Plant Physiol. (1993) 101: 477–483

Methotrexate Resistance in *Datura innoxia*[1]

Uptake and Metabolism of Methotrexate in Wild-Type and Resistant Cell Lines

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A wild-type *Datura innoxia* cell line (Px4) was used to select methotrexate-resistant cells through a stepwise procedure. Two independently selected cell lines, MTX161 and MTX132, were stable and shown to be 5 to 15 times more resistant to methotrexate than wild type. These methotrexate-resistant cells were similar to the wild-type cells in levels and kinetic properties of dihydrofolate reductase, the sensitivity of dihydrofolate reductase to methotrexate, the binding of [^{3}H]methotrexate to soluble proteins, and the formation of methotrexate polyglutamate derivatives. High performance liquid chromatographic analyses indicated that methotrexate polyglutamylation is only slight and may not be significant in the toxicity of methotrexate to *Datura* cells. The uptake of methotrexate was also investigated in the wild-type and resistant cells. The Px4 cells exhibited a linear uptake that lasted for 1 to 7 h. The uptake was saturable, pH and energy dependent, and had a K_m of 65.6 nM and a V_{max} of 12.5 nmol $h^{-1}g^{-1}$ fresh weight. Neither MTX161 nor MTX132 exhibited the sustained uptake of methotrexate shown by the Px4 cells. As a result, there were greatly reduced concentrations of intracellular methotrexate in resistant cells. Resistant cell lines had 2- to 3-fold higher K_m values for methotrexate uptake compared with Px4 cells. It is proposed that these cells became resistant as a result of a stable change in the membrane transport system for methotrexate.

DHFR (EC 1.5.1.3) catalyzes the reduction of DHF to tetrahydrofolate. Folate coenzymes are necessary in the biosynthesis of thymidylate, purine nucleotides, Ser, Gly, and Met. DHFR plays a key role in these processes because the generation of tetrahydrofolate is required for subsequent one-carbon transfer reactions (Cossins, 1980, 1987).

MTX, an analog of DHF, is a potent inhibitor of DHFR from various organisms including plants (Cossins, 1980). In cultured mammalian cells, increased levels of DHFR resulting from gene amplification have been identified as a common mechanism of acquired MTX resistance (Schimke, 1984). MTX-resistant plant cell lines have been isolated from various species (Mastrangelo and Smith, 1977; Shimamoto and Nelson, 1981; Barg et al., 1984, 1987; Cella et al., 1984, 1987; Lazar et al., 1989; Barg and Shabtai, 1991; Shabtai et al., 1992). At least three biochemical changes have been identified that are associated with acquired resistance to MTX in plants, namely (a) an increase in a MTX-binding protein(s) that is different from DHFR (Barg et al., 1987; Shabtai et al., 1992); (b) a decrease in the uptake of MTX (Cella et al., 1984; Barg and Shabtai, 1991); and (c) an increase in the target enzyme, DHFR (Shimamoto and Nelson, 1981; Cella et al., 1987; Lazar et al., 1989). However, no DHFR gene amplification in plant cells has been demonstrated.

In mammalian cells, MTX, like its physiological counterpart tetrahydrofolate, can serve as a substrate for FPGS and be converted into MTX polyglutamates (Jolivet and Schilsky, 1981). This not only increases the intracellular retention of MTX but also increases binding to DHFR (Fry et al., 1982; Pizzorno et al., 1989). Lack of such polyglutamylation has been implicated as a cause for the MTX resistance in some mammalian cells (Cowan and Jolivet, 1984). However, MTX polyglutamylation in plants has not been investigated.

Our initial interest was to investigate the possibility of MTX-induced gene amplification in plant cells. Stepwise selection against increasing MTX concentrations led instead to two MTX-resistant cell lines of *Datura innoxia* with reduced MTX uptake. These cell lines could provide a useful system for the investigation of folate transport and metabolism in plants. Here we report the isolation and characterization of these two MTX-resistant cell lines. The properties of uptake and the metabolism of MTX in wild-type and these resistant cell lines were also investigated.

MATERIALS AND METHODS

Cell-Suspension Cultures

A predominantly haploid (>90%) wild-type cell-suspension culture of *Datura innoxia* P. Mill. was used for the isolation of MTX-resistant cell lines. Cell lines were maintained as previously described (Saxena and King, 1988).

Variant Selection

MTX was dissolved in 0.1 N NaOH, made up to a final concentration of 10^{-2} M, filter sterilized, and stored at −20°C. A stepwise procedure was used for selection of MTX-resistant

[1] This work was supported by grants-in-aid of research from the Natural Sciences and Engineering Research Council of Canada to J.K. and E.A.C. K.W. is grateful for a University of Saskatchewan Graduate Scholarship.

