Purification and Properties of Dihydrofolate Reductase from Cultured Mammalian Cells

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Dihydrofolate reductase was purified quickly and simply from small quantities of cultured mammalian cells by affinity chromatography. On gel electrophoresis of the purified enzyme, multiple bands of activity resulted from enzyme–buffer interaction at low but not high buffer concentration. A Ferguson plot (Ferguson, 1964) showed that this heterogeneity was due to a charge difference with no alteration in the size of the enzyme. Stimulation of enzyme activity by KCl, urea and *p*-hydroxymercuribenzoate, and inhibition by methotrexate and trimethoprim, showed only minor differences between the various enzymes.

The enzyme dihydrofolate reductase (tetrahydrofolate dehydrogenase; EC 1.5.1.3) has been purified from viral (Erickson & Mathews, 1971), bacterial (Hillcoat & Blakley, 1966; Nixon & Blakley, 1968; Sirotinak & Salser, 1971; Gundersen *et al.*, 1972; Poe *et al.*, 1972), protozoan (Diggins *et al.*, 1970), avian (Kaufman & Gardiner, 1966) and mammalian (Greenberg *et al.*, 1966; Perkins *et al.*, 1967) sources by a variety of techniques, which usually begin with relatively large quantities of material. Recent reports describe the use of affinity chromatography in which tight-binding inhibitors of the enzyme have been coupled to agarose (Erickson & Mathews, 1971; Hillcoat *et al.*, 1971; Newbold & Harding, 1971; Poe *et al.*, 1972) and other supports (Mell *et al.*, 1968*b*). We here report the use of this technique to purify dihydrofolate reductase from extracts of small quantities of cultured cell lines to compare the properties of various mammalian enzymes.

Experimental

Materials

Reagents were of the highest grade available from commercial sources. We prepared dihydrofolate by reducing folate with dithionite (Blakley, 1960). Myoglobin and soya-bean trypsin inhibitor were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) and Worthington (Winley–Morris Co., Montreal, Canada), respectively, and Sepharose 4B was from Pharmacia (Montreal, Canada). Methotrexate (amethopterin, 4-amino-*N*¹⁰-methylpteroylglutamic acid) was a product of Lederle (Montreal, Canada) and trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine] was a gift from Dr. G. Elion (Burroughs Wellcome, New York, N.Y., U.S.A.). Media for cell culture were purchased from Grand

Island Biological Co. (Grand Island, N.Y., U.S.A.). The cell lines used included the LM4 and LS2 clonal sublines of the L1210 murine lymphoma, isolated in our laboratory (Hillcoat, 1971); a human haematoblastoid line (RPMI 4265) originally supplied by Dr. G. Moore (Roswell Park Memorial Institute, Buffalo, N.Y., U.S.A.) and maintained by us for several years; the HTC (rat hepatoma line) provided by Dr. G. Tomkins (Department of Biochemistry, University of California, San Francisco, Calif., U.S.A.); and the B1A2A (Syrian Hamster line), a gift from Dr. J. Littlefield, (Massachusetts General Hospital, Boston, Mass., U.S.A.).

Methods

Cell culture. The LS2, LM4 and RPMI 4265 lines grew as suspension cultures in RPMI 1640 medium supplemented with 10% foetal calf serum, and the HTC and B1A2A lines as monolayers in Swim's and Eagle's minimum essential media respectively with 10% foetal calf serum. Harvested, washed cells were stored frozen until used.

Enzyme purification. We purified the enzyme by affinity chromatography on Sepharose 4B to which the carboxyl group(s) of methotrexate had been coupled to the amino groups of aminoethyl-substituted agarose by standard procedures (Gauldie & Hillcoat, 1972). Purification was carried out in a manner similar to that described previously (Hillcoat *et al.*, 1971). Cells (1×10^8 – 6×10^8) were suspended in 2.0–3.0 ml of 0.05 M-Tris–HCl buffer, pH 7.5, containing 10 μ M-NADPH and sonicated at 4°C for two periods of 20 s each at a setting of 30 on a Biosonic 3 apparatus (Bronwill Scientific, Rochester, N.Y., U.S.A.). The solution was centrifuged at 17000g for 15 min at 4°C and the precipitate discarded. We applied the supernatant to a column (3 cm $\times$ 0.5 cm)

