2	3.15	0.22	1.8
10-Benzyl-aminopterin	3450.0	2.8	3.18	0.21	34.0
10-Phenethyl-aminopterin	1910.0	6.3	3.04	0.22	30.0
3-Deaza-methotrexate	31.0	3.0	2.95	0.23	43.6

* Method of enzyme extraction and the determination of inhibition by various folate analogs are described in the legend of Fig. 1. Values for K_i were derived by the method of Henderson [22] for titration inhibition or, otherwise, by the method of Haynes [23]. Average of three to five separate determinations (S.D. = < 13 per cent).

† Cells, removed as ascites suspensions from the peritoneal cavity of $BD2F_1$ mice, were washed once in cold (0°) 0.14 M NaCl plus 0.01 M potassium phosphate (pH 7.4) and resuspended in a solution of buffered salts [8] with 2 mM glucose but not serum. Initial influx measurements were made with various external concentrations of [^{3}H]methotrexate; the incubation time was adjusted at each concentration so that the intracellular accumulation did not exceed the dihydrofolate reductase drug-binding capacity. A double reciprocal plot of the data (v/[drug]) was constructed to obtain values for maximum velocity (V_{max}) and the apparent Michaelis constant (K_m). After influx measurements, incubation was terminated by a 10-fold dilution of cells in cold (0°), buffered, isotonic saline solution and washing three times with the same cold solution. No loss of drug occurs at this temperature [8]. Since drug accumulation is nonexchangeable because of binding to dihydrofolate reductase, no loss would be expected to occur. [^{3}H]Methotrexate uptake was determined by scintillation counting of radioactivity. Intracellular accumulation was determined after correction for cell surface absorption [8]. Average of three to five separate determinations (S.D. = < 15 per cent).

§ Logarithmic phase L1210 cells (10^4 cells/ml) in RPMI 1640 medium (formulated with 2.2 μM folic acid), 10% with respect to fetal bovine serum, were dispensed into 16 × 125 mm culture tubes (final volume 5.0 ml) containing various concentrations of each folate analog. Cell growth in tubes with and without folate analogs was monitored every 24 hr to verify that the growth pattern was normal. After 72 hr, the cell density in all experimental groups was determined with a model ZBI Coulter Counter. Cell counts were averaged; the means were plotted on full logarithmic paper against the concentration of reduced folate to determine the amount necessary to produce a 50 per cent reversal of inhibition. Cell counts from triplicate culture tubes within the same experimental group agreed within 10 per cent. Average of three to five separate determinations (S.D. = < 18 per cent).

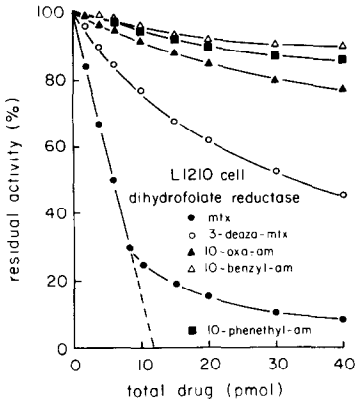

Fig. 1. Inhibition of L1210 cell dihydrofolate reductase by folate analogs. Enzyme preparations were made from L1210 cell suspensions ($A_{600} = 30$; 2.43×10^8 cell/ml) in 0.05 M Tris–HCl (pH 7.4) with 0.001 M EDTA by sonication (Heat Systems-Ultrasonics, Inc; Plainview, NY) at 0° for 30 sec. Cellular debris was removed by centrifugation, and supernatant fluid stored at −70° until used. In a modification [8] of a previously published [3] method, various amounts of drug were added to tubes containing 100 nmoles NADPH/ml and 1 mg/ml of mercaptoethanol in 0.05 M potassium phosphate buffer (pH 7.3). Enough enzyme preparation was added to give a change in absorbance (A_{340}) of 0.3 in control tubes after the addition of 100 nmoles/ml of dihydrofolate and incubation of 10 min at 37°. The total volume of the reaction mixture was 2.5 ml. Methotrexate (100 μg) was added at the end of the incubation period to stop the reaction prior to the absorption measurement. Average of four experiments (S.D. = < 12 per cent).

