*Proc. Natl. Acad. Sci. USA*
Vol. 85, pp. 3145–3149, May 1988
Medical Sciences

# Conversion of encapsulated 5-fluoro-2′-deoxyuridine 5′-monophosphate to the antineoplastic drug 5-fluoro-2′-deoxyuridine in human erythrocytes

(bioreactors/deoxyribonucleotidase/fluoropyrimidine drugs/encapsulation)

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*Communicated by Rita Levi-Montalcini, November 23, 1987*

**ABSTRACT** **The fluoropyrimidine deoxyribonucleotide 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP) was encapsulated in human erythrocytes by a procedure based on hypotonic hemolysis and isotonic resealing. Encapsulated FdUMP (up to 9 μmol/ml of packed erythrocytes) did not affect erythrocyte metabolism or morphology. Hemolysates were found to catalyze efficient dephosphorylation of FdUMP to yield nearly stoichiometric amounts of the corresponding deoxyribonucleoside 5-fluoro-2′-deoxyuridine (FdUrd), an antineoplastic drug showing selective cytotoxicity toward liver metastases from colorectal carcinomas. The dephosphorylation reaction had an apparent $K_m$ of 7.7 ± 1.2 mM FdUMP at pH 7.4 and was remarkably slower at pH 8.2. ATP, GTP, and UTP inhibited both the disappearance of FdUMP and the formation of FdUrd in hemolysates. The enzyme responsible for the FdUMP-to-FdUrd conversion was identified with the deoxyribonucleotide-specific isozyme of erythrocyte pyrimidine 5′-nucleotidase (EC 3.1.3.5). Intracellular formation and subsequent release of FdUrd were observed in intact erythrocytes loaded with FdUMP. Inhibition of FdUrd release from these erythrocytes was obtained by raising the pH intracellularly and, alternatively, by coencapsulation of ATP. Autologous FdUMP-loaded erythrocytes might be used as endogenous bioreactors designed for time-programmed and liver-targeted delivery of FdUrd.**

Human and animal erythrocytes can be engineered to behave as "passive" vehicles of encapsulated chemicals (1, 2) or as bioreactors performing either unusual or improved biological functions (3). In the first case, the manipulated erythrocytes merely represent an alternative to other transport systems (e.g., liposomes), the main advantages being biocompatibility and stability (4) and the disadvantage being the difficulty of large-scale utilization. In the second type of application, intrinsic functional properties of erythrocytes (mostly related to specific proteins) can be exploited to construct "bioreactors." These may be used both for *in vitro* studies and, when suitable to retransfusion, for wide biomedical applications (5–9).

A strategy still to be fully developed concerns encapsulation within erythrocytes of suitable drug precursors (prodrugs) tailored to be converted by resident erythrocyte enzymes to membrane-diffusible active drugs. Advantages over other passive transporters are: (*i*) slow and time-programmable drug delivery (e.g., by influencing intraerythrocytic enzyme activities) to ensure long-lasting plasma concentrations and to meet specific pharmacokinetic requirements, and (*ii*) the possibility of overcoming low- or zero-encapsulation potential of a drug by entrapping a related prodrug with higher encapsulation potential (10).

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We report here on manipulation of human erythrocytes designed to obtain the intracellular production and the controlled release of a potent antineoplastic drug—i.e., 5-fluoro-2′-deoxyuridine (FdUrd). This result was achieved by encapsulating in erythrocytes the phosphorylated precursor 5-fluoro-2′-deoxyuridine 5′-monophosphate [FdUMP, which also represents a major pharmacologically active metabolite in target neoplastic cells (11)]. The kinetics of FdUrd production and exit from the FdUMP-containing erythrocytes and the properties of the loaded erythrocytes are compatible with the medical applicability of this "active" delivery system.

## MATERIALS AND METHODS

**Materials.** FdUMP (sodium salt) and FdUrd (crystalline) were obtained from Sigma. ATP, ADP, AMP, phospho*enol*pyruvate, and 2,3-bisphosphoglycerate were purchased from Boehringer Mannheim. GTP, UTP, UMP, and β-glycerophosphate were from Fluka. Two preparations of pyrimidine 5′-nucleotidase (PyrNase, 5′-ribonucleotide phosphohydrolase, EC 3.1.3.5), partially purified from human erythrocytes during a study on nucleotidases, were kindly made available by S. Gini and P. L. Ipata (University of Pisa, Italy). Their specific activities, as assayed in the experimental conditions described for hemolysates—namely at 37°C in 10 mM Tris·HCl (pH 7.4) with 5 mM substrate (either UMP or FdUMP)—were 1.32 (UMP) and 1.5 (FdUMP) units (μmol of nucleoside released per hr) per mg of protein (first preparation), and 1.86 (UMP) and 5.52 (FdUMP) units per mg of protein (second preparation), respectively.