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Abbreviations: DHF, dihydrofolate; DHFR, dihydrofolate reductase; DNP, dinitrophenol; FPGS, folylpolyglutamyl synthetase; MTX, methotrexate; $MTX(Glu)_2$, methotrexate diglutamate; PCV, packet cell volume.

variants. Five grams of 3-d-old wild-type cells were passed through a 300-μm screen (Albert Godde-Bedin Sales, Elmsford, NY) collected on Miracloth (Chicopee Mills, Inc., New York, NY), and resuspended in 50 mL of fresh culture medium containing 10^{-9} M MTX. The medium was replaced once every 7 to 14 d. The MTX concentration was raised only when significant increases in PCV were observed. Two suspensions were subject to selection, and two independently selected MTX-resistant cell lines were obtained by a stepwise procedure in which MTX concentrations were raised from 10^{-9} to 10^{-7} M in 10 steps over a period of 7 months.

Growth of the cells was measured by both a filter paper assay (Horsch et al., 1980) and increase in PCV.

Extraction and Assay of DHFR

A 3-d-old suspension culture was homogenized in 50 mL of KH_2PO_4 buffer (0.1 M, pH 7.0) with 10% glycerol and 25 mM 2-mercaptoethanol. The homogenate was centrifuged at 30,000*g* for 15 min and the supernatant was treated with $(NH_4)_2SO_4$. The 30 to 60% $(NH_4)_2SO_4$ precipitate was then dissolved in 1 to 2 mL of KH_2PO_4 buffer and used for enzyme assay.

The DHFR assay used was that of Huennekens et al. (1976). The following components were added to a 1-mL cuvette: 0.25 mL of 0.1 M KH_2PO_4 buffer (pH 7.0), 0.1 mL of 1.5×10^{-3} M NADPH, 0.01 to 0.02 mL of enzyme, and various concentrations of MTX. The reaction mixture was incubated for 5 min before the reaction was initiated by the addition of 0.1 mL of DHF. The specific activity was expressed as nmol of DHF reduced mg^{-1} protein min^{-1} estimated from a molar extinction coefficient of 12,300 mol^{-1} cm^{-1}. Protein concentrations were determined according to Bradford (1976).

[^{3}H]MTX Binding Assay

The assay was performed essentially as described by Flintoff and Essani (1980) and by Cella et al. (1983). The 30 to 60% $(NH_4)_2SO_4$ precipitated proteins (0.5–1.0 mg) were suspended in 1 mL of the enzyme reaction mixture described above and binding was initiated by addition of 1 μCi [^{3}H]-MTX. The reaction mixture was allowed to incubate for 20 min at room temperature to ensure that binding was at equilibrium. To separate MTX bound to proteins from unbound MTX, gel filtration using Bio-Gel P-6DG gel (Bio-Rad) was used. Any drug bound to protein was excluded from the column, whereas free drug was bound to the column and was eluted after the void volume. Fractions of 0.5 mL were collected, added to 4 mL of Phase Combining System scintillation fluid, and then counted.

[^{3}H]MTX Uptake and Efflux

Three-day-old cells were collected by filtration on a nylon mesh and washed with fresh culture medium. Aliquots (200 mg) were resuspended in 10 mL of medium in a 25-mL flask and the suspensions were incubated at 25°C on a horizontal gyratory shaker (150 rpm). After the addition of [^{3}H]MTX, 0.5-mL duplicate aliquots were pipetted at the indicated times onto discs of 2.4-cm diameter filter paper (Whatman, GH/A grade) and washed three times using 5 mL of distilled water containing unlabeled MTX at a concentration 10 times higher than that of labeled MTX in the medium. The washed cells and filters were placed in scintillation vials with 4 mL of Phase Combining System and counted.

Efflux of radiolabeled MTX was measured as follows. Cells were exposed to different concentrations of [^{3}H]MTX for 1 h. These cells were then quickly washed and resuspended in MTX-free medium for 24 h. At intervals, aliquots of cells were harvested at different times and intracellular [^{3}H]MTX was measured. All uptake and efflux measurements were corrected for zero-time nonspecific binding of radioactivity. Any variation in experimental protocol is indicated in the text.

MTX Polyglutamate Assay

Five grams of cell suspension were incubated in the culture medium with [^{3}H]MTX (μCi mL^{-1}) for 24 or 48 h. For the extractions of MTX and its derivatives, cells were collected on Miracloth, washed with ice-cold distilled water, and then resuspended in 10 mL of 20 mM KH_2PO_4 buffer (pH 7.0) containing 50 mM 2-mercaptoethanol and heated at 100°C for 10 min. The suspension was thoroughly homogenized by using a pestle and mortar, then centrifuged at 10,000*g* for 10 min. The pellets were washed with 3 mL of KH_2PO_4 buffer and recentrifuged. The supernatant solutions were collected and stored at −20°C until HPLC analysis.

