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In vivo decline of methotrexate and methotrexate polyglutamates in age-fractionated erythrocytes*

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Summary. Methotrexate (MTX) accumulates in erythrocytes (ery) during weekly MTX administration, and the ery-MTX concentration reaches a steady state after 4–6 weeks. In order to study MTX accumulation and metabolism to polyglutamate derivatives in different age populations of red blood cells, we took erythrocytes from 12 children with ALL who were receiving maintenance treatment with MTX and 6-MP and separated them according to age on a discontinuous Percoll gradient. When the erythrocytes of these children were separated according to specific gravity a normal distribution was obtained. Age fractionation was confirmed by the exponential decline of the erythrocyte aspartate aminotransferase (ery-ASAT) and by the reticulocyte counts. The ery-MTX declined with increasing red blood cell age in an exponential manner no different from the decline of the ery-ASAT. The youngest population of red blood cells contained 2.3–5.9 (mean 3.8) times more MTX than the oldest population. By linear regression analysis the t½ of the ery-MTX was 19–79 days (mean 37 days). The ery-MTX t½ seemed to be directly related to the amount of MTX which had been metabolized to MTX-glu_{3-5}. The decline of the ery-MTX was predominantly due to selective disappearance of MTX-glu_{1+2}, whereas MTX-glu_{3-5} changed to a much lesser extent with advancing red blood cell age. The present investigation showed that steady-state ery-MTX concentration was determined by (1) the amount of MTX added to the circulation by the reticulocytes, (2) the in vivo loss predominantly of MTX with low numbers of glutamyl derivatives from erythrocytes, and (3) the loss of MTX from destroyed red blood cells. The observed in vivo disappearance of MTX from erythrocytes offers a possible explanation of the observation that the ery-MTX steady state was reached after 4–6 weeks of unaltered weekly MTX treatment.

Introduction

Methotrexate (MTX) has been shown to accumulate in red blood cells during low-dose weekly MTX treatment [8, 12, 13, 24, 25]. The MTX concentration in erythrocytes (ery-MTX) continues to rise during unchanged weekly MTX administration until a socalled steady-state concentration is reached [8]. This steady state ery-MTX has been shown to correlate roughly with the weekly dose of MTX [24, 26].

Like the natural folates MTX undergoes polyglutamylation in the bone marrow cells [14], which increases the intracellular half-life of the drug. Increased intracellular retention of the MTX-polyglutamates in normal hematopoietic cells may enhance the myelotoxic action of the drug.

There is pharmacokinetic evidence that MTX in erythrocytes has been incorporated into the red cell precursors of the bone marrow [5, 16, 21, 24, 30]. Providing there is a normal life span of 120 days of the MTX-containing erythrocytes and providing that the youngest and oldest red blood cells contain the same amount of MTX, the steady-state ery-MTX would be expected to be reached after 16–17 weeks. In the only study dealing with this problem, however, steady-state ery-MTX concentrations were reached after as little as 4–6 weeks of weekly oral MTX administration [8]. One explanation of this discrepancy might be that the MTX content of the red blood cells had decreased with increasing cell age. The aim of this study was to investigate how aging of the erythrocytes affected the accumulation of MTX and its polyglutamyl derivatives in this cell compartment.

Material and methods

Patients. Twelve children with ALL were included in the study. They were all in their first complete remission and had received oral maintenance therapy consisting of MTX 20 mg/m^2 per week and 6-mercaptopurine (6-MP) 75 mg/m^2 per day for 12–30 months. The doses of MTX and 6-MP had been constant for at least 8 weeks at the time of study. No reinduction treatment or intrathecal MTX had been given during the previous 3 months. The patients' MTX doses are seen in Table 1.

