A critical intracellular concentration of fully reduced non-methylated folate polyglutamates prevents macrocytosis and diminished growth rate of human cell line K562 in culture

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(Received 7 March 1983/Accepted 22 April 1983)

Growth rate of human leukaemic cell line K562 was independent of intracellular folate concentration when this was greater than $1.5\mu\text{M}$. When intracellular folate concentration was less than $1.5\mu\text{M}$, the rate of growth was proportional to the logarithm of intracellular concentration of non-methylated fully reduced folates, but not to the logarithm of the intracellular concentration of N^5 -methyltetrahydropteroylglutamate. Intracellular folate concentration sufficient to support an optimal growth rate was maintained by either DL- N^5 -formyltetrahydropteroylglutamate or DL- N^5 -methyltetrahydropteroylglutamate at a 100-fold lower concentration than pteroylglutamate. Addition of hypoxanthine to culture medium partially restored growth of folate-depleted cells: thymidine had no effect on growth rate either alone or in combination with thymidine. Folate-depleted cells with diminished growth rate were larger than replete cells, but did not have megaloblastic morphology. The mitotic index was not decreased in cultures with diminished growth rate. The rate of growth and cell size of K562 cells is thus dependent on a critical intracellular concentration of non-methylated tetrahydrofolates, which may be maintained by different concentrations of either reduced folates or pteroylglutamate.

Folate is required by all cells. The relationship between intracellular folate content and growth of mammalian cells in culture has been investigated in detail only in human fibroblasts (Hilton *et al.*, 1979). Folate deficiency prevents growth of cells and may produce disturbed morphology such as in megaloblastic anaemia (Chanarin, 1969). This can be corrected by folate or by providing products of folate metabolism (McBurney & Whitmore, 1974). We have undertaken to determine the intracellular folate required for cell growth in an erythropoietic cell and the effect of folate deficiency on these cells.

Cell line K562 was derived from a patient with

Abbreviations used: PteGlu, pteroylglutamate (folic acid); PteGlu₅, pteroylpentaglutamate; 5-CHO-H₄PteGlu, N^5 -formyltetrahydropteroylglutamate; 5-CH₃-H₄PteGlu, N^5 -methyltetrahydropteroylglutamate; 5-CH₃-H₄PteGlu_n, N^5 -methyltetrahydropteroylglutamate with a γ -glutamyl chain of unspecified length.

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chronic myelogenous leukaemia in blast crisis (Lozzio & Lozzio, 1975) and appears to be descended from either an erythroid stem cell (Anderson *et al.*, 1979; Rutherford *et al.*, 1979) or a stem cell that retains potential for both erythroid and myeloid differentiation (Marie *et al.*, 1981). This cell line is the human cell line in continuous culture most similar to the erythroid stem cell.

The megaloblastic state in bone marrow *in vivo* is characterized by macrocytosis, specific morphological features, an increased ratio of cells in the G2- and M-phases to cells in the G1-phase, and by ineffective erythropoiesis with intramedullary death of cells (Finch *et al.*, 1956; Messner *et al.*, 1969; Wickramasinghe *et al.*, 1969). We also investigated the changes in cell morphology and kinetics that occurred when K562 cells were deprived of folate to determine if such deprivation induced the megaloblastic state in culture as reported in mouse erythroleukaemia cells treated with antifolate drugs in culture (Steinberg *et al.*, 1980).

Materials and methods

Materials

PteGlu, DL-5-CHO-H₄PteGlu, DL-5-CH₃-H₄PteGlu and cyanocobalamin were purchased from the Sigma Chemical Company, [3',5',7,9-³H]PteGlu (sp. radioactivity 45 Ci/mmol) and [⁵⁷Co]cyanocobalamin (sp. radioactivity 0.30 Ci/mol) was from Amersham/Searle Corp., tissue culture media were from Grand Island Biological Company and the foetal-calf serum was from Flow Laboratories.

Cell culture

Cell line K562 was provided by Dr. A. K. Sullivan of the Division of Haematology, Royal Victoria Hospital, and the McGill Cancer Center. Cells were grown in suspension culture in Eagle's minimum essential medium plus non-essential amino acids supplemented with 10% foetal-calf serum; this medium contained 2.3 μ M-PteGlu. Cultures were split to relieve crowding and medium was replaced at 3-day intervals. Cell number was monitored by counting in a haemocytometer. The rate of cell growth was expressed as doublings per day calculated from cell numbers over a 3-day interval of growth; growth during these periods was shown to be exponential by examining cell numbers in culture at daily intervals.