MTX derivatives were separated by ion-exchange chromatography (Clarke and Waxman, 1987) and by HPLC using the chromatographic procedure of Shane (1986). MTX (4-amino-N^{10}-methylpteroyglutamic acid, sodium salt from Cyanamid of Canada Limited) and MTX$(Glu)_2$ (4-amino-N^{10}-methylpteroyldi-γ-L-glutamic acid, ammonium salt from Dr. B. Schircks Laboratories, Jona, Switzerland) were used as standards. In the former method, derivatives were eluted from a column (0.75 × 16 cm) of Whatman DE-52 cellulose by a linear gradient of 50 to 400 NaCl in 5 mM K_2HPO_4 (pH 7.0) and detected by their absorbance at 302 nm. HPLC analysis involved elution from a 4.6 × 250 Partisil 10 SAX column (flow rate 1 mL min^{-1} at 35°C) connected to a Varian 5000 liquid chromatography system. The column was eluted isocratically for 10 min with 25 mM ammonium phosphate (pH 6.5), followed by a gradient of ammonium phosphate at pH 6.5 (25–275 mM for 30 min and 275–500 mM for 45 min). MTX and MTX$(Glu)_2$ standards were detected at 302 nm. The identity of labeled MTX polyglutamates was based on comparing their retention times with those of authentic standards.

RESULTS

MTX Sensitivity

Two independently selected MTX-resistant cell lines were isolated by a stepwise procedure. The sensitivity of the wild-type (Px4) and the resistant (MTX161 and MTX132) cell lines to MTX are shown in Figure 1. Whereas the growth of the wild type was nearly completely inhibited by 10^{-8} M MTX, the resistant cell lines required 10^{-6} M for nearly complete inhibition of their growth. The I_{50} values for Px4, MTX132, and MTX161 were 5×10^{-9} M, 2.5×10^{-8} M, and 7.5×10^{-8}

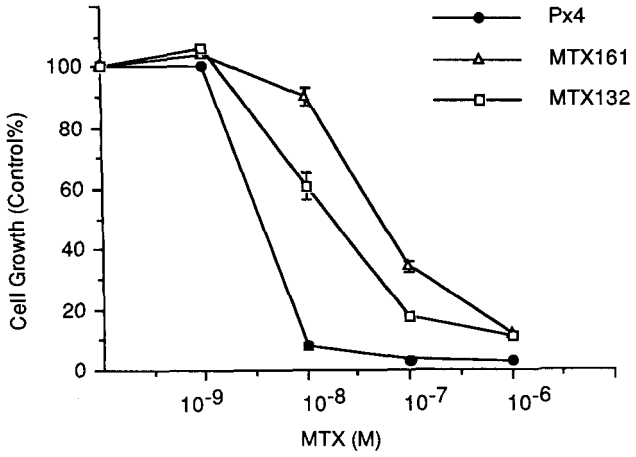

Figure 1. Inhibition of the growth of wild-type (Px4) and resistant (MTX161 and MTX132) cell lines of *Datura* by MTX.

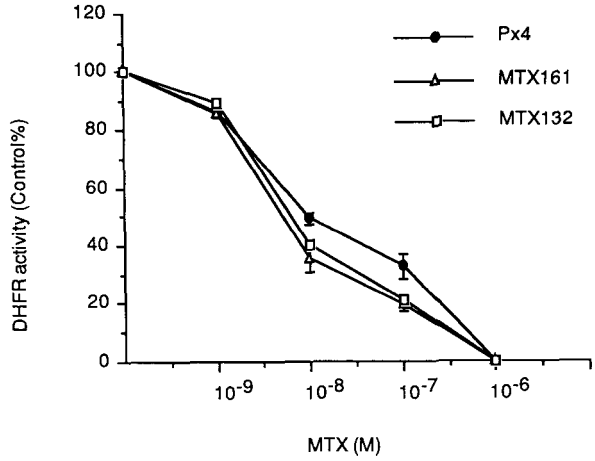

Figure 2. Effect of MTX on the specific activity of DHFR from wild-type (Px4) and resistant (MTX161 and MTX132) *Datura* cell lines. See Table I for the specific activity of uninhibited enzymes.

M, respectively. Thus, the two resistant lines were approximately 5- and 15-fold more resistant to MTX than the wild-type cell line. The MTX-resistant phenotypes have remained stable after subculturing in a medium without MTX for more than 1 year (data not shown).

DHFR Activity

The specific activity of DHFR and the apparent K_m for NADPH and DHF are shown in Table I. There was no significant difference between wild-type and resistant cell lines. The inhibition of DHFR activity by MTX was comparable for all cell lines (Fig. 2).

[^{3}H]MTX Binding

The levels of binding of [^{3}H]MTX to proteins of wild-type and resistant cell lines were 923, 1102, and 1084 cpm of [^{3}H]MTX mg^{-1} protein, respectively. No significant difference was found in the ability of [^{3}H]MTX to bind to proteins in these cell lines.