Erythrocyte age fractionation. A 12- to 15-ml sample of EDTA blood was taken from each child 6–7 days after the last MTX administration, at which time the serum-MTX concentration was below the detection limit of the MTX assay. Once the blood sample had been filtered through an α-cellulose filter to remove the leukocytes, the red blood cells were suspended in cold NaCl and layered on top of a

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Table 1. MTX and $t^1/_2$ of MTX in age-fractionated red blood cells

Pt. no.	MTX dose mg/m² per week	Ery-MTX nmol/mmol Hb in fraction				Recovery	In vivo $t^1/_2$ (days)
		0	1	7	fr. 1/fr. 7		
1	19.2	9.2	29.6	5.0	5.9	72%	19
2	19.2	7.2	19.1	5.1	3.8	95%	31
3	17.0	6.6	25.0	5.0	5.0	109%	36
4	18.4	4.7	9.6	2.3	4.2	108%	42
5	18.9	5.5	7.8	2.9	2.7	93%	40
6	18.2	6.1	20.0	4.5	4.4	101%	28
7	17.4	6.5	17.1	4.1	4.2	100%	31
8	18.5	8.0	14.1	5.0	2.8	92%	47
9	19.4	7.0	10.2	4.5	2.3	94%	79
10	19.2	5.9	12.3	4.3	2.9	96%	37
11	19.2	4.5	15.8	3.1	5.1	92%	24
12	14.4	7.8	12.2	4.8	2.5	94%	33
Mean		6.6		4.2	3.8	96%	37

Fraction 0, unfractionated blood; fractions 1 and 7, youngest and oldest population of red blood cells, respectively
Recovery: cumulated MTX content in the seven erythrocyte fractions relative to the MTX content of unfractionated blood

discontinuous Percoll gradient consisting of six solutions differing by 3% increments in gravity and prepared as described in detail elsewhere [24].

After centrifugation at 4°C, × 1000 *g* for 10 min, 6–7 distinct fractions of erythrocytes were harvested and washed three times with ice-cold NaCl. Before the final centrifugation each fraction was assayed for hemoglobin to calculate the percentages of red cells in the individual fractions.

After the last washing appropriate volumes of each fraction were removed to determine reticulocyte count and erythrocyte-aspartate-aminotransferase activity (ery-ASAT) to allow evaluation of the age differences among the fractions. The remainder of the red blood cells in each fraction was hemolyzed in four volumes of a buffer containing NADP-EDTA-mercaptoethanol [4] for determination of the ery-MTX. The ery-ASAT and ery-MTX were expressed as units per millimole of hemoglobin and nanomoles per millimole of hemoglobin.

Since the MTX content of erythrocytes declined with increasing red blood cell age we wanted to investigate whether this could be explained by a selective loss of MTX with low glutamyl numbers. Because a larger volume of red blood cells was necessary for the MTX-polyglutamate determinations the procedure was altered slightly. A gradient consisting of three Percoll solutions differing in density by 9% increments was employed. This gave three suitable fractions of erythrocytes of increasing mean age containing approximately 10%, 70%, and 20%, respectively, of the cells loaded on the gradient. After centrifugation, washing and removal of aliquots for determination of hemoglobin, reticulocyte counts, and ery-ASAT, the remaining erythrocytes were hemolyzed in an alkaline mercaptoethanol buffer (50 m*M* Tris, 150 m*M* mercaptoethanol, 10 m*M* EDTA, pH 8.3 at 25°C). This buffer has been shown to inhibit the degradation of MTX-polyglutamates to MTX-monoglutamate by the enzyme pteroyl-glutamyl-γ-glutamyl peptidase (conjugase) (B. A. Kamen, personal communication 1985).

Methotrexate assay. The erythrocytes were boiled and centrifuged, and the MTX concentrations were assayed in the clear supernatant using a sequential radioligand-binding assay, with bovine dihydrofolate reductase as binder, slightly modified from that described by Kamen et al. [11]. The assay had a range of 1–8 pmol/ml and a sensitivity of 1 pmol/ml. Concentrations below 1 pmol/ml were disregarded. The assay allowed linear dilution of MTX-containing erythrocytes of hemoglobin values between 4.0 and 0.3 mmol/l. This method yielded reliable MTX concentrations in the erythrocyte fractions even when they constituted about 1% of the red blood cells separated on the gradient. All MTX concentrations were given per millimole of hemoglobin.