Folate depletion studies utilized Eagle's minimum essential medium with non-essential amino acids but lacking PteGlu, supplemented with 10% foetal-calf serum that had been dialysed for 24 h at 4°C against a 10-fold volume of 0.9% NaCl with two changes and with 1 μ M-cyanocobalamin. Folate content of medium so supplemented was 1 nM. Studies of the effects of folate derivatives and products of folate metabolism on cell growth utilized folate-free medium supplemented with 10% dialysed foetal-calf serum, and 1 μ M-cyanocobalamin and the growth factor to be tested (PteGlu, DL-5-CHO-H₄PteGlu, DL-5-CH₃-H₄PteGlu, thymidine and/or hypoxanthine at the indicated concentration). Media containing DL-5-CH₃-H₄PteGlu were also supplemented with 50 μ g of sodium ascorbate/ml to maintain the folate in its reduced state. Sodium ascorbate at this concentration had no effect on growth of cell line K562.

In common with several transformed cells (Halpern *et al.*, 1974; Hoffman & Erbe, 1976) cell line K562 was found to have an absolute requirement for methionine that could not be replaced by homocysteine. These cells did not proliferate when the L-methionine concentration of the culture medium was less than 10 μ M. All experiments reported in the present paper utilized medium containing 0.1 mM-L-methionine.

Extraction of intracellular folate

Cells were removed from suspension by centrifugation, washed twice with phosphate-buffered NaCl and counted. The cell pellet was resuspended in 0.1 M-potassium phosphate buffer, pH 6, containing 1.5 mg of ascorbic acid/ml, vortex-mixed, boiled for 10 min and centrifuged to remove precipitate. The supernatant was incubated for 90 min at 37°C with 10% (v/v) charcoal-treated human serum to convert folate polyglutamate forms into monoglutamate (conjugase treatment) (Rosenblatt *et al.*, 1979).

Folate determinations

Folate was assayed microbiologically with *Lactobacillus casei* A.T.C.C. 7469 and *Pediococcus cerevisiae* A.T.C.C. 8081 using aseptic addition of samples to sterile medium already inoculated with assay organisms as described previously (Baker *et al.*, 1971; Cooper & Jonas, 1973).

Fractionation of folates on DEAE-Sephadex A-25 was as described previously using a linear gradient (0.1–0.8 M) of potassium phosphate, pH 6 (Nixon & Bertino, 1971). Folate content of samples of eluent was determined by microbiological assay. Folate derivatives were identified by comparison of the conductivity of the eluting buffer with that of standard folate coenzymes.

To determine the intracellular distribution of folate polyglutamates and monoglutamates, cells were grown for 24 h in medium supplemented with 0.34 μ M-[³H]PteGlu. Cells were then harvested, washed and resuspended in 0.1 M-potassium phosphate buffer, pH 9.2, containing 10 mM-2-mercaptoethanol. The extract was boiled, centrifuged and immediately filtered through Sephadex G-25 with 0.1 M-NH₄HCO₃ (Buehring *et al.*, 1974; Hilton *et al.*, 1979). The column was standardized with Blue Dextran and ³H₂O. Radioactivity of the eluent was measured by liquid-scintillation spectroscopy. PteGlu and reduced folate monoglutamates were identified by co-elution with authentic standards and folate polyglutamates were identified by co-elution with authentic standards of PteGlu, (kindly supplied by Dr. C. M. Baugh, Department of Biochemistry, University of Southern Alabama, Mobile, AL, U.S.A.) and a folate polyglutamate extract prepared from cultures of *L. casei* (Buehring *et al.*, 1974), and by shift of mobility on Sephadex G-25 after treatment with the serum conjugase preparation.

Preparation of cells for microscopy

Smears of K562 cells grown in suspension culture were prepared by using a Cytospin and air dried on glass slides. The slides were fixed in absolute methanol and stained metachromatically with Wright's stain and Giemsa stain. Diameter of cells

prepared in this manner was determined by using an ocular micrometer calibrated with a haemocytometer counting chamber.