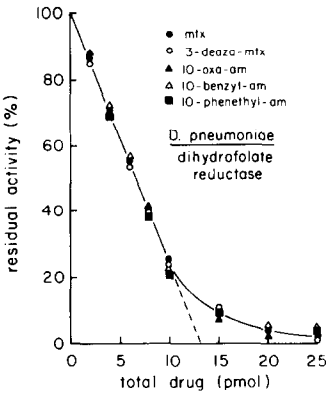

Fig. 2. Inhibition of *D. pneumoniae* dihydrofolate reductase by folate analogs. Enzyme preparations were made [8, 11] by distrupting suspensions of *D. pneumoniae* in 0.05 M Tris–HCl (pH 7.3) with 0.001 M EDTA. The conditions employed during the enzyme assay and for measurements of inhibition by folate analogs are described in the legend of Fig. 1. Average of three experiments (S.D. = < 9 per cent).

for aminopterin and the other N^{10} analogs; values for IC_{50}, however were only 2- to 50-fold greater than aminopterin.

Details relating to the kinetics of inhibition of dihydrofolate reductase in cell-free extract by methotrexate, 3-deaza-methotrexate, and the 10-oxa, 10-benzyl and 10-phenethyl derivatives of aminopterin are presented in Fig. 1 through 3. The ineffective inhibition of the L1210 cell enzyme (Fig. 1) observed with these four analogs is in sharp contrast to the partially stoichiometric inhibition by methotrexate and the other N^{10} analogs (data not shown). A similar result was obtained (data not shown) in the same assay employing dihydrofolate reductase in cell-free extracts of Sarcoma 180 and Ehrlich cells. When the source of dihydrofolate reductase employed in the assay was microbial (*D. pneumoniae*), rather than mammalian, however, inhibition by all of the analogs exhibited the same (partially stoichiometric) kinetics. Data for some of these analogs are shown in Fig. 2.

Evidence that the ineffective inhibition of L1210 cell dihydrofolate reductase by 3-deaza-methotrexate, or by 10-oxa-, 10-benzyl- or 10-phenethyl-aminopterin was actually related to the enzyme–drug interaction itself, and not to other factors, was derived in the following manner. First, titration inhibition of L1210 cell dihydrofolate reductase by methotrexate in the range of 0–10 pmoles, as in Fig. 1, could be demonstrated in the presence of larger amounts of 3-deaza-methotrexate (10 pmoles), 10-oxa-aminopterin (100 pmoles) or 10-benzyl-aminopterin (200 pmoles). These results tend to eliminate the possibility that some impurity in the samples of these three analogs was responsible for the relatively ineffective inhibition of this enzyme. Second, results identical to those shown in Fig. 1 were obtained with each analog when cell-free extract was derived from an L1210 cell variant that overproduces dihydrofolate reductase 12-fold. Third, when each analog was incubated with L1210 cell extract under the same conditions used in the enzyme assay and, then, this mixture was used to titrate the *D. pneumoniae* enzyme, results identical to those seen in Fig. 2 were obtained. The results of these two control experiments appear to eliminate the possibility that factors in the L1210 cell-free extract other than the enzyme, itself, were responsible for the results observed. Finally, it can be seen from a comparison of the data in Fig. 3 that, despite the fact that the 10-oxa and

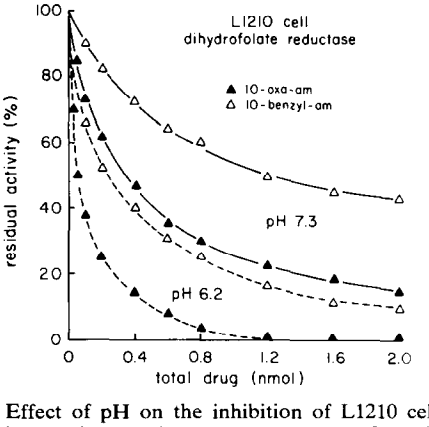

Fig. 3. Effect of pH on the inhibition of L1210 cell dihydrofolate reductase by folate analogs. Methods of enzyme isolation and details of the enzyme inhibition assay are given in the legend of Fig. 1. Average of three experiments (S.D. = < 15 per cent).

10-benzyl analogs were poor inhibitors of L1210 cell dihydrofolate reductase, a pH dependence could be demonstrated for this inhibition that is characteristic of the pH dependence (reviewed in Refs. 6 and 7) reported for other 4-amino-folate analogs. Similar data (not shown) were derived for 3-deaza-methotrexate and 10-phenethyl-aminopterin.