Analysis of MTX Polyglutamates

Following exposure to [^{3}H]MTX, the intracellular accumulation of MTX and its polyglutamate derivatives was analyzed by HPLC in the wild type (Px4) and one resistant cell line (MTX161). After 24 h of incubation with 5×10^{-9} M MTX, ion-exchange chromatography revealed a labeled derivative in extracts of both cell lines, which was eluted before authentic MTX and MTX(Glu)$_2$ (data not shown). This early-eluting product (retention time 5 min) was also detected when these extracts were examined by HPLC (Fig. 3). After 24 h of incubation, this product accounted for a significant amount of the ^{3}H recovered from wild-type cells. In contrast, extracts of MTX-resistant cells contained very little radioactivity in this compound (Fig. 3C). After 48 h, label in this compound increased in the MTX-resistant cells (Fig. 3D) but declined in the wild type (Fig. 3B). Earlier studies of MTX metabolism by mammalian cells also revealed a derivative that was eluted prior to MTX and MTX polyglutamates (Jolivet and Schilsky, 1981). This product, although unidentified, did not inhibit mammalian DHFR. It remains to be determined whether the compound formed by *Datura* cells is like that observed by these earlier workers.

These extracts (Fig. 3) also contained two other distinct peaks of radioactivity (retention times of 15 and 25 min, respectively). Based on a comparison of these retention times with those of authentic standards, we conclude that peak 2 is MTX and peak 3 is MTX(Glu)$_2$. In the wild type, the amounts of MTX polyglutamate was no greater after 48 h of incubation than at 24 h (Fig. 3, A and B). Significant amounts of radioactivity were not detected in higher polyglutamates of MTX. In the resistant cells, MTX(Glu)$_2$ was not detected after 24 h of incubation (Fig. 3C), but small amounts of this derivative were found after 48 h (Fig. 3D). Considering the levels of tritium in these products, we conclude that MTX161 cells took up less [^{3}H]MTX during these incubation periods but were not necessarily deficient in their ability to form MTX polyglutamates.

Table I. *Kinetic constants of DHFR from the wild-type (Px4) and resistant (MTX161 and MTX132) cell lines of Datura*

Cell Lines	Specific Activity	K_m of NADPH	K_m of DHF
	nmol mg^{-1} protein min^{-1}	*μM*	*μM*
Px4	17.37 ± 3.52	44.2 ± 3.3	83.2 ± 16.7
MTX161	18.96 ± 3.45	49.8 ± 7.1	88.5 ± 16.3
MTX132	21.34 ± 2.16	42.1 ± 6.2	71.4 ± 14.1

Uptake of [^{3}H]MTX

The uptake rates of [^{3}H]MTX throughout the cell-growth cycle were measured (Fig. 4, A and B). Uptake increased to a peak during the period of most rapid growth, then decreased to a rate equal to that at the time of subculturing (Fig. 4B). The [^{3}H]MTX uptake rates of the two resistant cell lines were significantly lower than that of the wild type during the entire growth cycle.

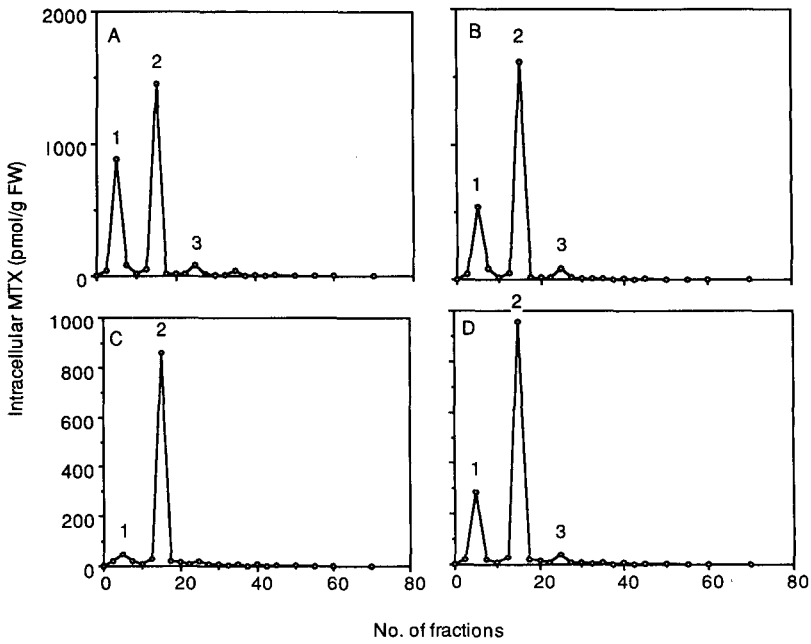

Figure 3. HPLC of intracellular [^{3}H]MTX in *Datura* cells. Cells were incubated in the medium with 5 nM [^{3}H]MTX for 24 (A and C) and 48 h (B and D). A and B, Wild-type cells (Px4); C and D, resistant cells (MTX161). 1, Unidentified derivative; 2, MTX; 3, MTX(Glu)$_2$.