of Sepharose-methotrexate at 4°C, which had been equilibrated with Tris-HCl buffer (0.05M, pH7.5, with 10 μ M-NADPH), washed away unwanted protein with Tris-glycine buffer (0.2M, pH9.5, 2.0M in KCl with 10 μ M-NADPH) and eluted the enzyme in 0.5ml fractions with Tris-glycine buffer (0.2M, pH9.5, 2.0M in KCl with 10 μ M-dihydrofolate). Some fractions of purified enzyme of high activity from the LM4 cells were desalted and the NADPH was removed by passage through a small column of Sephadex G-10 which had been equilibrated with 0.5M-Tris-HCl buffer, pH7.5, and the enzyme was then used for electrophoretic studies. After this, NADPH was added to stabilize the remainder. Other active fractions were pooled and concentrated in a dialysis sac covered with solid sucrose, then dialysed against 0.05M-Tris-HCl buffer, pH7.5, containing 10 μ M-NADPH.

Enzyme assay. Enzyme activity was recorded on a Gilford spectrophotometer as the rate of change in E_{340} with dihydrofolate (50 μ M) and NADPH (100 μ M) as substrates at 37°C (Hillcoat *et al.*, 1967); 1 unit of activity represents 1 μ mol of substrate reduced/h and specific activity is expressed in units/mg of protein. Protein was measured by the method of Lowry *et al.* (1951). During purification of the enzymes, assays of dihydrofolate reductase activity were performed at pH8.5 in the presence of 0.15M-KCl. The effect of different conditions on enzyme activity was measured by adding increasing concentrations of KCl, 2M-urea or 0.5mM-*p*-hydroxymercuribenzoate to the assay mixture at pH7.0 (0.05M-Tris-HCl buffer). In the latter case, 2-mercaptoethanol was omitted initially from the assay mixture, which contained buffer and NADPH. At 1 min after the addition of enzyme, 2-mercaptoethanol and dihydrofolate were added to begin the reaction. The inhibition of dihydrofolate

reductase activity by methotrexate is pH-dependent (Bertino *et al.*, 1964), so the inhibition of the enzyme by methotrexate and trimethoprim was compared at pH6.0 (0.05M-potassium phosphate buffer) and pH8.5 (0.05M-Tris-HCl buffer).

Polyacrylamide-gel electrophoresis. Routine electrophoresis was performed with modifications of the method of Ornstein and Davis (Ornstein, 1964; Davis, 1964) at 4°C in a Canalco apparatus (International Scientific Co., Ottawa, Canada) by using gels of 7.5% polyacrylamide with riboflavin as catalyst.

The enzyme solution (0.2ml) in 12% (w/v) sucrose was layered directly on top of the separating gels. The tank buffer was Tris-glycine (0.038M, pH8.5) and the gel buffer was Tris-HCl (0.38M, pH8.9). A current of 1mA/tube was applied for 30min then increased to 2mA/tube for a further 1½h. The gels were then removed and incubated in the dark at 25°C in 2.0ml of assay mixture to localize the enzyme as blue bands formed on reduction of a tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] by the tetrahydrofolate formed (Gunlack *et al.*, 1968; Mell *et al.*, 1968a; Hiebert *et al.*, 1972). The position of the bands was recorded as previously described (Hiebert *et al.*, 1972). Ferguson plots (Ferguson, 1964) were obtained with LM4 enzyme by using a series of gels of different polyacrylamide concentrations, as described by Chrambach & Rodbard (1971). The mobility (R_F) of bands of dihydrofolate reductase activity were recorded at gel concentrations from 7.0 to 17.5% and the log R_F was plotted against gel concentration. Bromophenol Blue, soya-bean trypsin inhibitor and myoglobin were run as well, on separate gels. We determined the R_F values of the enzyme from the other cell preparation by using 7.5% gels with high concentrations of gel

(1.9M) and tank (0.38M) buffers for reasons given below.

Results

Enzyme activity in cell lines

The dihydrofolate reductase activity of unpurified extracts varied considerably in the different cell lines on different occasions (Table 1). The latter variation probably reflected the dependence of enzyme activity on the age of the culture (Hillcoat, 1971). The LS2 and RPMI 4265 lines had low enzyme activity/cell and low specific activity. The growth of both cell lines is inhibited by low concentrations of methotrexate (Hillcoat, 1971; B. L. Hillcoat, unpublished work). The LM4 and B1A2A lines possessed high enzyme activity and are relatively resistant to methotrexate (Hillcoat, 1971; Littlefield, 1969). The HTC line had an intermediate activity of enzyme/cell but a low specific activity.