In the remaining experiments to be described, we sought information on the basis for the discrepancies observed between the data on growth and that on enzyme inhibition, with 3-deaza-methotrexate, 10-oxa-aminopterin, and 10-benzyl-aminopterin. In these experiments, we attempted to determine the relative extent to which each of these analogs was actually bound to dihydrofolate reductase within the cell, by delineating the nonexchangeable fraction of accumulated drug. The parental L1210 cell-line (L1210$\bar{V}$) was incubated at 37° in the presence of a 5μM concentration of each analog for a period of 20 min to allow for the accumulation of drug to intracellular levels in excess of the dihydrofolate reductase binding capacity. The cells were then washed once at 0° and resuspended in cold (0°) drug-free transport medium. The washed suspensions were then incubated at 37° to allow drug to efflux from the cell. The time-course for efflux is shown in Fig. 4. It can be seen that all four analogs effluxed at the same rate. Most importantly, loss of each analog ceased at a level approximately equivalent to the tight binding fraction of the intracellular dihydrofolate reductase. Moreover, when this experiment was repeated with L1210 cells (L1210$\bar{V}/C_1$) that had a 2- to 3-fold higher level of dihydrofolate reductase, the result (Fig. 4) was essentially identical except that efflux of each analog ceased at the higher level, i.e. approximately equivalent to the tight binding fraction of dihydrofolate reductase characteristic of these cells.

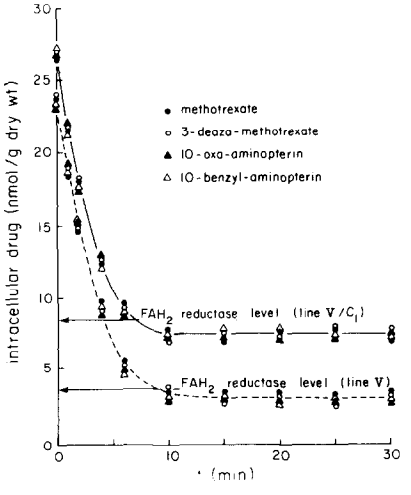

Fig. 4. Nonexchangeable levels of intracellular drug in L1210 cells following accumulation and efflux of various folate analogs. L1210$\bar{V}$ or L1210$\bar{V}/C_1$ cells were washed once in cold (0°) 0.14 M NaCl plus 0.01 M potassium phosphate (pH 7.4) and resuspended in cold transport medium [8] containing 107 mM NaCl, 5.3 mM KCl, 26.2 mM $NaHCO_3$, 1.9 mM $CaCl_2$, 1 mM $MgCl_2 \cdot 6H_2O$, 10 mM glucose, and 10 mM Tris–HCl (pH 7.4). No serum was added. Five μM concentrations in 50 μl of each analog were added to 10 ml aliquots and incubated for 20 min at 37°. The cells were then washed once in cold (0°) transport medium and resuspended in 1 ml of cold transport medium without drug and reincubated at 37°. Aliquots were removed at the times indicated, cooled to 0° and diluted 10-fold in cold (0°) 0.14 M NaCl plus 0.01 M potassium phosphate (pH 7.4). After washing twice at 0° in the same buffer, drug was extracted [8, 12, 13] and the concentration in each extract was determined by titration of *D. pneumoniae* dihydrofolate reductase [8, 13]. The level of intracellular dihydrofolate reductase was determined by a titration–inhibition assay of enzyme activity [5, 8] in a supernatant fraction prepared from a sonicated suspension of L1210 cells. Average of three experiments (S.D. = < 16 per cent).

In another experiment, L1210 cells were incubated for 20 min with 2.5 μM [^{3}H]methotrexate alone and in the presence of 2.5 μM unlabeled methotrexate or one of the other analogs. The cells were then washed twice at 0° and resuspended in cold (0°) drug-free medium. These washed suspensions were then incubated at 37° for 20 min to allow for efflux of all exchangeable (non-enzyme bound) drug. Since all four analogs would be expected to accumulate at the same rate (K_m and V_{max} values for influx are essentially the same; see Table 1), any differences in the values for the nonexchangeable fraction of [^{3}H]methotrexate obtained in the presence of the unlabeled analog should reflect differences in the extent of competition at the level of the intracellular dihydrofolate reductase. From these results, shown in Fig 5, it can be seen that 10-oxa-aminopterin was