MTX-polyglutamate fractionation. In order to remove impurities the boiled erythrocyte supernatants were applied to a DEAE-cellulose column and eluted with 1 *M* ammonium bicarbonate. The samples were lyophilized and reconstituted in 300µl sterile H_2O and filtered through a 0.45-µm millipore filter as described by Kamen and Winick [10]. Of each sample, 5–25 pmol was applied to a µBondapak C18 column (part no. 086684, Waters Ass.) for high-performance liquid chromatography (HPLC). Two buffers were used to generate the 20% and 40% acetonitrile gradients: buffer A [5 m*M* tetrabutyl ammonium phosphate (Pic A Waters part no. 85101) in H_2O, pH 7), and buffer B (5m*M* tetrabutyl ammonium phosphate in 40% acetonitrile and 60% H_2O, pH 7). Initially an isocratic system of the two buffers was used. From 5 to 25 min a convex gradient was generated to 100% buffer B (Waters automated gradient controller). The flow rate was 1 ml/min. Fractions (300 µl) were collected at 18-s intervals and assayed for MTX with the sequential radioligand-binding assay as described above. The MTX content was expressed as picomoles per fraction. This HPLC method in combination with the MTX assay allowed quantitative recovery (80%–120%) of 5–25 pmol MTX applied to the column. The individual MTX-polyglutamate peaks, measured by the MTX assay, were identified by comparison with the retention times of MTX-polyglutamate standards (MTX-$glu_{1+2+3+4+6+7}$) detected spectrophotometrically at 304 nm. (The MTX polyglutamate standards were a generous gift from Dr B. A. Kamen, Dallas, Tex. The sensitivi-

ty of the MTX-polyglutamate analysis was 1 pmol/ml (= 0.3 pmol/fraction).

Data analysis. The theoretical basis of the analysis of data obtained by age fractionation of erythrocytes has been described previously [18, 19, 29].

It has been shown that when normal erythrocytes are fractionated according to specific gravity a normal distribution is obtained. Even though the mean specific gravity is rather constant in any one individual, the interindividual variation is large [20, 29]. Therefore, when several persons are studied the cumulative distribution function (CDF), which is based on the sample percentile of each erythrocyte fraction, must be used for pooling data.

The sample percentile of each fraction (f_n) was calculated from the formula:

$$\frac{H_{(f1)}}{H_{(t)}}+\frac{H_{(f2)}}{H_{(t)}}+..+\frac{H_{(fn-1)}}{H_{(t)}}+\frac{H_{(fn)}}{H_{(t)}}\times ½\times 100, \qquad \text{(Eq. 1)}$$

where $H_{(f)}$ = hemoglobin content of each fraction (mmol), $H_{(t)}$ = total hemoglobin content of all fractions (mmol), $f_{(1)}-f_{(n)}$ = consecutive fractions of erythrocytes from top to bottom. The enzymatic activity and the MTX concentrations may be related to the hemoglobin concentration of each fraction, because the mean cell hemoglobin (MCH) of the erythrocyte is constant throughout its life span and thus the hemoglobin concentration of each fraction is proportional to the number of cells [4].

Because red blood cells are distributed normally in the gradient the probit of the percentile position of the individual red blood cell fractions in the gradient is related to cell age [29]. The decline of the intracellular ery-ASAT activity in the age related fractions is approximately given by:

$$E_{(1)} = E_o \times e^{-\alpha t} \qquad \text{(Eq. 2)}$$

where E_o = ery-ASAT U/mmol Hb at mean erythrocyte age and α = velocity constant of the ery-ASAT decline.

Thus the logarithm of any age-related parameter should decline linearly among the fractions from top to bottom, and the value read at the 50% point of the regression line should equal that of the unfractionated blood sample. Since this was observed for both the ery-ASAT and the ery-MTX determinations in the individual patients (data not shown), it seems highly probable that the erythrocytes from the patients were normally distributed in the gradient and that no random red blood cell destruction took place.

The extrapolated slope of the decline of the gradient for reticulocytes, when plotted as a function of the CDF, intercepted the 100% level at −4 SD on the probability paper. Other workers [18, 19, 29] have found the slope of the regression line for the reticulocytes to intercept the 100% level at −3.9 SD. This point was set at 0 days and, since the cells are distributed normally, those of 120 days should be equidistant from the midpoint of the gradient. MTX and ASAT values at 0 and 120 days had to be obtained by extrapolation since the method did not allow sufficient amounts of cells for the MTX and ASAT analyses at the extremes of the gradient. For determination of the regression lines of the ASAT activity and the MTX concentrations the CDF was converted to the corresponding mean cell age by inserting a linear age scale along the *x*-axis of the probability paper in which 0 days was set at −4 SD, 60 days at 0 SD, and 120 days at +4 SD (Fig. 1).