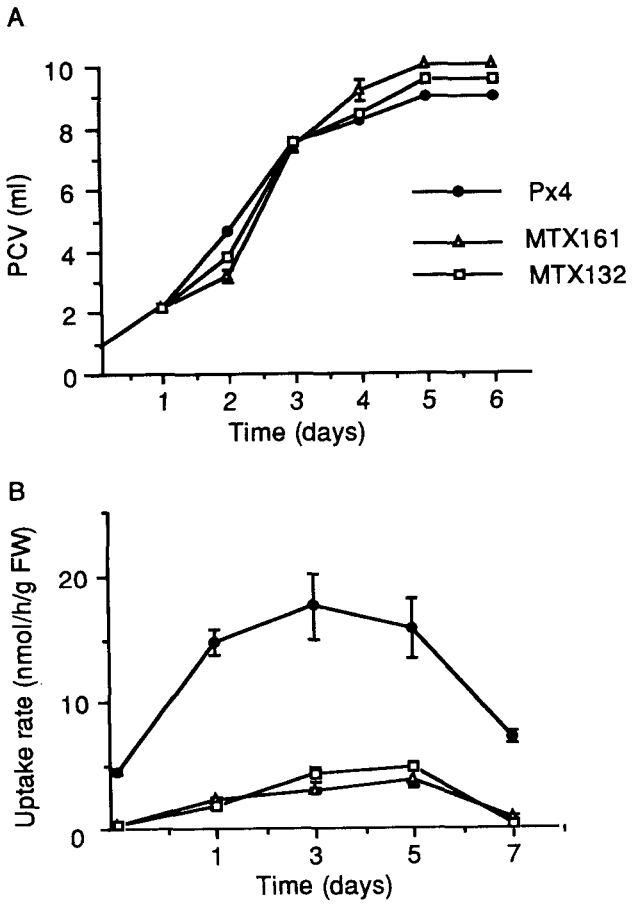

Figure 4. Increase in packed cell volume (A) and MTX uptake rate (B) during the growth cycles of wild-type (Px4) and resistant (MTX161 and MTX132) cell lines of *Datura*.

When the concentration of MTX in the medium was 2.42 $\times$ 10^{-8} M, the wild-type cells exhibited a rapid accumulation of MTX that persisted for about 1 h and that was followed by a longer period of several hours during which MTX accumulated more slowly (Fig. 5, A and B). At a high level of extracellular MTX (2.42 $\times$ 10^{-7} M), a rapid accumulation of MTX lasting 7 h was observed in the wild type (Fig. 5C).

Neither MTX161 nor MTX132 exhibited the sustained uptake of MTX shown by wild-type cells. Markedly lower amounts of intracellular [^{3}H]MTX in resistant cells were found. To identify if these lower amounts of intracellular MTX are due to reduced uptake or enhanced efflux of MTX, efflux of radiolabeled MTX was measured after 1 h of exposure to [^{3}H]MTX (24.2 and 242 nM). In all three cell lines, the levels of [^{3}H]MTX decreased in the 1st h, but thereafter remained constant (data not shown). This suggests that increased efflux was not the main reason for lower amounts of intracellular [^{3}H]MTX.

The effects of extracellular pH and metabolic inhibitors on the uptake of MTX were examined. There was a significant decrease in uptake rate above pH 5 (Fig. 6). Metabolic inhibitors DNP and sodium azide reduced uptake significantly (Table II).

The K_m and V_{max} values of MTX uptake in these cell lines were calculated from linear regressions of uptake by cells exposed to between 15 and 100 nM concentrations of MTX (Table III). Although the V_{max} values of the three cell lines were similar, the K_m values for uptake of MTX by the two resistant cell lines were 2- to 3-fold higher than that of the wild type.

DISCUSSION

The similarity of the growth curves of the wild-type and resistant cell lines growing in a MTX-free medium suggests that the latter were not defective in their ability to grow. The acquired MTX resistance in the resistant cell lines seems to

