Europ. J. Cancer Vol. 16, pp. 1539–1546

0014-2964/80/1201-1539 $02.00/0

A Methotrexate Insensitive Variant of Folate Reductase Present in Two Lines of Methotrexate-Resistant L5178Y Cells

J. H. GOLDIE,*†‡ G. KRYSTAL,§ D. HARTLEY,† G. GUDAUSKAS† and S. DEDHAR†

†*Division of Advanced Therapeutics, Cancer Control Agency of British Columbia,* §*Department of Medical Biophysics, B. C. Cancer Research Center, and* ‡*Department of Medicine, University of British Columbia, Canada*

Abstract—*Utilizing affinity chromatography with MTX–sepharose, a variant form of folate reductase has been isolated from two lines of MTX-resistant L5178Y cells. This variant enzyme differs from the principal form of the enzyme present in the cells by being markedly insensitive to MTX inhibition. No detectable amounts of the insensitive reductase were found in the MTX-sensitive cells. Characterization of the insensitive reductases from the MTX-resistant cells indicated that their* Km *for folic acid was essentially unchanged from that of the principal form of the enzyme. However, the* Ki *for MTX of the insensitive reductase had undergone a market increase of approximately* 100,000*-fold. This large increase in* Ki *is likely an important mechanism in conferring a high degree of MTX resistance in these cells.*

INTRODUCTION

THE PRINCIPAL mechanisms of resistance to the folic acid antagonist methotrexate (MTX) that have been reported in experimental tumour systems include (1) increased levels of the enzyme folate reductase (E.C.1.5.1.3) [1–5], (2) impaired transport of methotrexate [2, 6, 7] and (3) an increase in *Ki* for methotrexate of the reductase [6, 8]. The alterations in *Ki* that have been reported have been in the order of a 10–50-fold increase.

We have investigated this latter mechanism of increased *Ki* for MTX in two lines of L5178Y lymphoma cells, both highly resistant to MTX (1300-fold and 300,000-fold increase in resistance, respectively). Affinity chromatography with MTX–sepharose was used to separate out forms of folate reductase on the basis of their affinity for MTX. The principal form of reductase present in the resistant cells is markedly sensitive to MTX, and binds firmly to the affinity column. There is present in addition, however, in both lines of MTX resistant cells, a variant form of folate reductase that does not bind to the affinity column. This second form of reductase is present in much smaller amounts than is the sensitive enzyme, and is characterized by an extreme alteration in *Ki* for MTX (approximately 10^5 increase). This variant form was not detected in the parent MTX sensitive cell line.

Accepted 17 April 1980.
*Address: Cancer Control Agency of British Columbia, 2656 Heather Street, Vancouver, B.C., Canada V5Z 3J3.

MATERIALS AND METHODS

Cell culture

Three lines of L5178Y lymphoma cells were employed in these studies. The properties of these cells are summarized in Table 1. The parent sensitive line was designated S and the two resistant sub-lines which were derived from it were designated R_3 and R_4. The resistant lines were obtained by sub-culturing the cells with progressively increasing sub-lethal concentrations of MTX until the desired level of resistance had been achieved. Both MTX-resistant lines were maintained in

Table 1. *Properties of lymphoma cell lines*

Cell line	ID_{50} (MTX) (M)	Folate reductase Sp. act—U/mg protein)
L5178Y (S)	3×10^{-8}	1.0
L5178Y (R_3)	4×10^{-5}	7.0
L5178Y (R_4)	1×10^{-2}	8.6

MTX-containing medium; the R_3 cell line grew continuously in 10^{-5} M MTX and the R_4 lines in 10^{-3} M MTX. The cells were grown at 37°C as suspension cultures in plastic flasks or tubes (Falcon) under an atmosphere of 5% CO_2 in air. The growth medium was Fisher's medium supplemented with 10% dialyzed horse serum, 1% glutamine and 100 U/ml penicillin—100 μl/ml Streptomycin (Grand Island Biological Co., Grand Island, New York). Cell stocks were diluted every 1 or 2 days with fresh medium to maintain the cultures in exponential growth. Under these conditions the doubling time for all three cell lines was approximately 12 hr. Total cell numbers were determined by counting on a Model Z_{B1} Coulter Counter (Coulter Electronics, Hialeah, Fla.).