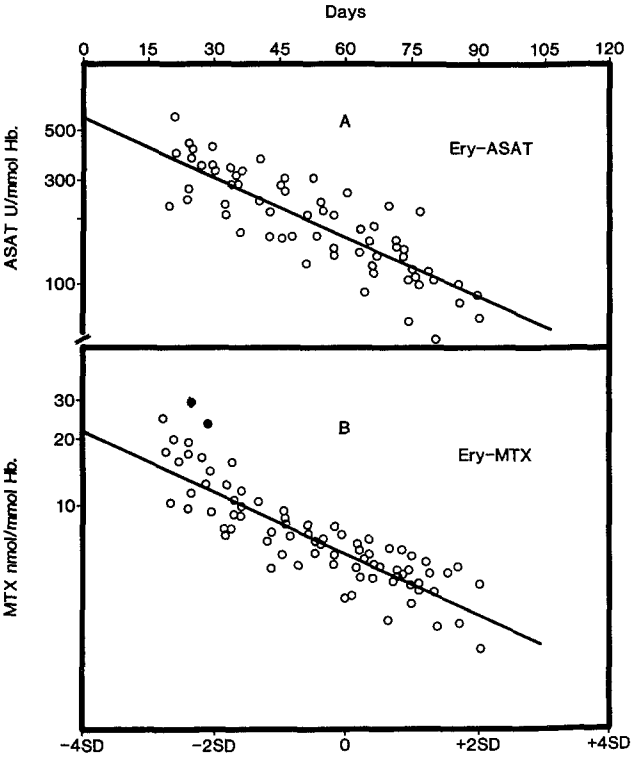

Fig. 1A, B. Rate of decline of erythrocyte-ASAT activity **(A)** and erythrocyte-MTX concentration **(B)**. The slope of the regression line was obtained by least squares regression of the $\log_{10}$ ery-ASAT and the $\log_{10}$ ery-MTX, respectively, vs the cumulative percentile of the age-separated fractions converted to erythrocyte mean age, as described under "data analysis"

To calculate the in vivo t½ of MTX and ASAT activity in age-fractionated erythrocytes the slope from the regression equation was determined for each of the 12 children in the study.

Results

Table 1 shows that the ery-MTX concentrations in the youngest red cells were 2.3–5.9 times (mean 3.8) higher than in the oldest red cell populations (fr 1/fr 7). The MTX contents of the erythrocytes declined between adjacent fractions from the top to the bottom of the gradient in all patients. Figure 1B shows the pooled data of the log ery-MTX concentrations as a function of the CDF in the 12 children. Two points in Fig. 1B (●) seemed to indicate that the loss of MTX was not monoexponential. This might have been caused by an admixture, to the reticulo-

Table 2. In vivo t½ of erythrocyte MTX and MTX-polyglutamate distribution in red blood cells

	BML	GE	JGJ
MTX-glu$_1$[a]	35	36	14
MTX-glu$_2$	34	18	13
MTX-glu$_3$	27	36	46
MTX-glu$_4$	4	10	22
MTX-glu$_5$	–	–	5
t½ ery-MTX[b]	19	31	79

[a] Percentage of total MTX-glu
[b] Days

Table 3. Distribution of MTX and MTX-polyglutamates in age-fractionated erythrocytes

	Patient 1				Patient 7				Patient 2				Patient 9			
Fraction no.	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3
MTX-Glu_n	7.5	15.2	9.3	5.2	5.0	9.9	5.3	3.4	5.8	14.6	6.1	4.4	6.6	11.0	6.6	5.4
MTX-Glu 1	2.6	10.6	3.9	1.2	1.8	3.6	1.7	1.1	2.2	10.8	2.1	<0.2	1.0	5.4	0.8	0.5
MTX-Glu 2	2.6	3.0	2.2	1.3	1.3	3.3	1.4	0.8	1.1	3.4	1.2	0.8	0.9	1.8	1.6	0.7
MTX-Glu 3	2.0	1.6	2.7	2.4	1.6	2.4	2.1	1.4	2.0	<0.2	2.3	2.8	2.7	3.1	2.6	2.8
MTX-Glu 4	0.3	<0.2	0.4	0.3	0.3	0.3	0.2	0.2	0.6	<0.2	0.5	0.8	1.5	0.9	1.5	1.3
MTX-Glu 5													0.3	<0.2	0.2	0.2
Recovery (%)	88	99	98	82	89	89	103	77	98	120	105	110	88	77	106	94