Measurement of intracellular cobalamins

Intracellular cobalamin derivative distribution of K562 cells grown for 3 days in medium supplemented with 25 pg of [^{57}Co]cyanocobalamin/ml previously bound to transcobalamin 2 in normal human serum was determined by extraction in hot ethanol and chromatography on SP-Sephadex (Mellman *et al.*, 1979).

Measurement of volume of intracellular water

The mean intracellular water volume was determined by measurement of $^3\text{H}_2\text{O}$ space in K562 cells, with correction for extracellular space with [^{14}C]poly(ethylene glycol) (Hilton *et al.*, 1979), and comparison of these data with cell numbers determined by haemocytometer counting. Comparison of cell volume measured by this technique with those calculated from slides of K562 cells, assuming that the diameter of cells measured on the slide was equal to one-half the circumference of the intact cell, showed that the measured and calculated volumes were approximately equal.

Results

During maintenance of cell line K562 in Eagle's minimum essential medium plus non-essential amino acids with $2.3\text{ }\mu\text{M}$ -PteGlu, the cell population doubled once every 30.6 h (standard deviation 5.7 h). The mean intracellular water content of cells so maintained was $1.65 (\pm 0.77)$ pl/cell (mean $\pm$ S.D.) (6.06×10^8 cells/ml of cell water).

Intracellular folates and cobalamins of cell line K562 grown under basal conditions ($2.3\text{ }\mu\text{M}$ -PteGlu)

The folate content of the cell extracts, determined by microbiological assay with *L. casei*, was $4.81 \pm 1.11\text{ ng}/10^6$ cells (mean $\pm$ S.D.), or $6.48 \pm 1.51\text{ }\mu\text{mol/litre}$ of cell water. Of this, $3.87 \pm 1.00\text{ }\mu\text{M}$ also supported growth of *P. cerevisiae*, indicating that this was fully reduced folate other than $5\text{-CH}_3\text{-H}_4\text{PteGlu}_n$. Filtration through Sephadex G-25 of extracts of K562 cells grown in [^3H]PteGlu for 24 h revealed that more than 85% of intracellular folate was polyglutamate, 7% was reduced monoglutamate folate and 6% was PteGlu.

When conjugase-treated extracts of K562 cells grown under basal conditions were eluted from DEAE-Sephadex A-25 with a potassium phosphate gradient, 20% of intracellular folate other than PteGlu was eluted as $10\text{-CHO-H}_4\text{PteGlu}$, 32% as $5\text{-CHO-H}_4\text{PteGlu}$, 45% as $5\text{-CH}_3\text{-H}_4\text{PteGlu}$ and 3%

as unidentified tetrahydrofolates with high affinity for the resin, which supported growth of *L. casei* and *P. cerevisiae*.

Intracellular cobalamins of K562 cells incubated for 3 days in medium containing [^{57}Co]cyanocobalamin included 20% methylcobalamin and 24% $5'$ -deoxyadenosylcobalamin.

Effect of folate depletion upon growth and folate content of cell line K562

K562 cells that had been maintained in basal medium containing $2.3\text{ }\mu\text{M}$ -PteGlu were subcultured into medium lacking folate. During the period of depletion, cultures were split and medium was replaced at 3-day intervals. The rate of growth of cells did not change significantly during the first 6 days of culture in folate-free medium (Table 1), but decreased during the 6–9-day growth interval. After 12 days of folate depletion, no significant change in cell number was observed.

Microscopic examination of stained smears of cells during folate depletion revealed that average cell diameter increased from $27.0 \pm 3.6\text{ }\mu\text{M}$ (mean $\pm$ S.D.) before subculture in folate-free medium to $32.1 \pm 4.4\text{ }\mu\text{M}$ after 14 days. The standard deviation of cell diameter was about 14% of mean cell diameter in both folate-depleted and replete cells. Morphology of cells at this time was not recognizable as megaloblastic by a trained haematologist. The mitotic index of folate-depleted cells was 2–3%, no different from that of control cells despite decreased cell growth rate.

Folate content of conjugase-treated extracts of K562 cells that had been subcultured in folate-free medium for various periods was determined by microbiological assay. Both *L. casei*-active and *P. cerevisiae*-active folate content decreased exponentially under these conditions (Table 2), although cell growth rate did not change during the first 6 days of incubation in folate-free medium.