Assay for folate reductase activity

Folic acid reductase activity was measured using the radiolabelled folic acid method of Littlefield [9]. The 100 μl incubation mixture consisted of 20 μl 0.01 M potassium acetate buffer, pH 5.0; 30 μl 3.33 mM NADPH; 30 μl of 330 μM [2-^{14}C] folic acid (Sp. Act.: 55 mCi/mmole), or [3′, 5′, 7, 9-^{3}H] folic acid (Sp. Act.: 29 Ci/mmole) (Amersham Corp., Oakville, Ontario) and 20 μl enzyme preparation. Inhibitors (10 μl) were incubated for 5 min at room temperature with the enzyme preparation prior to starting the reaction with folic acid. After incubation at 37°C for 20 min, the reaction mixture was chilled on ice and 20 μl 0.027 M unlabelled folic acid was added. Unreduced folic acid was precipitated by the addition of 20 μl 0.17 M zinc sulphate and 5 μl glacial acetic acid. After 10 min in ice the mixture was centrifuged at 1000 ***g*** for 15 min at 4°C. Fifty μl of the supernatant fraction was added to 10 ml PCS scintillation cocktail (Amersham Corp.) and counted in a Searle Mark III liquid scintillation spectrometer. All assays were corrected for a blank reaction mixture lacking enzyme which showed less than 2% of the added radio-activity. One unit of activity is defined as that amount of enzyme reducing 1 nmole of folic acid in 20 min. Protein was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Chemical Division, Technical Bulletin 1051, April 1977).

Separation of specific folate reductases by affinity chromatography

The methods employed here were a modification of the method of Gauldie and Hillcoat [10, 11].

Preparation of extracts. Approximately 10^9 cells in logarithmic growth were removed from suspension by centrifuging at 250 ***g*** at 4°C for 6 min. (Prior to precipitation of cell extracts R_3 and R_4 cells were grown in MTX-free medium at 37°C for 24 hr to remove all enzyme-bound MTX.) The cells were washed twice in ice-cold PBS (0.15 M NaH_2PO_4 H_2); 0.15 M Na_2HPO_4 pH 7.2 in 0.9% NaCl) and then resuspended in 4–6 ml ice-cold 0.05 M Tris–HCl pH 7.5. The cells were disrupted by sonicating at 20 KHz for 30 sec at 4°C (Branson Sonifier, cell disrupter·350, Branson Sonic Power Co., Connecticut). The lysate was then centrifuged at 100,000 ***g*** for 1 hr at 4°C in a Beckman L5 Ultracentrifuge. The supernatant was termed 'crude lysate'.

MTX–Sepharose affinity chromatography. AH-sepharose-4B (5 g) was swollen and washed with 110.5 M NaCl on a sintered glass filter (G3). The gel was then washed with 500 ml of distilled water (made pH 4.5 with 1 N HCl). The gel was resuspended in 25 ml distilled water (pH 4.5) MTX (10 mg for S cells; and 50 mg for R_3 and R_4 cells) and 1-ethyl-3 (3-dimethyl-aminopropyl)-carbodiimide HCl (88 and 440 mg respectively) were added to the gel and incubated for 36 hr at room temperature on a multipurpose rotator. The MTX–sepharose was then washed with 0.05 M Tris–HCl, pH 8.0; 2 M KCl and poured into a 1 × 8 cm glass column. The packed column was then equilibrated with 200 ml 0.05 M Tris–HCl pH 7.5 containing 10^{-5} M NADPH. 'Crude lysate' (4 ml) was then applied to the column and chromatography was carried out using 20 ml 0.05 M Tris–HCl pH 7.5; 10^{-5} M NADPH (buffer 1), followed by 30 ml 0.2 M Tris–glycine buffer pH 9.5; 2 M KCl, 10^{-5} M NADPH (buffer 2) and the folate reductase was then eluted with 0.2 M Tris–glycine pH 9.5, 2 M KCl and 0.5 mM dihydrofolic acid (buffer 3). Fractions (3 ml) were collected automatically on a Gilson micro fractionator and scanned for protein (U.V. absorbance at 280 nM) and folic acid reductase activity.