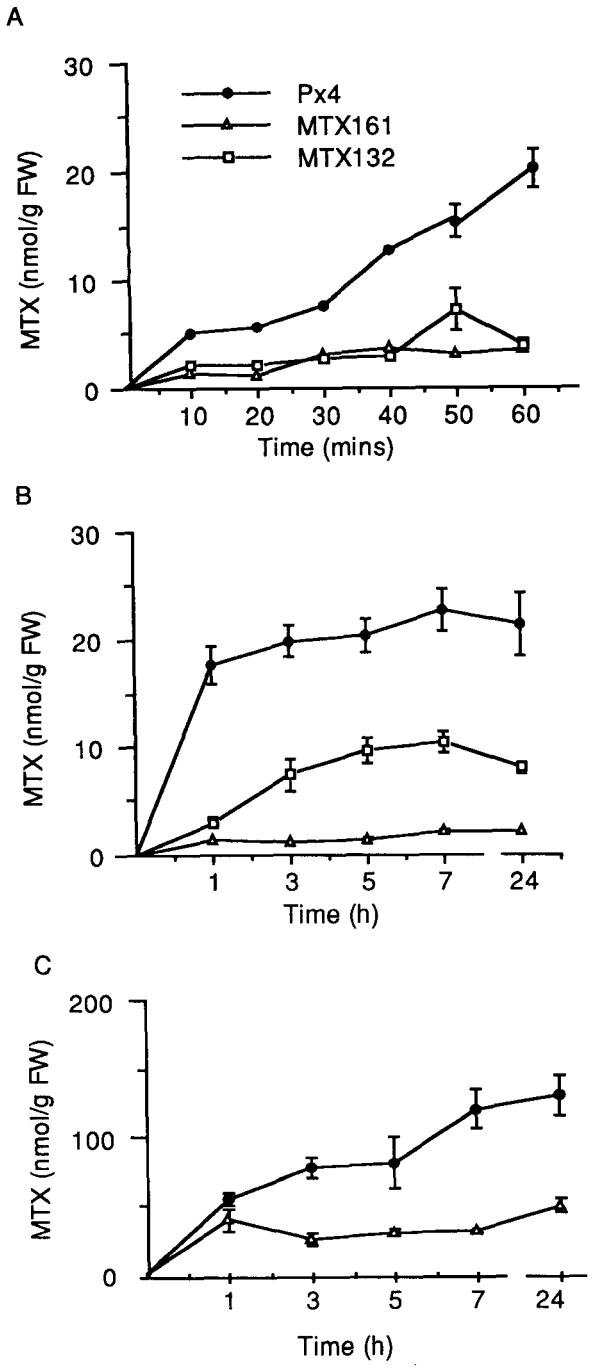

Figure 5. Time course of MTX uptake into wild-type (Px4) and resistant (MTX161 and MTX132) cell lines of *Datura*. Cells were incubated in the media with 24.2 nM (A and B) or 242 nM (C) [^{3}H]MTX.

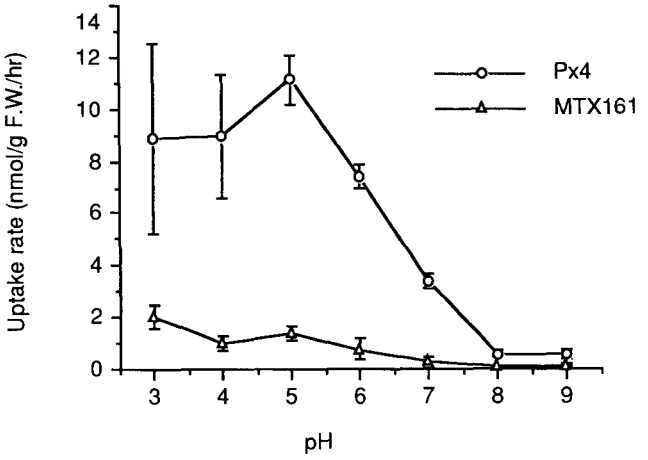

Figure 6. The effect of pH on MTX uptake. Intracellular [^{3}H]MTX was determinated after 0.5 h of incubation.

The analyses of DHFR suggest that resistance is not due to increased DHFR activity or altered DHFR with decreased affinity for MTX. There was also no significant difference in the ability of MTX to bind to soluble proteins. The resistance was apparently correlated to intracellular levels of MTX in these cell lines.

Both short-term and long-term [^{3}H]MTX uptake were shown to be different in wild-type and resistant cell lines. This result is different from that in tobacco MTX-resistant cells (Barg and Shabtai, 1991), in which reduced uptake was detectable only after 10 to 12 h of incubation. These differences apparently reflect different mechanisms of exclusion or elimination of [^{3}H]MTX from the cells. In mammalian cells, resistance resulting from both reduced influx and enhanced efflux of MTX has been identified (Sirotnak, 1985). Resistance in the MTX161 and MTX132 cell lines in *Datura* seems to result from reduced influx. Increased K_m values for uptake were evident in both resistant cell lines compared with the wild type. The increase in K_m indicates a reduction in the affinity of some component of the cell transport system for MTX. The folate transport proteins of the prokaryotic organism *Lactobacillus casei* (Fan et al., 1990) and mammalian cells (Freisheim et al., 1990) have been shown to transport MTX also. Whether the *Datura* MTX-resistant cell lines have a defective folate transport remains to be determined. Two types of folate uptake systems in mammalian cells have been identified (Fan et al., 1990). One system has high affinity for

be stable because the ability of MTX to inhibit their growth was not changed after more than 1 year of subculture in a MTX-free medium (data not shown). This is in contrast with the selected MTX-resistant cell lines of petunia (Barg et al., 1984) and carrot (Lazar et al., 1989), which were more unstable.