**Blood Samples, Enzyme Assays, and Metabolic Analyses.** Blood samples were obtained after informed consent from normal volunteers (both males and females). Heparin was used as anticoagulant, and leukocytes and residual platelets were removed as described by Beutler *et al.* (12). Blood samples were processed either immediately or within 4 days from withdrawal after storage in autologous plasma at 4°C. GSH, ATP, ADP, and AMP were estimated as described by Beutler (13). The hexose monophosphate shunt activity of intact erythrocytes was estimated as reported (14). The rate of lactate production was recorded after a 60-min incubation at 37°C as described (13). The activity of the PyrNase isozyme specific for ribonucleotides was assayed by the method of Paglia and Valentine (15) with UMP as substrate. Osmotic fragility curves were determined as described by Sprandel and Zöllner (16).

Abbreviations: FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; FdUrd, 5-fluoro-2′-deoxyuridine; PyrNase, pyrimidine 5′-nucleotidase (EC 3.1.3.5); SEM, scanning electron microscopy.

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**Dialysis Encapsulation.** This was achieved by the hypotonic dialysis and isotonic resealing method (17) essentially by the procedure of Ropars and coworkers (18) as described (19). A "two-step" procedure was used, consisting of a first dialysis of washed, packed (80% hematocrit) erythrocytes against 15 volumes of hemolysis buffer (56 milliosmolar NaCl) for 30 min at 4°C under gentle rotation. This step was followed by addition of the compound to be encapsulated (usually 40 mM FdUMP) inside the dialysis bag and further dialysis for 30 min at 4°C against the same hemolysis buffer. In some experiments 20 mM FdUMP was also added to the hemolysis buffer during the second incubation to reduce loss of FdUMP from the dialysis bag into the surrounding buffer, thus enhancing the amount of encapsulated drug. The opened erythrocytes were then recovered and resealed by addition of 0.1 volume of a solution (18, 19) of 5 mM adenine, 100 mM inosine, 100 mM sodium pyruvate, 100 mM sodium phosphate, 100 mM glucose, and 12% NaCl. After incubation for 5 min at 37°C in resealing solution (this time interval being sufficient to ensure satisfactory resealing), erythrocytes were extensively washed with ice-cold isotonic NaCl in a refrigerated centrifuge at 500 × *g*. Average recoveries of erythrocytes were 80 ± 7%, irrespective of the presence or absence of added FdUMP. Subsequent incubation of the native, unloaded (i.e., processed as for the encapsulation technique, yet without addition of FdUMP), and FdUMP-loaded erythrocytes was carried out at 37°C at a 15% hematocrit in isotonic NaCl buffered with 5 mM Tris·HCl (pH 7.4) and supplemented with 5 mM glucose.

For encapsulation at higher pH values, erythrocytes were opened for 30 min at 4°C by dialysis against 40 mM *N*-[2-hydroxyethyl]-piperazine-*N*'-3-propanesulfonate(EPPS)buffer (40 milliosmolar; pH 8.7). The pH of the resulting hemolysates was then adjusted to 8.0 with 0.1 M NaOH, and hemolysates were incubated for an additional 30 min at 4°C in the presence of 40 mM FdUMP. Resealing was obtained as described above, but at pH 8.4.

**Extraction and Analysis of FdUMP, FdUrd, 5-fluorouracil, UMP, and Uridine.** Analyses were carried out on neutralized perchloric acid extracts of aliquots from the incubated erythrocyte suspensions or hemolysates. The intraerythrocytic content of FdUMP was obtained by difference between the values actually found in erythrocyte suspensions and those determined in the corresponding supernatants. HPLC analyses were carried out in a Waters model 510 solvent delivery system equipped with a μBondapak $C_{18}$ column, with 0.1 M sodium phosphate buffer (pH 3.6) as eluant at a flow rate of 1.5 ml/min. The retention times (in minutes) for the various nucleotides, nucleosides, and nucleobases were: GTP, 2.1; UTP, 2.5; UMP, 2.9; ATP, 3.3; 5-fluorouracil, 3.5; ADP, 3.7; FdUMP, 4.3; uridine, 5.1; AMP, 5.5; adenine, 6.8; FdUrd, 9.8; and inosine, 11.4. Detection of eluted compounds was performed with a Hewlett-Packard 1040A high-speed spectrophotometric detector set at 260 nm (reference wavelength, 550 nm) and identification of the various peaks was accomplished both by coelution with known standards and comparison of UV absorption spectra with computer-stored standards. Integration of peak areas was obtained by means of a Hewlett-Packard 79996A analytical workstation, and quantitation of results was carried out by relating integrated peak areas to peaks obtained with high-purity standard compounds run separately. The concentrations of metabolites were referred to as μmol contained in or released by 1.0 ml of packed erythrocytes.

**Scanning Electron Microscopy (SEM).** Fixation of erythrocytes for SEM was obtained as reported by Sansone and Chiappara (20).