Decrease of intracellular folate in K562 cells

Table 1. Growth rate of cell line K562 during folate depletion

Results are means $\pm$ S.D.

Period of folate depletion	Number of observations	Growth rate (doublings/day)
Control	7	0.78 ± 0.15
Day 0–day 3	4	0.63 ± 0.05
Day 3–day 6	4	0.76 ± 0.11
Day 6–day 9	4	$0.54 \pm 0.12^*$
Day 9–day 12	4	$0.24 \pm 0.13^\dagger$
Day 12–day 15	3	$0.09 \pm 0.09^\dagger$

* Growth rate significantly less than that of control cultures at $P < 0.05$ by Student's *t* test.

† Growth rate significantly less than that of control cultures at $P < 0.001$ by Student's *t* test.

Table 2. Intracellular folate content of K562 cells grown in folate-free medium

All values are means $\pm$ s.d.

Time of depletion (days)	<i>n</i>	<i>L. casei</i> -active folate (μ mol/litre of cell water)	<i>P. cerevisiae</i> -active folate (μ mol/litre of cell water)	5-CH ₃ -H ₄ -PteGlu _n * (μ mol/litre of cell water)
0	7	6.62 $\pm$ 1.48	3.88 $\pm$ 1.05	2.75 $\pm$ 1.10
3	4	2.72 $\pm$ 0.65	1.67 $\pm$ 0.35	0.78 $\pm$ 0.12
6	4	1.05 $\pm$ 0.75	0.82 $\pm$ 0.65	0.24 $\pm$ 0.10
9	4	0.44 $\pm$ 0.14	0.33 $\pm$ 0.10	0.11 $\pm$ 0.08
12	4	0.22 $\pm$ 0.11	0.14 $\pm$ 0.05	0.08 $\pm$ 0.07

* [(*L. casei*-active) - (*P. cerevisiae*-active)]folate. Includes about 6% PteGlu.

Fig. 1. Growth rate of folate-depleted cells subcultured into media containing different concentrations of PteGlu (○) or the biologically-active isomer of 5-CHO-H₄PteGlu (●) as a function of extracellular folate concentration. Points shown are means of three to four individual experiments.

grown in folate-free medium was exponential ($r = 0.998$, 0.999 and 0.977 for the logarithms of *L. casei*-active folate, *P. cerevisiae*-active folate and the difference between these respectively versus duration of depletion). This rate of decrease was consistently less than the rate of cell accumulation in the cultures; cells growing at a rate of 0.63–0.78 doublings per day increased their numbers by about 50% per day, whereas folate associated with them decreased by a mean of 25% per day. During folate depletion, growth probably was progressively restricted to cells with greater intracellular folate content.

Extracellular folate requirements of folate-depleted K562 cells

To determine the minimum concentration of extracellular folate required to support growth of K562 cells, cells that had been grown in folate-free medium for 12 days were subcultured into media containing different concentrations of PteGlu, DL-5-CHO-H₄PteGlu or DL-5-CH₃-H₄PteGlu for three days and growth rate was determined. As illustrated in Fig. 1, cells subcultured into concentrations of PteGlu lower than 10 nM or of DL-5-CHO-H₄PteGlu lower than 0.2 nM did not grow. Growth rate was

optimal in 1 μ M-PteGlu or 20 nM-DL-CHO-H₄-PteGlu, and was the same as that of cells grown in basal medium. Cells subcultured in media containing 20 nM-DL-5-CH₃-H₄PteGlu grew at the same rate as did cells grown in the same concentration of DL-5-CHO-H₄PteGlu (results not shown), and DL-5-CH₃-H₄-PteGlu at concentrations of 0.2 nM or less did not support cell growth. K562 cells were thus shown to have a finite extracellular folate requirement for growth, and this requirement was 100 times less for biologically-active isomers of reduced folate derivatives than for PteGlu.