High pressure liquid chromatography of reduced folates

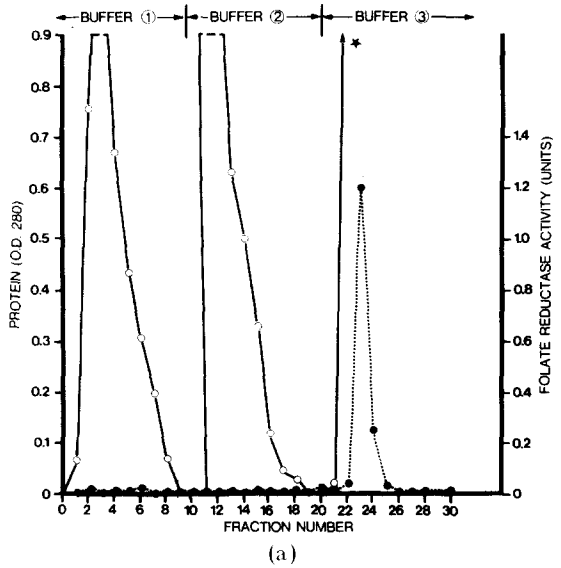
The products of the radioactive folate reductase assay were determined by HPLC. The method used was a modified form of that used by Stout *et al.* for the separation of substituted pteroyl monoglutamates and pteroyl oligo-L-glutamates [12].

Tetrahydrofolates, dihydrofolate and fully oxidised folic acid were separated by anion-

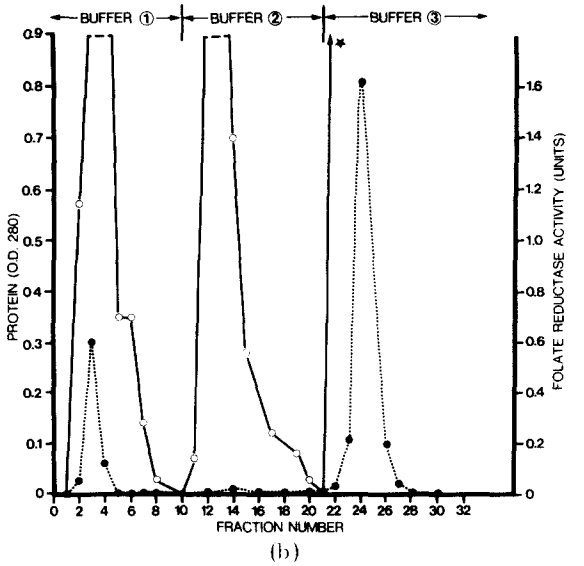
exchange chromatography on a Model 7000 B chromatograph (Micromeritics, Norcross, Ga., U.S.A.) using a Reeve-Angel Partisil-10 SAX column 25 × 4.6 mm i.d. × $\frac{1}{4}$ in o.d. (Whatman, Clifton, N.J., U.S.A.). The eluent (0.16 M NaCl solution containing 4 mM $NaNH_4P_4$ pH 6.5) was formed by mixing distilled water (primary reservoir) and 2 M NaCl containing 0.05 M $NaNH_4HPO_4$ pH 6.5 (secondary reservoir). The run was carried out isocratically with the secondary reservoir set at 8%. The column temperature and the flow-rate were maintained at 29°C and 2.0 ml/min, respectively. Before the chromatographic run, the column was equilibrated with 0.6 M NaCl obtained by the use of a 30% setting for the secondary reservoir. Standard reduced and oxidised folates were detected in the column effluent by their U.V. absorbance at 254 nM, using a Chromonitor 785 flow spectrophotometric detector (Micrometrics). One millilitre fractions of the column effluent were also collected automatically into scintillation vials on a Gilson fraction collecter (Gilson Medical Electronics, Middleton, Wi., U.S.A). Ten ml PCS scintillation fluid (Amersham Corp.) was added to each vial and the radioactivity counted on a Searle Mark III liquid scintillation spectrometer.

RESULTS

Figure 1a shows the affinity chromatogram of the sensitive L5178Y cells. The *y* axis shows the protein estimation (solid line) per fraction and the reductase activity (broken line) per fraction. The *x* axis indicates the number of fractions collected. A peak of folate reductase activity can be seen in the late fractions after dihydrofolate buffer elution. There was no



(a)