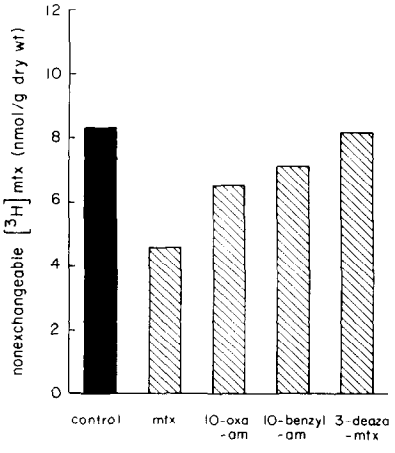

Fig. 5. Nonexchangeable levels of intracellular [^{3}H]methotrexate following efflux after accumulation in the presence of methotrexate and various analogs. Washed L1210$\bar{V}/C_1$ cells were incubated for 10 min with 2.5 μM [^{3}H]methotrexate in the presence or absence of a 2.5 μM concentration of one of the following: methotrexate (MTX), 10-oxa-aminopterin (10-oxa-AM), 10-benzyl-aminopterin (10-benzyl-AM) or 3-deaza-methotrexate (3-deaza-MTX) for 20 min at 37° in transport medium. After washing at 0°, the cells were resuspended in cold (0°) transport medium and reincubated at 37° for 20 min. [^{3}H]Methotrexate was extracted [8] from the cells and the radioactivity was determined by scintillation counting. Additional experimental details are provided in the text and the legends of Figs. 4 and 5. Average of three experiments (S.D. = < 12 per cent).

less effective than methotrexate, as a competitive inhibitor of enzyme binding of [^{3}H]methotrexate; 10-benzyl-aminopterin and 3-deaza-methotrexate were even less effective.

Further evidence for reduced intracellular binding of 3-deaza-methotrexate, 10-oxa-aminopterin, and 10-benzyl-aminopterin to L1210 cell dihydrofolate reductase in comparison to methotrexate was also derived in the following experiment. L1210 cells were loaded with unlabeled methotrexate or one of the other three analogs (20-min incubation at 27° in 5 μM drug). Then the cells were washed twice with cold (0°) medium, resuspended in cold (0°) medium, and reincubated at 37° for 20 min to allow efflux of drug to enzyme level. [^{3}H]Methotrexate (2.5 μM) was then added to each aliquot of cells exposed to drug as well as to control cells that had been incubated without unlabeled drug; incubation was continued for another 20 min. These cells were then washed and allowed to efflux; and the level of nonexchangeable [^{3}H]methotrexate was determined. The data shown in Fig. 6 give evidence of complete exchange on the enzyme by [^{3}H]methotrexate in the case of cells preloaded with 3-deaza-methotrexate, 10-oxa-aminopterin, or 10-benzyl-aminopterin, but only partial exchange in the case of methotrexate. A similar extent of exchange between [^{3}H]methotrexate bound to intracellular dihydrofolate reductase and nonradioactive methotrexate was reported in an earlier study [24].

From the results of these experiments, we conclude that binding of 3-deaza-methotrexate, 10-oxa-, 10-benzyl- and probably 10-phenethyl-aminopterin to dihydrofolate reductase is appreciably greater *in situ* than was indicated by the results of the cell-free enzyme inhibition assay. Although binding of these three analogs to this enzyme does not appear to be quite as strong as that of methotrexate, the extent of enzyme binding of these analogs demonstrated *in situ* more closely reflected the net effect of each on cell growth. These findings also prompt a note of caution when interpreting the results of experiments measuring the inhibition of dihydrofolate reductase in cell-free extracts. Determinations of the relative inhibitory potency of specific analogs should probably be accompanied by data on membrane transport and growth inhibition for the same cell-line from which the target enzyme was isolated. Even so, we have no explanation at the molecular level for these aberrancies in mammalian cell dihydrofolate reductase inhibition by these four analogs in this cell-free system. It is possible that localized changes in tertiary structure, which would affect binding, could occur during the extraction of this enzyme and that these changes might manifest themselves in an analog specific manner. In this connection, it is of interest that the methyl, ethyl and propyl substituents at the 10 position, but not the larger aromatic substituents, were as effective as aminopterin in binding to cell-free dihydrofolate reductase. There are other possible explanations, however, which have not been eliminated by the results. Further, the aberrancies observed with the mammalian cell enzymes were not observed with the microbial enzyme. Finally, although our observations relate only to modification of the N^3 and N^{10} positions of the folate molecule, it is possible that modification elsewhere may result in a similar effect on binding by this mammalian enzyme in conventional cell-free extracts.