MTX-Glu_n, Total MTX (nmol/mmol Hb) in erythrocytes of age fractions 0–3 before HPLC separation; MTX Glu 1–5, nmol/mmol Hb

cyte-rich fraction of these two patients, of neutrophils that had not been removed by the filtering process, since neutrophils contain 10–15 times more MTX than erythrocytes [25]. The extrapolated regression line of the ery-MTX of Fig. 1B intercepted the day 0 axis at 22.4 nmol MTX/mmol Hb, corresponding to the MTX concentration of a 100% pure reticulocyte population. This implies that newly released reticulocytes contain 3–4 times more MTX than unfractioned erythrocytes.

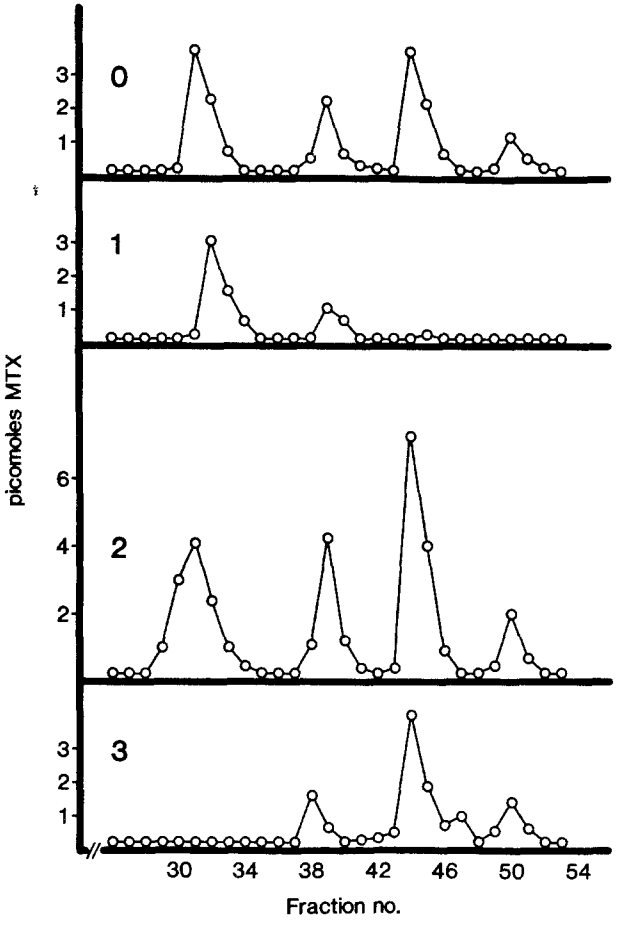

Fig. 2. MTX-polyglutamate distribution in unfractionated red blood cells (0) and in three age-related fractions (1–3) of erythrocytes in one patient. The peaks of the individual MTX-polyglutamates eluted in the following fractions: MTX-glu_1 in fractions 30–32, MTX-glu_2 in fractions 38–39, MTX-glu_3 in fractions 44–45 and MTX-glu_4 in fraction 50

When linear regression analyses were applied to the MTX data of each patient, the t½ of the ery-MTX, representing the in vivo decline of MTX, could be calculated. Table 1 shows that the in vivo t½ of MTX in red blood cells was 19–79 days (mean 37.3 ± 15.3).

In three patients with short, intermediate, and long in vivo half-lives of intraerythrocytic MTX the MTX-polyglutamate profiles of the unfractionated erythrocytes were analyzed. In the patient with a t½ of 19 days, 69% of the MTX existed as MTX-glu_{1+2}, and in the child with a t½ of 79 days only 27% of the MTX was in the form of glu_{1+2}. In the third patient, with an intermediate in vivo t½ of ery-MTX, the MTX-polyglutamate distribution was also intermediate between those of the other two patients (Table 2).