Enzyme purification

Enzyme, with total activity 8.75–176.0 units, was applied to a column of Sepharose–methotrexate as described. The amount of enzyme retained did not

depend on the amount applied, as shown in Table 2. Recovery varied, being lowest (33.6%) for the preparation with the highest total activity. Concentration and dialysis caused loss of activity with the enzyme from LS2 and RPMI 4265 cells but not with that from HTC cells.

Electrophoretic properties

Under normal conditions of electrophoresis we had previously observed multiple bands of dihydrofolate reductase activity, some of which resulted from binding of the tracking dye, Bromophenol Blue, to the enzyme (Hiebert *et al.*, 1972). As well, since electrophoretic heterogeneity also resulted from binding of NADPH to the enzyme (Dunlap *et al.*, 1971), we have compared results with and without NADPH in the electrophoretic buffer. When NADPH was present, multiple peaks were still observed (Table 3), but when the buffer concentration was increased, a single peak resulted.

A series of gels of various polyacrylamide concentrations were run as suggested by Chrambach and Rodbard (Chrambach & Rodbard, 1971; Rodbard & Chrambach, 1971) to determine the nature of the observed heterogeneity in the presence of NADPH.

Table 1. *Dihydrofolate reductase activity of cell lines used*

Activity of the enzyme was determined in extracts of various cell lines as described in the text.

Cell line	Dihydrofolate reductase activity ($\mu\text{mol/h}$)	
	per 10^7 cells	per mg of protein
LM4	5.5–10.0	4.5–9.0
LS2	0.17	0.32
B1A2A	42.5–135.0	21.0–74.5
HTC	1.0	0.26
RPMI 4265	0.2	0.39

Table 2. *Purification of dihydrofolate reductase enzymes by affinity chromatography*

Enzyme from various cell lines was purified by affinity chromatography on Sepharose–methotrexate as described in the text. 'Percentage recovered' means after concentration and dialysis (see the text); —, denotes that concentration was not carried out.

Cell line	Enzyme applied (units)	Percentage not retained*	Percentage eluted	Percentage recovered
LM4	10.0	7	137	—
LS2	10.0	20	128	30
B1A2A	176.0	5	34	—
HTC	9.9	0	51	54
RPMI 4265	8.75	5	115	32

* Including washes.

Table 3. *Electrophoretic behaviour of dihydrofolate reductase from LM4 cells*

Purified enzyme from LM4 cells was subjected to electrophoresis on polyacrylamide gels by using different buffer concentrations with and without NADPH. The tank buffer was Tris-glycine, pH8.5, and the gel buffer, Tris-HCl, pH8.9. R_F values are expressed relative to Bromophenol Blue run in a separate gel.

Concn. of buffer (M)		Concn. of NADPH in tank buffer (μ M)	Electrophoretic pattern	R_F of major band
Tank	Gel			
0.038	0.38	0	Multiple peaks (3)	0.35
0.038	0.38	50	Multiple peaks (3)	0.53
0.38	1.9	0	Single peak	0.33
0.38	1.9	30	Single peak	0.32

Fig. 1. Ferguson (1964) plot of R_F versus gel concentration

R_F values (plotted on a logarithmic scale) were determined after polyacrylamide-gel electrophoresis as described in the text. Each line represents results for a different sample. Lines 1, 3 and 4 each show duplicate results. 1, Bromophenol Blue; 2, soya-bean trypsin inhibitor; 6, myoglobin; 3, 4, 5, most-, medium- and least-mobile forms of dihydrofolate reductase respectively.

The mobilities (R_F) of three dihydrofolate reductase activity bands were recorded at various gel concentrations on a Ferguson (1964) plot. Soya-bean trypsin inhibitor and myoglobin were included as standards. The results are shown in Fig. 1. The slope of the line (the retardation coefficient, $K_r = \log R_F / \text{polyacrylamide concentration}$) can be used as a physical parameter, peculiar to the molecule in question.

The parallel lines obtained for the three dihydrofolate reductase bands (constant K_r) indicate that the separation between these bands is caused by a difference in charge alone and is not due to a difference in molecular weight. The value of K_r (Table 4) obtained indicates a mean molecular radius of 2.0nm for dihydrofolate reductase [see Table 1 of Chrambach & Rodbard (1971)].