Effect of extracellular folate on growth and intracellular folate content of folate-replete K562 cells

K562 cells that had been maintained in basal medium containing 2.3 μ M-PteGlu were subcultured into media containing different concentrations of PteGlu or DL-5-CHO-H₄PteGlu. Cultures were split and medium was replaced at 3-day intervals. Cell number was monitored over a 12 day period. PteGlu at concentrations of 1 μ M or greater, and DL-5-CHO-H₄PteGlu at concentrations of 20 nM or greater, supported the growth of K562 cells at normal rates throughout the 12 day period (Table 3). The growth rate of cells subcultured in media containing 0.1 μ M-PteGlu or 2 nM-DL-5-CHO-H₄PteGlu was less than that of cells grown in basal medium. PteGlu at 10 nM or DL-5-CHO-H₄PteGlu at 0.2 nM was not sufficient to maintain normal growth rate, and growth rate of cells subcultured into these folate concentrations after 9 days was less than that in cells grown in basal medium.

K562 cells that had been subcultured for 12 days in 0.1 μ M- or greater PteGlu or in 2 nM- or greater DL-5-CHO-H₄PteGlu or for 9 days in 10 nM-PteGlu or 0.2 nM-DL-5-CHO-H₄PteGlu were harvested. Folate content of conjugase-treated extracts was determined by microbiological assay. Intracellular concentration of both *L. casei*-active and *P. cerevisiae*-active folate was proportional to the extracellular folate concentration (Table 3). Cells grown in medium containing DL-5-CHO-H₄PteGlu accumulated intracellular folate to a greater extent than did cells grown in medium containing PteGlu at the same concentration. A 100-fold greater extracellular concentration of PteGlu than of the biologically active isomer of 5-CHO-H₄PteGlu was required to support the same intracellular folate concentration. The proportion of intracellular *L. casei*-active folate that did not support growth of *P. cerevisiae* (most of which represented 5-CH₃-H₄PteGlu_n) was greater in cells grown in high concentrations of folate than in lower concentrations (Table 3).

Intracellular folate and cell growth

The growth rate of cells in folate-free medium was proportional to intracellular folate only when

Table 3. Growth rate and intracellular folate content of K562 cells grown in different concentrations of folate
Results are means $\pm$ S.D.

Extracellular folate (μM)	<i>n</i>	Growth rate (doublings/day)	<i>L. casei</i> -active intracellular folate (μM)	5-CH ₃ -H ₄ PteGlu (μM)
PteGlu				
10	4	0.78 $\pm$ 0.11	7.07 $\pm$ 1.82	2.34 $\pm$ 1.22
1	4	0.83 $\pm$ 0.09	3.69 $\pm$ 1.16	2.58 $\pm$ 1.07
0.1	4	0.69 $\pm$ 0.06*	0.61 $\pm$ 0.24	0.50 $\pm$ 0.19
0.01	3	0.34 $\pm$ 0.28†	0.45 $\pm$ 0.03	0.21 $\pm$ 0.16
DL-5-CHO-H ₄ PteGlu				
0.2	4	0.74 $\pm$ 0.09	6.02 $\pm$ 1.73	2.90 $\pm$ 1.24
0.02	4	0.71 $\pm$ 0.04	3.15 $\pm$ 0.66	2.73 $\pm$ 0.46
0.002	4	0.65 $\pm$ 0.12*	0.57 $\pm$ 0.23	0.51 $\pm$ 0.19
0.0002	2	0.38	0.49	0.42

* Growth rate differs from that of cultures grown in control medium (0.81 $\pm$ 0.16 doublings/day) at $P < 0.1$ by Student's *t* test.

† Growth rate differs from that of cultures grown in control medium at $P < 0.05$ by Student's *t* test.

intracellular folate (measured by microbiological assay was *L. casei*) was less than 1.5 μM , or when intracellular folate measured by microbiological assay with *P. cerevisiae* was less than 1.0 μM . Above this concentration growth rate was independent of intracellular folate concentrations (Fig. 2). Growth rate was proportional to the logarithm of intracellular *L. casei*-active folate below 1.5 μM ($r = 0.807$, $P < 0.001$) and of *P. cerevisiae*-active folate below 1.0 μM ($r = 0.848$, $P < 0.001$), but not proportional to the logarithm of calculated 5-CH₃-H₄PteGlu ($r = 0.141$, $P = 0.30$). Data from cells cultured in less than 1 μM -PteGlu or less than 0.02 μM -DL-5-CHO-H₄PteGlu were similar to these.