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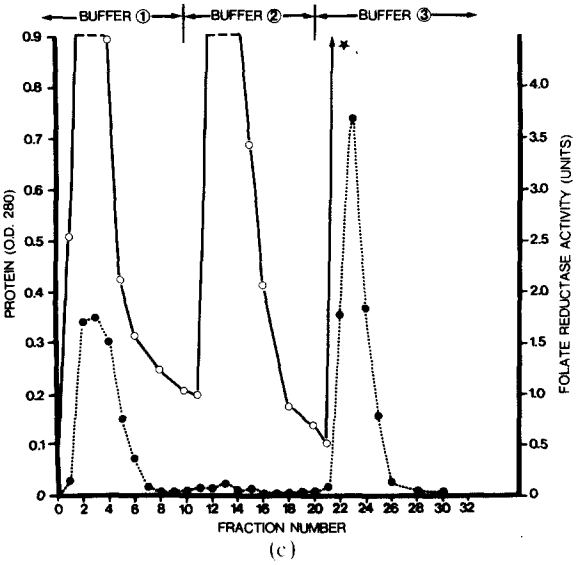


(c)

Figs. 1*a, b and c. Cellular lysates of L5178Y (S), L5178Y (R_3) and L5178Y (R_4) cells were subjected to chromatography on an MTX–sepharose affinity column; (a) L5178Y (S); (b) L5178Y (R_3); (c) L5178Y (R_4). In each case, eluting buffer 1 was* 0.05 M Tris-HCl pH 7.5 + 10^{-5} M NADPH. *Buffer 2:* 0.2 M Tris–glycine pH 9.5 in 2 M KCl + 10^{-5} NADPH. *Buffer 3:* 0.2 M Tris–glycine pH 9.5 in 2 M KCl + 5 mM *dihydrofolic acid.* x *axis: fraction number. y axis: protein estimation per fraction (solid line).* y *axis: folate reductase activity per fraction (broken line). *The high O.D. 280 values in the fractions eluted with buffer 3 are due to the presence of dihydrofolic acid in these fractions. The fractions were dialyzed against* 0.05 M Tris–HCl *to remove the dihydrofolic acid prior to assaying for enzyme activity.*

detectable folate reductase activity seen in the early fractions before the elution.

Figure 1b shows the affinity chromatogram of the R_3 cells. Its overall appearance is similar to that of the sensitive cells but there is now apparent a small peak of reductase activity in the early fractions before elution. There is in addition a second large peak of reductase activity in the later fractions after dihydrofolate buffer elution.

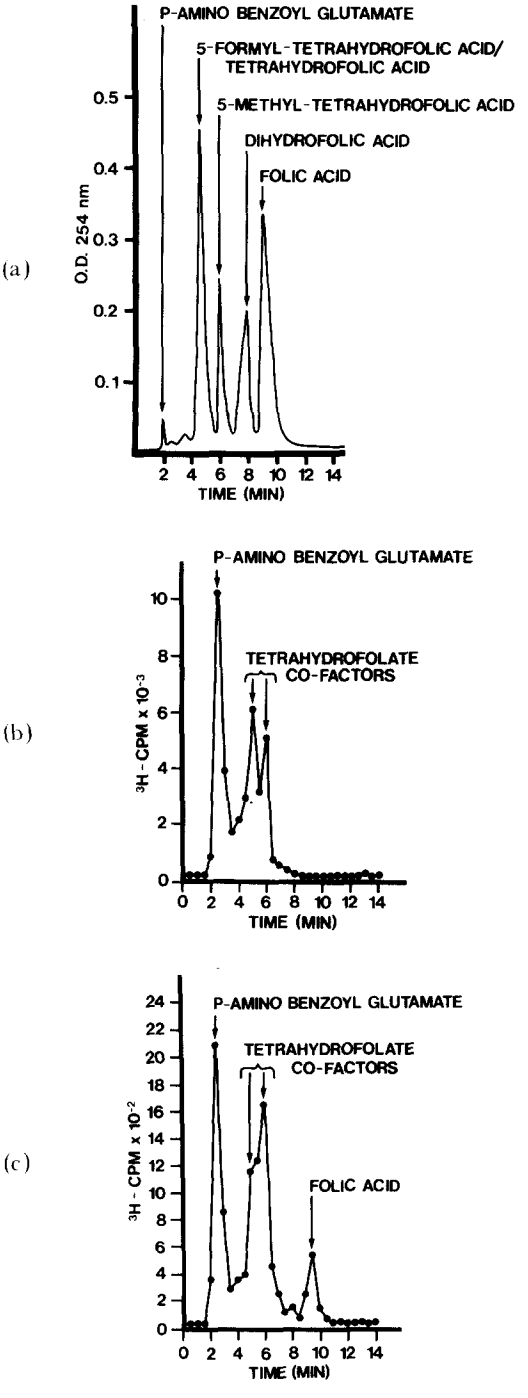
Figure 1c shows the affinity chromatogram of the R_4 cells and there is again noted a peak of early fraction activity before dihydrofolate buffer elution. The amount of this activity is greater than that which was seen in the R_3 cells. There is again a second larger peak after elution with dihydrofolate buffer.