Table 4. *Molecular parameters obtained from a series of Ferguson (1964) plots*

Results are given for molecular parameters derived from the Ferguson (1964) plots (Fig. 1). Marker compounds and dihydrofolate reductase were subjected to electrophoresis on gels of various concentrations of polyacrylamide (see the text). K_r is the retardation coefficient and r , the geometric mean radius; (1), (2) and (3) refer to the three forms of dihydrofolate reductase observed under these conditions.

	K_r	$\sqrt{K_r}$	r (nm)	Mol.wt.
Bromophenol Blue	0.0093	0.096	0.7	
Myoglobin	0.046	0.21	1.9	16890
Soya-bean trypsin inhibitor	0.051	0.022	2.0	22700
Dihydrofolate reductase (1), (2), (3)	0.054	0.023	2.0	18000

Table 5. *Effect of pH and additives on dihydrofolate reductase activity*

Dihydrofolate reductase, purified from the cell lines shown, was assayed in the absence and in the presence of the compounds shown. Activities are expressed as percentages of the activity at pH7.0 taken as 100%, which are in the order of cell lines given 15.5, 0.95, 57.5, 0.96 and 0.7 units.

		Activity (%)						
		None		KCl			Urea (2M)	<i>p</i> -Hydroxymercuri- benzoate (0.5mM)
Addition	...	8.5	7.0	0.05M	0.15M	0.5M	7.0	7.0
Cell line	pH	...	8.5	7.0	8.5	7.0	7.0	7.0
LM4		58	100	144	144	181	205	250
LS2		57	100	116	134	174	295	358
B1A2A		56	100	122	139	148	425	465
HTC		46	100	92	113	115	150	172
RPMI 4265		43	100	77	100	86	186	230

The electrophoretic heterogeneity thus results from association of the protein with various amounts of undissociated buffer acid, as described for other proteins by Cann (1970). By increasing the gel and tank buffer concentrations, dihydrofolate reductase was shown to migrate as a single sharp band with an R_F value (relative to Bromophenol Blue) of 0.33. The effects of buffer and NADPH concentrations are summarized in Table 3. The high buffer concentrations were used as a routine to analyse the dihydrofolate reductase isolated from the various cell lines, and the R_F values refer to the enzyme-buffer complex.

Under these conditions, the enzymes from LM4 and LS2 cells showed similar mobility (R_F 0.33 and 0.32 respectively). The enzymes from HTC and B1A2A cells also showed similar mobility (R_F 0.43 and 0.44 respectively), migrating faster than the previous pair. The R_F value of the human enzyme (cell line RPMI 4265) was intermediate (R_F 0.37).

Stimulation of enzymes

All enzymes were stimulated by urea and *p*-hydroxymercuribenzoate (Table 5). Those showing the highest stimulation by *p*-hydroxymercuribenzoate

(cell lines B1A2A, LS2 and LM4) also showed highest stimulation by urea and KCl. Those less stimulated by *p*-hydroxymercuribenzoate (cell lines HTC and RPMI 4265) were also less stimulated by urea and not significantly affected by KCl. All enzymes showed a similar decrease in activity at pH8.5 compared with that at pH7.0.

Inhibition of enzymes

In these studies, 0.024 unit of activity of each enzyme was used, and freshly diluted inhibitors were added. Under those conditions, the enzymes from cell lines RPMI 4265, LM4 and LS2 showed a similar pattern of inhibition at pH8.5 and 6.0, especially at 10 and 100 μ M-methotrexate (Fig. 2). With the enzymes from cell lines B1A2A and HTC at both pH values inhibition was less marked at lower concentrations of methotrexate. At pH6.0, all enzyme activities were inhibited to a greater extent by concentrations of methotrexate of 1 and 10 nM than at pH8.5. The HTC-cell enzyme was more sensitive to inhibition by trimethoprim at pH6.0 than were the other enzymes (Fig. 3).

Fig. 2. Inhibition of dihydrofolate reductase activity by increasing concentrations of methotrexate at pH 8.5 (a) and 6.0 (b)

Enzyme was assayed at each pH as described in the text, in the presence of the concentrations of methotrexate shown. Cell lines: ▲, LM4; △, LS2; □, B1A2A; ■, HTC; ●, RPMI 4265.