Partial replacement of folate requirement with thymidine and hypoxanthine

To determine whether these products of folate metabolism would permit growth of K562 cells after depletion of intracellular folate, cells grown in folate-free medium for 12 days were subcultured into media lacking folate but containing 40 μM -thymidine, 10 μM -hypoxanthine or both together. Addition of hypoxanthine alone to the culture medium resulted in a significant increase in growth rate ($P < 0.001$) compared with cells grown in folate-free medium with no additions, but this was only 47% of the growth rate observed in depleted cells subcultured into medium supplemented with 0.2 μM -DL-5-CHO-H₄PteGlu. Addition of thymidine to culture medium resulted in no significant increase in growth rate, nor did addition of thymidine and hypoxanthine together result in any increase in growth rate beyond that produced by hypoxanthine alone.

Discussion

Human leukaemic cell line K562 required folate for growth, and cell numbers in culture did not

Fig. 2. Growth rate of K562 cells growing in folate-free medium as a function of the intracellular concentration of (a) *L. casei*-active folate and (b) *P. cerevisiae*-active folate

increase at an optimal rate when intracellular folate was below a critical level. Although the cell in culture had an absolute requirement for methionine, neither excess folate nor methionine would replace each other. Growth rate was proportional to intracellular non-methylated reduced folate below 1 μM in intracellular water but was not proportional to intracellular 5-CH₃-H₄PteGlu. At concentrations of intracellular folate greater than

1.5 μM , an increasing proportion of intracellular folate accumulated as polyglutamates of 5-CH₃-H₄PteGlu, suggesting that this was a storage form in K562 cells under these conditions. Absence of an important function for 5-CH₃-H₄PteGlu_n was not unexpected because of the presence of L-methionine in the medium at a concentration that probably satisfied all intracellular requirements for methionine. Methionine auxotrophy of cell line K562 was probably of the type previously reported in several transformed cells (Halpern *et al.*, 1974; Hoffman & Erbe, 1976). In such cells, methionine synthase activity is in the same range as that observed in untransformed cells (Hoffman & Erbe, 1976). The presence in K562 of methylcobalamin, which is associated *in vitro* with this enzyme (Taylor & Weissbach, 1974), indicates the presence of methionine synthase enzyme in these cells.

The critical intracellular concentration of reduced folates other than 5-methyl forms for growth was achieved by exposure of cells to different folates but 100-fold less of the biologically active isomers of 5-CHO-H₄PteGlu or 5-CH₃-H₄PteGlu was required than PteGlu. The greater effectiveness of reduced folates in supporting intracellular folate levels and growth of K562 cells can most easily be explained by a greater affinity of the folate-transport system for reduced folates than for PteGlu. Most of the folate in human plasma is 5-CH₃-H₄PteGlu, and its normal concentration is about 0.1 μM , a concentration that supported maximum growth rate of cell line K562 in culture.

Folate-depleted K562 cells, which showed little or no increase in cell number, proliferated rapidly when subcultured into medium containing folate. The rate of increase of cell number under these conditions is consistent with all of the cells present participating in the proliferation. Proliferation was only partially restored by the addition of hypoxanthine to the culture medium, whereas addition of thymidine produced no effect. Failure of hypoxanthine to completely restore growth of folate-depleted cells may be due to limitation of salvage pathway capacity.

The effects of folate depletion on K562 cells were complex. Cells became larger than normal but the mitotic index remained constant at 2–3% of stained cells. This mitotic index is appropriate for a cell cycle of 30–35 h if mitosis requires about 1 h as reported for normal human erythroid cells (Rondanelli *et al.*, 1969). The persistence of this mitotic index in folate-depleted cells with effective doubling times more than four times the normal suggests either a balance between proliferation and death of depleted cells or a prolongation of the entire cell cycle, including prolongation of mitosis. Data are

not at present available to evaluate these possibilities.

We are grateful to Ms. S. Lue-Shing and Ms. N. Vera Matiaszuk for technical assistance, to Dr. A. K. Sullivan and Dr. R. Hand for the McGill Cancer Center for providing the cell culture facilities for these studies and to the Medical Research Council for support through grant MA802. D. W. received a Studentship from the Medical Research Council of Canada. B. A. C. is a Career Investigator of the Medical Research Council of Canada.

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