These results suggested that the resistant cells might be containing a second form of folate reductase that was not binding to the affinity column. However, the detection of this 'early fraction' reductase could have other explanations. The positive assay results in the early fraction material could be spurious due to nonspecific breakdown of the radiolabelled folic acid mimicking the action of the true reductase. Another possibility to be ruled out was that the activity in the void volume represented 'spill over' of the normal sensitive reductase due to saturation of binding sites on the column. If these possibilities could be excluded then one would have greater confidence in attributing the early fraction activity to a specific variant reductase which had low affinity for the MTX–sepharose column.

To determine that true enzyme activity was present in the early fraction material, two further tests were carried out. Firstly, it was noticed that no enzyme activity was detected if NADPH was excluded from the reaction mixture. Next, HPLC analysis of the products of the reaction mixture was performed. The purpose of this analysis was to determine whether the low affinity reductase present in the early fractions was generating tetrahydrofolate as its product. The supernatant of the folate reductase radioassay was subjected to chromatography by HPLC on an anion-exchange column (see Materials and Methods).

Figure 2a shows the separation of a standard mixture of N_5 formyl tetrahydrofolic acid (folinic acid), N_5 methyl tetrahydrofolic acid, tetrahydrofolic acid, *para*-aminobenzoyglutamate, *para*-aminobenzoic acid, dihydrofilic acid and folic acid.

Figure 2b represents the radioactive profile of the reaction products of the MTX-sensitive folate reductase present in the cell lysate from the L5178Y (S) cells. Three radioactive peaks are evident,co-chromatographing in the regions of *para*-aminobenzoic acid and the tetrahydrofolate cofactors. The retention time of peak 1 coincides with *para*-aminobenzoic acid and can be tentatively identified as such. Peaks 2 and 3 coincide with the regions where N_5 formyl tetrahydrofolic acid and methyl tetrahydrofolic acid and methyl tetrahydrofolic acid clute. Under these chromatographic conditions tetrahydrofolic acid and N_5 formyl tetrahydrofolic acid co-elute. The absolute



Figs. 2a, b and c. (*a*) *Separation of a mixture of folate compounds* (*as indicated*) *by high pressure liquid chromatography on an anion exchange column.* (*For experimental details, see Materials and Methods section*). (*b*) *and* (*c*): 20 µl *of the supernatant from the radiolabelled folate reductase assay of the L5178Y* (*S*) *lysate and L5178Y* (R_3) '*early fraction*' *reductase, respectively, were injected onto the anion exchange column and chromatographed by high pressure liquid chromatography under the same conditions used for the separation of the standard folate mixture* [*see* (*a*)]. (*For experimental details, see Materials and Methods section*).

identification of these peaks awaits further work, but they can tentatively be identified as one or more forms of tetrahydrofolate.

Figure 2c shows the radioactive profile of the products of the MTX-insensitive folate reductase from the L5178Y R_3 cells. The appearance is similar to the products of the L5178Y S lysate with there being two peaks in the region of the tetrahydrofolate cofactors. Nonreduced folic acid is present but there is a complete absence of dihydrofolic acid.

From these results we would conclude that in early fraction material there is present a true folate reductase enzyme which is capable of generating tetrahydrofolic acid from the reduction of folic acid.

If the early fraction reductase simply represented overflow from the affinity column, then the properties of this enzyme with respect to MTX sensitivity should be identical to that of the enzyme bound to the affinity column.

We next determined the sensitivity of the early fraction enzyme activity to MTX inhibition. Early fraction material from both resistant cells was pooled and lyophilized. The activity of each of the R_3 and R_4 early fractions was tested for its sensitivity to MTX inhibition. This is shown in Fig. 3 where the percentage inhibition of the enzyme is plotted against increasing concentrations of MTX. Included for comparison is the sensitivity of the folate reductases derived from the sensitive L5178Y cells (closed circles), and the late fraction (high affinity) reductase derived from the R_4 cells (open squares). It can be seen that both sensitive enzymes show virtually 100% inhibition by 10^{-6} M MTX. In contrast, the R_3 early fraction enzyme (solid circles) showed only approximately 50% inhibition by 10^{-3} M MTX and the R_4 early fraction enzyme showed less than 40% inhibition by 10^{-3} M MTX.