Since the MTX content decreased in aging erythrocytes, we investigated to what extent this observation could be explained by changes in the MTX-polyglutamate concentration in three erythrocyte fractions of increasing mean cell age in four children. A representative distribution of the MTX and MTX-polyglutamates in unfractionated blood (0) and in the three age-related fractions (1–3) is seen in Fig. 2. MTX in the youngest cell fraction (fraction 1) was predominantly in the form of MTX-glu_1 and -glu_2, whereas MTX-glu_3 and -glu_4 predominated in the oldest red cell population (fraction 3). Figure 3 shows that the amount of MTX-glu_{1+2} as a proportion of the total

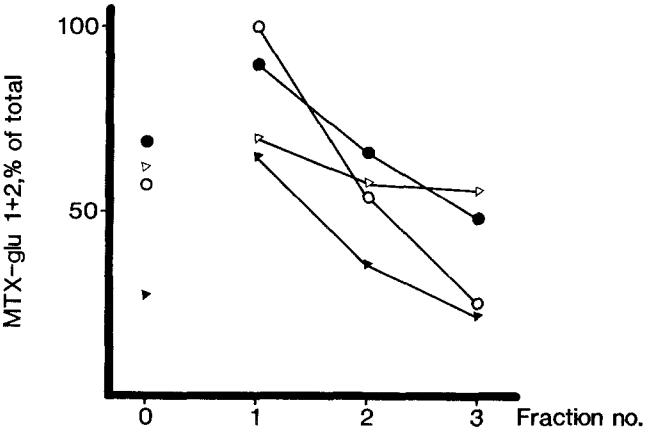

Fig. 3. Concentration of MTX-glu_{1+2} as percentage of the total ery-MTX concentration (MTX-glu_n) in three age-related fractions (1–3) in four patients. The ery-MTX concentration of unfractionated red blood cells is included for comparison. o, pt 2; ●, pt 1; Δ, pt 7; ▲, pt 9

MTX (MTX-glu_n) decreased with increasing red blood cell age in all four children who were investigated.

The MTX-glu_{1+2} concentrations (nmol/mmol Hb) decreased among all three age-related fractions of red blood cells in all the children, whereas the concentrations of MTX-glu_3 did not change uniformly among the fractions (Table 3). In two patients the amount of MTX-glu_3 seemed to increase, in one it was unchanged, and in one the amount decreased.

Discussion

After a single dose of MTX p.o., the serum MTX concentration (se-MTX) reaches a peak after 1–2 h. By 24 h later the se-MTX is below 10 nmol/l. A kinetically similar concentration curve is seen for MTX in the red blood cells, but with a lower peak value [8]. About 72 h after MTX administration the erythrocyte MTX concentration starts to rise at a time when the se-MTX is zero. Pharmacokinetic studies have suggested this to be caused by the release of reticulocytes that have accumulated MTX during their maturation process in the bone marrow [5, 16, 21, 24, 30].

During unchanged weekly MTX medication the ery-MTX continues to rise until a so-called steady-state is reached after 4–6 weeks [8]. The steady-state ery-MTX has been shown to be correlated with the weekly dose of MTX both in a group of psoriasis patients [26] and in a large group of children with ALL receiving maintenance treatment [27].

Animal studies have shown that MTX that has accumulated in various tissues, e.g., liver, kidney, brain, testes, in vivo has very long intracellular retention times, probably as a result of firm binding to intracellular proteins and/or extensive metabolism to polyglutamates, which are retained intracellularly for prolonged periods of time [15, 17, 23, 31].

MTX-polyglutamates have been demonstrated in circulating erythrocytes in few cases [2, 6, 12, 17]. So far no quantitation of the erythrocyte MTX-polyglutamates or the degree to which they are related to red blood cell age has been performed. Such studies will provide further understanding of some of the mechanisms determining the steady-state ery-MTX concentration.

The linear decline of the log of the ery-ASAT activity showed that a normal distribution was obtained of the age-fractionated red blood cells from MTX- and 6-MP-treated children in the discontinuous Percoll gradient, as was obtained when erythrocytes from normal healthy persons were fractionated [18, 19, 29]. The t½ values of ery-MTX and ery-ASAT from the pooled data of Fig. 1A, B were 34.8 and 34.1 days, respectively. This suggests that the ery-MTX declines with age in much the same way as intracellular catalytic enzyme systems and may be described by Eq. (2) (see data analysis).