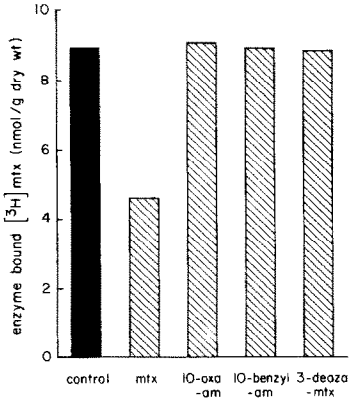

Fig. 6. Exchange of dihydrofolate reductase bound fraction of various folate analogs by [^{3}H]methotrexate. Washed L1210$\bar{V}$/C$_1$ cells were incubated for 10 min in a 2.5 μM concentration of one of the following analogs: methotrexate, 3-deaza-methotrexate, 10-oxa-aminopterin or 10-benzyl-aminopterin, washed (0°), and resuspended in cold (0°) transport medium and then incubated at 37° for 20 min to allow efflux of drug. The cells were washed and resuspended in cold (0°) medium again and reincubated at 37° for another 10 min in the presence of 2.5 μM [^{3}H]methotrexate. Each aliquot of cells plus control cells that were only incubated with [^{3}H]methotrexate were reincubated at 37° for another 20 min to allow efflux of drug. Cells were then cooled (0°), diluted 10-fold, and washed (0°) prior to extraction for determination of radioactivity. Further details are given in the text and in the legends of Figs. 4 and 5. Average of three experiments (S.D. = < 14 per cent).

Acknowledgements—The authors gratefully acknowledge the able technical assistance of Lydia Goutas, Ellen Wong, and Mary Beth Swerz during the course of these studies. This work was supported, in part, by grants CA 08748, CA 18856, CA 22764, and CA 25236 from the National Cancer Institute and Grant CH-26 from the American Cancer Society.

REFERENCES

1. C. A. Nichol and A. D. Welch, *Proc. Soc. exp. Biol. Med.* **74**, 403 (1950).
2. S. Futterman, *J. biol. Chem.* **228**, 1031 (1957).
3. M. J. Osborn and F. M. Huennekens, *J. biol. Chem.* **233**, 969 (1958).
4. S. F. Zakrzewski, *J. biol. Chem.* **235**, 1776 (1960).
5. W. C. Werkheiser, *J. biol. Chem.* **236**, 888 (1961).
6. B. L. Baker, *Design of Active Site-Directed Irreversible Enzyme Inhibitors*, pp. 192–263. John Wiley, New York (1967).
7. R. L. Blakley, *The Biochemistry of Folic Acid and Related Pteridines*, pp. 139–81. John Wiley, New York (1969).
8. F. M. Sirotnak, *Pharmac. Ther.* **8**, 71 (1980).
9. P. L. Chello and J. W. Bruckner, *Antimicrob. Agents Chemother.* **10**, 185 (1976).
10. A. Hoshino, A. M. Albrecht, J. C. Biedler and D. J. Hutchison, *Cancer Res.* **26**, 1397 (1966).
11. F. M. Sirotnak and S. L. Hachtel, *Genetics* **61**, 293 (1969).

12. F. M. Sirotnak and R. C. Donsbach, *Cancer Res.* **36**, 1151 (1976).
13. F. M. Sirotnak and R. C. Donsbach, *Cancer Res.* **32**, 2120 (1972).
14. H. E. Sauberlich and C. A. Baumann, *J. biol. Chem.* **176**, 165 (1948).
15. M. May, T. J. Bardos, F. L. Barger, M. Lansford, J. M. Ravel, G. L. Sutherland and W. J. Shive, *J. Am. Chem. Soc.* **73**, 3067 (1951).
16. R. D. Elliott, C. Temple, Jr., J. F. Frye and J. A. Montgomery, *J. org. Chem.* **36**, 2818 (1971).
17. M. G. Nair and P. T. Campbell, *J. med. Chem.* **19**, 825 (1976).
18. J. A. Montgomery, J. R. Piper, R. D. Elliot, C. Temple, E. Roberts and Y. F. Shealy, *J. med. Chem.* **22**, 862 (1979).
19. J. C. Parham, M. A. Templeton, P. L. Chello and F. M. Sirotnak, *J. heterocyclic Chem.* **16**, 1645 (1979).
20. F. M. Sirotnak, P. L. Chello, J. R. Piper and J. A. Mongomery, *Biochem. Pharmac.* **27**, 1821 (1978).
21. F. M. Sirotnak, P. L. Chello, J. R. Piper, J. A. Montgomery and J. I. DeGraw, in *Chemistry and Biology of Pteridines* (Eds. R. L. Kisliuk and G. M. Brown) p. 597. Elsevier-North Holland, New York (1979).
22. P. J. F. Henderson, *Biochem. J.* **135**, 101 (1973).
23. C. S. Haynes, *Biochem. J.* **26**, 1406 (1932).
24. M. Cohen, R. A. Bender, R. Donehower, C. E. Myers and B. A. Chabner, *Cancer Res.* **38**, 2866 (1978).