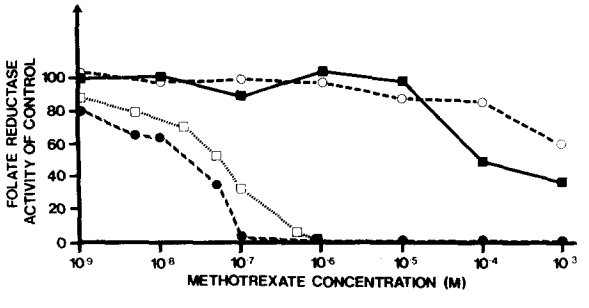


Fig. 3. Enzyme preparations indicated below were assayed for folate reductase activity in the presence of MTX concentrations shown above. The enzyme preparation was incubated with NADPH and MTX at 37°C for 5 min. The reaction was started by the addition of 2-^{14}C folic acid. (--●--) *L5178Y (S) lysate,* (—□—) *L5178Y (R_4) High MTX-affinity reductase.* (—■—) *L5178Y (R_3) early fractions reductase.* (--○--) *L5178Y (R_4) early fractions reductase.*

Thus it is seen that the early fraction reductases have markedly diminished sensitivity to MTX inhibition compared with both the L5178Y S enzyme and the late fraction high affinity reductase from the resistant cells. This great difference in MTX sensitivity would preclude the possibility that the early fraction reductase was simply spill over of the 'normal' sensitive reductase.

The *Km* for folic acid and approximate *Ki* for MTX were determined for the folate reductase activities present in the L5178Y S. R_3, and R_4 lysates (Table 2). *Km* values for folic acid were determined from Lineweaver–Burk plots [13]. The slope and the intercepts on the *x* and *y* axes were determined with the aid of a linear regression program written in BASIC language on a PDP 11-34 computer.

Table 2. Km *and* Ki *of folate reductase*

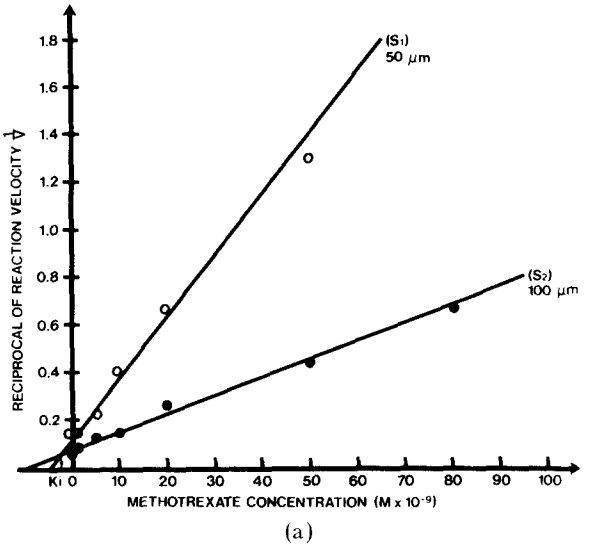
Cell lysate	*Km* folic acid (M)	**Ki* for methotrexate (M)	ID_{50} (MTX) (M)
L5178Y (S)	1.72×10^{-5}	2.6×10^{-9}	2.3×10^{-8}
L5178Y R_3	2.8×10^{-4}	1.5×10^{-8}	6.0×10^{-8}
L5178Y (R_4)	4.8×10^{-4}	1.9×10^{-8}	3.0×10^{-7}

*The *Ki* values for methotrexate under these conditions are only approximate because of the tight binding nature of the inhibitor, and because of the different molar concentrations of enzyme in the R_3 and R_4 cells.