Discussion

Dihydrofolate reductase is specifically inhibited by methotrexate, which is clinically useful in the therapy of malignant disease. For this reason, the enzyme has been widely studied from a variety of sources. Its purification from malignant tissue, however, has not been reported often, owing to the limited material available and the relatively complex steps hitherto required in its purification. Affinity chromatography now provides a means whereby small amounts of material can yield highly purified enzyme in one or two simple steps. We have shown the usefulness of this method by purifying and studying dihydrofolate reductase from small amounts of cultured cells. With several cell lines the recovery was greater than 100%, indicating activation of the enzyme or removal of an inhibitor. The purification procedure gave consistent results, as shown in the several preparations of enzyme from LS2 cells (Table 6).

Differences in electrophoretic properties were shown to exist between several of the buffer-enzyme complexes studied. Although definite, those differences were not great and may reflect either a close similarity between the various reductase enzymes or differences that have been partially masked by combination of enzyme with buffer. We found no multiple forms of the enzyme in the various cell lines studied. Our results do reflect the caution necessary in interpreting multiple forms of enzymes observed on electrophoresis when protein-buffer interaction can occur. Also, another possibility is that the enzyme and tracking dye (Bromophenol Blue) may interact to produce multiple forms of the enzyme on gel electrophoresis, as we recently reported (Hiebert *et al.*, 1972). Variation of the buffer concentration and omission of tracking dye can overcome these problems and it can be determined by a Ferguson (1964) plot whether the molecular size or charge of the

Fig. 3. Inhibition of dihydrofolate reductase activity by two concentrations of trimethoprim at pH 8.5 (1) and 6.0 (2)

For details see the text. Concns. of trimethoprim; ▨, 10 μM; ■, 0.1 mM. The enzyme activity is given as the percentage of that at pH 8.5 (1) or 6.0 (2) in the absence of trimethoprim.

Table 6. Purification of dihydrofolate reductase from LS2 cells

Three independent purification procedures were carried out with extracts of LS2 cells (see the text).

Preparation	Enzyme applied (units)	Percentage not retained*	Percentage eluted
1	10.0	20	128
2	7.0	22	187
3	7.6	26	140

* Including washes.

various forms differ. A tenfold increase in buffer concentration produced one instead of several enzyme species. Intermediate concentrations of buffer were not tested but probably a concentration lower than 1.9M gel buffer would have a similar effect.

On the whole, the effect of pH and additives on the enzyme activities were similar. An exception was the enzyme from rat hepatoma cells (HTC), which was only slightly stimulated by KCl, urea and *p*-hydroxymercuribenzoate. The enzyme from human cells also showed little effect from KCl and urea. As expected, the properties of the enzyme from two lines of the L1210 tumour of mice (LS2 and LM4) were alike, but differed from those of the enzyme from the hepatoma cells of the rat (HTC).

This pattern did not apply to the inhibition observed with methotrexate and trimethoprim except that the enzymes from LS2 and LM4 cells were again similar. All, however, showed the expected inhibition

by methotrexate at low concentrations of the drug (1 mM), especially at the lower pH value. A noteworthy observation was that the lowest concentration of methotrexate (10 μM) produced no inhibition of the enzyme from HTC or B1A2A cells at either pH values nor of that from RPMI 4265 cells at pH 8.5. That this was not due to loss of inhibitor, e.g. by absorption on to glass, was shown by the inhibition observed with enzymes from other cell lines. The general shape of the inhibition curves at pH 8.5 resembled that of those enzymes not inhibited by 10 μM-methotrexate at pH 6.0 (cell lines HTC and B1A2A). The reasons for this effect are unknown. The enzyme from HTC cells was also exceptional in its greater inhibition by trimethoprim. The very different properties of this enzyme may result from its organ of origin, liver, rather than its species, rat. The other cell lines were of lymphoid (LS2, LM4, RPMI 4265) or fibroblast (B1A2A) origin.

The properties of the various reductase enzymes were similar, although there were minor differences, e.g. in the inhibition pattern by the antifolate drugs methotrexate and trimethoprim. Such differences might be important in the development and use of inhibitors of this enzyme. Affinity chromatography should prove a useful method for purifying dihydro-folate reductase from small amounts of normal and malignant tissue to permit such studies to be carried out on highly purified enzyme.

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