Table II. *Effects of metabolic inhibitors on uptake of MTX into wild-type (Px4) cells of Datura*

Inhibitors	Uptake Rate	Percent Inhibition
	nmol h^{-1} g^{-1} fresh wt	
None	13.4 ± 0.34	
DNP (10 μM)	0.64 ± 0.04	94.9
DNP (100 μM)	0.10 ± 0.01	99.3
Sodium azide (100 μM)	0.36 ± 0.02	97.3
Sodium azide (1000 μM)	0.16 ± 0.01	98.8

Table III. *Kinetic constants of MTX uptake by wild-type (Px4) and resistant (MTX161 and MTX132) cell lines of Datura*

Cell Lines	K_m	V_{max}
	nm	*nmol h^{-1} g^{-1} fresh wt*
Px4	65.6 ± 2.3	12.0 ± 0.92
MTX161	214.3 ± 31.8	11.6 ± 1.06
MTX132	115.4 ± 12.7	11.3 ± 1.77

5-substituted reduced folate and MTX, but has a low affinity for folic acid. The other prefers folate as a substrate, while reduced folate and MTX are poor substrates. It would be interesting to investigate the properties of folate transport systems in plant cells. The isolated MTX-resistant cell lines in *Datura* could serve as a useful system for such investigations.

The transport of MTX into plant cells has not been widely investigated. Creason and Gonzales (1988) reported that uptake of MTX into tobacco cells is saturable, with a K_m of 50 μM and a V_{max} of 1 nmol $h^{-1}g^{-1}$ fresh weight. N^5-Formyltetrahydrofolate (leucovorin) inhibited MTX uptake competitively. Our results showed that MTX transport into *Datura* cells was an energy- and pH-dependent, saturable process. We calculated a K_m of about 65 nM in the wild-type cell line, which is about 1000 times lower than that observed in tobacco cells. The V_{max} for *Datura* cells was about 12.5 nmol $h^{-1}g^{-1}$ fresh weight, which is about 10 times higher than that in tobacco cells. These differences may partly explain the different sensitivities of the two species to MTX. *Datura* cells are at least 100 times more sensitive to MTX than tobacco cells, the latter requiring 10^{-6} M MTX for complete inhibition of growth (Shabtai et al., 1992).

The presence of the metabolic inhibitors DNP and sodium azide prevented the accumulation of [^{3}H]MTX in *Datura* cells. Sodium azide has been shown to enhance the rate of MTX uptake by suppressing efflux components of the transport system in mammalian cells (Dembo et al., 1984). It seems that the plant MTX transport system has different properties when compared with that of animal cells. It was demonstrated that there are two distinct carrier systems involved in the mediation of MTX transport into mammalian cells (Sirotnak, 1985). One of these carrier systems was solely responsible for mediating the inward flux of MTX; the other was an actively driven efflux "pump." We did not observe significant MTX efflux from *Datura* cells. The intracellular [^{3}H]MTX that we detected is more likely to be nonexchangeable (MTX bound to DHFR and other possible targets). Higher concentrations of [^{3}H]MTX may be required in the medium to achieve exchangeable (osmotically active) intracellular [^{3}H]-MTX for efflux studies.

Polyglutamylation plays an important role in MTX action in mammalian cells (Fry et al., 1982). We detected only low concentrations of MTX polyglutamates in all our *Datura* cells after feeding with [^{3}H]MTX, nor was MTX polyglutamation increased by longer periods of [^{3}H]MTX feeding. This implies that MTX polyglutamates are in a state of turnover. In comparison, of the MTX taken up by mammalian cells, a higher percentage is converted to polyglutamate forms and the conversion is also more rapid (Jolivet and Schilsky, 1981; Cowan and Jolivet, 1984). It is possible that the *Datura* enzyme responsible for polyglutamation (FPGS) has a much narrower specificity for folate substrates than the comparable enzymes of mammals. In studies of pea FPGS, MTX had only 3% of the activity shown by tetrahydrofolate monoglutamate when both were provided as substrates of FPGS (Imeson and Cossins, 1991). MTX polyglutamate formation in MTX161 cells was detectable only after 48 h of incubation compared with 24 h in the wild type. This may reflect a low intracellular MTX level in the resistant cells rather than a deficiency in FPGS activity, a possibility that we will investigate in future work. Based on the present studies, it appears that MTX polyglutamylation in *Datura* cells is only slight and may not be significant in MTX toxicity.

Received August 12, 1992; accepted October 7, 1992.