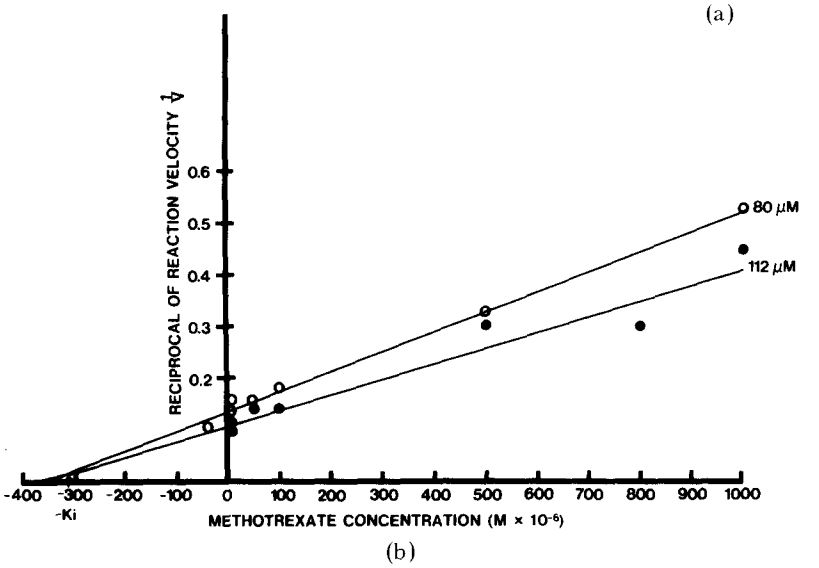
The *Ki* values for MTX with respect to the substrate, folic acid, were determined from Dixon plots of 1/VI against MTX concentration at different substrate concentrations [14]. The slopes and the *x* and *y* intercepts were determined as described for the *Km* values. Because of the tight binding inhibition by MTX, and because of differing molar concentration of enzyme in the different cell lines, this method does not yield a completely accurate estimate for *Ki*, though the values are useful for comparative purposes. Included also are the ID_{50} concentrations of MTX for the enzyme (ID_{50} = molar concentration of drug required to reduce the enzyme activity by 50%). It is noted that there was a moderate increase in the *Km* for folic acid and ID_{50} for MTX in the two resistant cell lines as compared with the sensitive cell line. The values for the two resistant cell lines were very similar.

Next, the *Km* and *Ki* of the folate reductase in the early fraction material were determined. These values are given in Table 3, the *Km* for folic acid is essentially identical in the R_3 and R_4 early fraction enzymes. This in turn is very close to the values that were determined for the whole cell lysate. Marked differences are seen in the *Ki* for MTX which in the R_3 early fraction enzyme is now increased to 3.2×10^{-4} M and in the R_4 enzyme where there has been a further increase to 7.5×10^{-4} M. In the case of the R_4 enzyme this represents an increase in *Ki* for MTX of approximately 2×10^5.

To further illustrate the magnitude of the shift in *Ki* that has occurred in the early fraction reductases, the Dixon plots for the reductases from the L5178Y sensitive cell lysate, and the early fraction reductases from the R_3 and R_4 cells are displayed in Figs. 4a, b and c.



(a)



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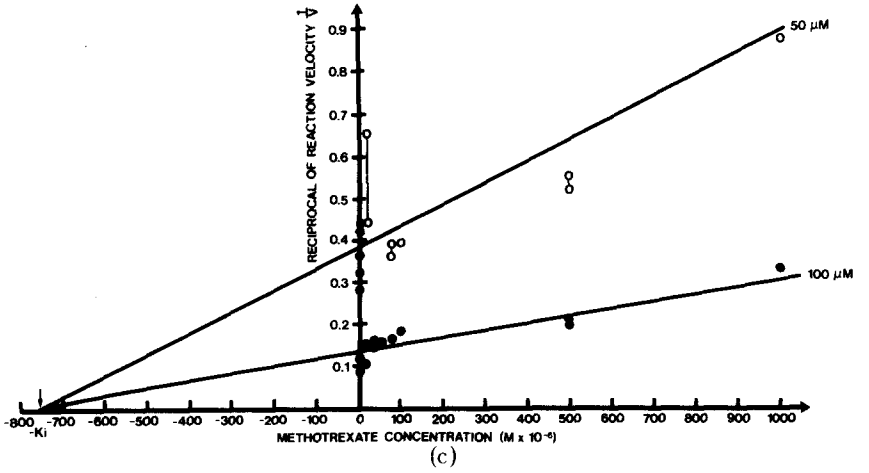

(c)