Our results have shown that most of the MTX present in the reticulocytes was lost from the red blood cells as they aged (Fig. 1B). The present methods demanded a hemoglobin concentration of 0.3–0.6 mmol/l for the MTX and ASAT analysis, so that values at the extremes of the curve had to be obtained by extrapolation. When the extrapolated values of ery-MTX were used, a 100% pure reticulocte population of day 0 was estimated to contain about 3–4 times more MTX than the red blood cells at the estimated midpoint of their life span. These observations, hitherto unreported, offer a reasonable explanation for the fact that the steady-state ery-MTX is reached after 4–6 weeks, rather than at 16–17 weeks as might have been expected. At the other extreme seen in Fig. 2B extrapolation of the regression line to 120 days or +4 SD is probably not justified, since we demonstrated that the amount of intraerythrocytic MTX-glu_{3-5} only changed to a minor extent with advancing red blood cell age (Table 3). Thus, the MTX concentration would probably only change a little, if at all, if erythrocytes beyond a certain age could be analyzed [28].

When so-called steady-state ery-MTX concentration has been reached the daily amount of MTX added to the circulation via the reticulocytes must be equal to the amount of MTX lost per day. This implies that at steady-state ery-MTX concentration the amount of MTX delivered to the circulation by the reticulocytes must be equal to the in vivo disappearance from intact erythrocytes plus the amount lost by the destruction of senescent red blood cells.

Our data showed an approximately five-fold variation in the t½ of the ery-MTX. The in vivo MTX decline seemed to be inversely correlated with the amount of MTX polyglutamates with 3–5 glutamyl molecules. The loss of MTX could virtually exclusively be explained by the loss of MTX-glu_{1+2}, whereas the concentration of MTX-glu_{3-5} varied considerably less in the different age populations of the red blood cells. This finding is in agreement with the finding of Schalhorn et al. [22] that all MTX in the red blood cells existed as MTX-polyglutamates 6 weeks after high-dose MTX infusions. Since the present investigation we have shown that all MTX-glu_1 and all MTX-glu_2 had disappeared from the erythrocytes three and six weeks, respectively, after discontinuation of MTX maintenance therapy [28]. Moreover, MTX with low numbers of glutamyl residues has been shown to efflux from other cells much more easily than MTX with higher glutamyl numbers (MTX-glu_{3-5}) [1, 9]. In addition, Benesh et al. [3] showed that MTX with high numbers of glutamyl residues seemed to bind much more strongly to hemoglobin in erythrocytes than MTX with lower numbers of glutamyl molecules and therefore might be expected to be retained in erythrocytes for much longer time periods. The ery-MTX kinetics described here are thus consistent with an efflux of MTX-glu_{1+2} from red blood cells, although this has not been demonstrated directly. In vitro demonstration of MTX efflux from red blood cells would demand long incubations in MTX-free media, since the erythrocyte half-life of MTX-glu_1 was shown to be 3–9.5 days [22].

Concerning the kinetics of MTX-glu_3 the results were not uniform. In two of the patients the concentration of this metabolite seemed to increase with red blood cell age (Table 3), implying that the circulating erythrocytes (presumably reticulocytes or very young erythrocytes) were able to metabolize MTX to polyglutamate forms to a small extent in these two patients. However, a study of the changes of the MTX-polyglutamate distribution in erythrocytes during three to four months following cessation of maintenance therapy failed to demonstrate MTX polyglutamylation to take place in red blood cells in vivo [28]. In another study this has been assumed to take place [7]. Krakower and Kamen [16], however, were not able to demonstrate polyglutamate formation in mature erythrocytes, but they studied red blood cells from rats 4 days after a single MTX

injection, and not after repetitive weekly doses as in the present study.

The calculations of the t½ values for ery-MTX and ery-ASAT were based on the assumption that the erythrocytes of these patients have a life span of 120 days. The demonstration of MTX in erythrocytes 13–15 weeks after cessation of therapy was compatible with an approximately normal life span of the erythrocytes in these children [28]. The observed t½ of ery-MTX was made up of the t½ values of the individual polyglutamates, which seemed to increase with increasing polyglutamate number in erythrocytes too (Table 3). However, the data from the present four patients did not allow meaningful calculations of the t½ of the individual erythrocyte MTX-polyglutamates.

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