LITERATURE CITED

Barg R, Peleg N, Perl M, Beckmann JS (1984) Isolation and characterization of methotrexate-resistant cell lines in *Petunia hybrida* upon stepwise selection procedure. Plant Mol Biol **3:** 303–311

Barg R, Perl M, Beckmann JS (1987) Elevated amounts of methotrexate-binding protein, different from normal dihydrofolate reductase in a petunia MTX^R-cell line. Plant Mol Biol **8:** 87–94

Barg R, Shabtai S (1991) Reduced intracellular content of methotrexate in an isolated cell line of *Nicotiana plumbaginifolia*. Plant Cell Rep **10:** 269–272

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Cella R, Albani D, Biasini MG, Carbonera D, Parisi B (1984) Isolation and characterization of a carrot cell line resistant to methotrexate. J Exp Bot **158:** 1390–1397

Cella R, Carbonera D, Etteric L, Maestri E, Parisi B (1987) Selection and characterization of methotrexate-resistant cell lines in *Daucus* by protoplast fusion. J Plant Physiol **127:** 135–146

Clarke L, Waxman DJ (1987) Human liver folylpolyglutamate synthetase: biochemical characterization and interaction with folate and folate antagonists. Arch Biochem Biophys **256:** 585–596

Cossins EA (1980) One-carbon metabolism. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants. Vol 2. Academic Press, New York, pp 365–418

Cossins EA (1987) Folate biochemistry and the metabolism of one-carbon units. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants. Vol 11. Academic Press, New York, pp 317–352

Cowan KH, Jolivet J (1984) A methotrexate resistant human breast cancer cell line with multiple defects, including diminished formation of methotrexate polyglutamates. J Biol Chem **259:** 10793–10800

Creason GL, Gonzales RA (1988) N^5-Formyltetrahydrofolate counteracts methotrexate toxicity in tobacco cell culture. Plant Cell Rep **7:** 182–185

Dembo M, Sirotnak FM, Moccio DM (1984) Effects of metabolic deprivation on methotrexate transport in L1210 leukemia cells: further evidence for separate influx and efflux systems with different energetic requirements. J Membr Biol **78:** 9–17

Fan J, Pope LE, Vitols KS, Huennekens FM (1990) Visualization of folate transport proteins by covalent labeling with flurescein methotrexate. *In* G Weber, ed, Advance in Enzyme Regulation. Vol 30. Pergamon Press, Oxford, pp 3–12

Flintoff WF, Essani K (1980) Methotrexate-resistant Chinese hamster ovary cells contain a dihydrofolate reductase with an altered affinity for methotrexate. Biochemistry **119:** 4321–4327

Freisheim JH, Price EM, Ratnam M (1990) Folate coenzyme and antifolate transport proteins in normal and neoplastic cells. *In* G Weber, ed, Advances in Enzyme Regulation, Vol 29. Pergamon Press, Oxford, pp 13–26

Fry DW, Yalowich JC, Goldman ID (1982) Rapid formation of polyglutamyl derivatives of methotrexate and their association with dihydrofolate reductase as assessed by high pressure liquid chromatography in the Ehrlich ascites tumor cell in vitro. J Biol Chem **257:** 1890–1896

Horsch RB, King J, Jones GE (1980) Measurement of cultured plant cell growth on filter paper discs. Can J Bot **28:** 2402–2406

Huennekens FM, Vitols KS, Whiteley JM, Neef VG (1976) Dihydrofolate reductase. *In* H Busch, ed, Methods in Cancer Research, Vol 13. Academic Press, New York, pp 199–225

Imeson HC, Cossins EA (1991) Higher plant folylpolyglutamate synthetase. II. Some major catalytic properties of the enzyme from *Pisum sativum* L. J Plant Physiol **138:** 483–488

Jolivet J, Schilsky RL (1981) High-pressure liquid chromatography analysis of methotrexate polyglutamates in cultured human breast cancer cells. Biochem Pharmacol **30:** 1387–1390

Lazar G, Toth I, Haakonson L, Goodman HM (1989) Coregulation of dihydrofolate reductase and thymidine synthase in overproducer cell lines of wild carrot. Plant Physiol **91:** 1168–1173

Mastrangelo IA, Smith HH (1977) Selection and differentiation of aminopterin resistant cells of *Datura innoxia*. Plant Sci Lett **10:** 171–179

Pizzorno G, Chang Y, McGuire JJ, Bertino JR (1989) Inherent resistance of human squamous carcinoma cell lines to methotrexate as a result of decreased polyglutamylation of this drug. Cancer Res **449:** 5275–5280

Saxena PK, King J (1988) Herbicide resistance in *Datura innoxia*. Cross-resistance of sulfonylurea-resistant cell lines to imidazolinones. Plant Physiol **86:** 863–867

Schimke RT (1984) Gene amplification in cultured animal cells. Cell **37:** 705–713

Shabtai S, Ziv M, Barg R (1992) Isolation of MTX-resistant cell line NP-19 of *Nicotiana plumbaginifolia*: phenotypic, genetic and biochemical study. J Exp Bot **43:** 471–478

Shane B (1986) Identification of folylpoly-r-glutamate chain length by cleavage to and separation of p-aminobenzoylpoly(r-glutamates). Methods Enzymol **122:** 323–330

Shimamoto K, Nelson OE (1981) Isolation and characterization of aminopterin-resistant cell lines in maize. Planta **153:** 436–442

Sirotnak FM (1985) Obligate genetic expression in tumor cells of fetal membrane property mediating "folate" transport: biological significance and implications for improved therapy of human cancer. Cancer Res **45:** 3992–4000