Figs. 4a, b and c. Dixon plots of the reciprocal of initial velocity of reaction against MTX concentration at two substrate (folic acid) concentrations. Two lines with different slopes are obtained and intersect at a point to the left of the ordinate axis. This point lies at a value of −Ki, *which can thus be read directly from the graph* [13]. *The assays for the determination of* Ki *values were carried out using the radiolabelled folic acid method described in the text. The concentration of NADPH was kept constant for all determinations. (a) L5178Y (S) Lysate. (b) L5178Y (R_3) early fractions reductase. (c) L5178Y (R_4) early fraction reductase.*

Table 3. Km *and* Ki *of folate reductases in the 'early fractions'*

Early fraction from affinity column	*Km* for folic acid (M)	*Ki* for methotrexate (M)
L5178Y (R_3)	4.26×10^{-4}	3.2×10^{-4}
L5178Y (R_4)	4.76×10^{-4}	7.5×10^{-4}

DISCUSSION

The results described above indicate that the resistant sub-lines of L5178Y cells contain two forms of folate reductase. The principal form is similar to the enzyme present in the MTX-sensitive cells in that it binds firmly to the affinity column and is very sensitive to MTX inhibition. The second form of the enzyme is present in much lower amounts and is characterized by poor binding with MTX–sepharose and by a marked insensitivity to MTX inhibition. The insensitive enzymes retained their affinity for the substrate folic acid, suggesting the capability of physiological function in the intact cell. In the parent MTX-sensitive cells there were no detectable amounts of the insensitive reductase present. In the R_4 cells, which are the most resistant subline, there has been an increase both in the amount of the low affinity reductase and in the value for the *Ki*, with respect to the R_3 low affinity reductase. Thus, in the three cell lines studied there is a correlation between the degree of MTX resistance and the presence and amount of the insensitive reductase.

The marked insensitivity to MTX of the early fraction enzymes from the two resistant cell lines would seem to provide the basis as to why these enzymes do not bind to the affinity column. Binding to the column is dependent on there being a high affinity between the enzyme and the pteridine portion of the MTX molecule. The usual forms of folate reductase are characterized by very high affinity and by tight binding with MTX and hence the utility of MTX–sepharose as a means of separating out and purifying folate reductase. The variant folate reductase has markedly diminished sensitivity to MTX and thus would not be expected to bind appreciably to the column.

In other respects the low affinity folate reductases appear to be similar to that of the high affinity form. They carry out the NADPH-dependent reduction of folic acid to tetrahydrofolate and the *Km* for folic acid of the enzyme is not markedly different from that of the high affinity variant.

The next question that has to be addressed is whether the presence of these variant low affinity forms of folate reductase constitute an important mechanism of MTX resistance in these two cell lines. The total amount of folate reducing activity associated with the low affinity enzymes is approximately 2% of total activity in the R_3 cells and about 10% in the R_4 cells, bearing in mind that the total amount of enzyme activity in these two cells has increased 7–8-fold as compared with the parent sensitive line. If one arbitrarily assigns a value of folate reductase activity in the S cells of 100%, then the values of activity in the R_3 and R_4 cells are 15 and 70% respectively, using the amount of activity in the sensitive cells as the standard. It has been estimated [15, 16] that in murine tumour cells no more than 5% of the folate reductase activity is required to generate sufficient tetrahydrofolate cofactors to maintain cell viability. Thus, the amounts of low affinity enzyme present in these resistant cells could in theory be sufficient to maintain folate metabolism, even if all of the sensitive enzyme were inactivated. As the titration curves of the enzyme show (Fig. 3), this inhibition of sensitive enzyme would be complete at drug concentrations that have no significant effect on the insensitive enzymes. Significant inhibition of the low affinity insensitive enzyme will only occur at drug concentrations greatly in excess of that required to inhibit the sensitive enzyme. This is consistent with the response of the intact cell *in vitro*, which are killed only by very high extracellular MTX concentrations.

It remains to be seen whether markedly insensitive reductases of the type described in this paper are frequently present in highly resistant cell lines and hence constitute a general mechanism of MTX-resistance, or whether their presence is confined to only a small number of experimental tumour cells. In this connection, preliminary work in our laboratory (data not shown) has indicated the presence of an early fraction low afffinity reductase similar to the ones described above, that is present in MTX-resistant L1210 cells.

If low affinity reductases similar to those described above are commonly present in MTX-resistant cells, then it would be reasonable to assume that they are a significant factor in conferring phenotypic resistance to MTX in the cell. In such circumstances detailed characterization of the enzyme and a search for more effective inhibitors of it might prove profitable with respect to an improved pharmacological control of MTX-resistant tumours.

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