## RESULTS

**Encapsulation of Fluoropyrimidine Compounds in Human Erythrocytes.** As shown in Table 1 and as expected from occurrence of a nucleoside transporter in erythrocyte membranes (21), FdUrd was not retained after its encapsulation. The same result was observed with the free base, 5-fluorouracil. Accordingly, human erythrocytes cannot be used as passive delivery systems for either fluoropyrimidine drug. However, the corresponding deoxynucleotide, FdUMP, showed a reasonably high encapsulation potential. Average amounts of 1.5 mg of FdUMP per ml of packed erythrocytes were entrapped by standard procedures, and concentrations as high as 3 mg/ml were obtained by adding FdUMP to the hemolysis buffer during dialysis. Similar results were obtained with the corresponding natural nonfluorinated compounds uracil, 2'-deoxyuridine, and UMP (not shown).

**Properties of the FdUMP-Loaded Erythrocytes.** SEM did not reveal appreciable differences in morphology between unloaded and FdUMP-loaded erythrocytes (Fig. 1).

A number of metabolic properties of the FdUMP-loaded erythrocytes are reported in Table 2, which also shows the corresponding parameters of unloaded cells for comparison. The procedure of encapsulation per se did not produce significant alterations of metabolic properties, as indicated by the close similarity with those of native erythrocytes (not shown). These properties were investigated during the first 60 min after encapsulation of FdUMP (5 μmol per ml of erythrocytes), this time interval being sufficient for transformation of as much as 40–60% of encapsulated FdUMP (see below). The results obtained seem to exclude any major perturbation of biochemical and morphological properties of erythrocytes as related to encapsulation of FdUMP and to its metabolism.

**Dephosphorylation of FdUMP by Erythrocyte Lysates.** The metabolic interaction of FdUMP with human erythrocytes was first investigated by using hemolysates. These were found to catalyze rapid dephosphorylation of FdUMP, yielding nearly stoichiometric amounts of FdUrd (Fig. 2). The reaction was proportional to the amount of hemolysate (not shown) and was almost completely blocked at pH 8.2 (Fig. 2). Formation of 5-fluorouracil was not observed. At all FdUMP concentrations except those above 20 mM, the rate of FdUrd formation was found to decline with time (up to 60 min), indicating steady exhaustion of substrate. However, interpolation of initial velocities recorded at increasing FdUMP concentrations allowed a $K_m$ value of 7.7 ± 1.2 mM FdUMP to be calculated. The average level of FdUMP-dephosphorylating activity measured under the experimental conditions described in *Materials and Methods* at 20 mM FdUMP was 3.0 ± 0.4 μmol/ml of hemolysate per 10 min.

A number of metabolites were tested for their effects on the activity of the phosphatase system acting on FdUMP (Table 3). The nucleoside triphosphates UTP, GTP, and ATP were found to be strong inhibitors of FdUrd production

Table 1. Encapsulation potential of fluoropyrimidine compounds in human erythrocytes

| | Average % encapsulation* | Amount encapsulated, μmol/ml of RBC |
|---|---|---|
| 5-Fluorouracil | 0 | 0 |
| FdUrd | 0 | 0 |
| FdUMP | 42 ± 7 | 4.5 ± 0.7 |

Data are means ± SD of six different experiments. RBC, erythrocytes.

*Calculated as the percent ratio of the erythrocyte-encapsulated compound to the amount of the same compound present in the hemolysate just before resealing.

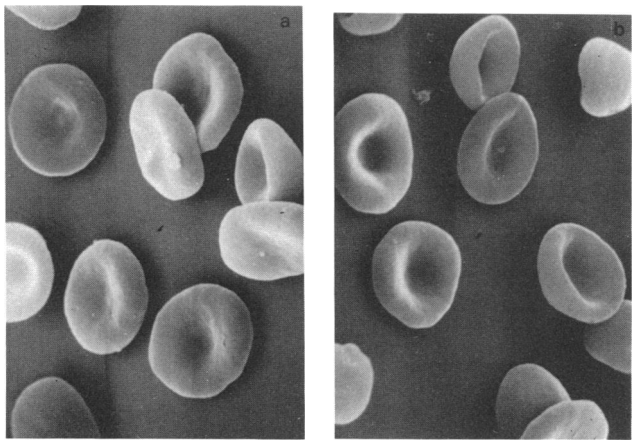

FIG. 1. SEM of unloaded (*a*) and FdUMP-loaded (*b*) human erythrocytes. (×1400.)

by hemolysates. Such an effect could result, alternatively, from direct inhibition of the dephosphorylation reaction or from a kinase-mediated phosphorylation of FdUrd back to FdUMP. However, the latter possibility, which could be suggested by the moderate shift of the [ATP]/[ADP][AMP] ratio after encapsulation of FdUMP (Table 2), was excluded by a number of experiments in which supplementation of hemolysates with ATP failed to determine conversion of FdUrd to FdUMP in a variety of conditions (e.g., over a wide range of $Mg^{2+}$ concentrations, incubation at pH 8.0 to minimize the dephosphorylation reaction, and use of labeled FdUrd to enhance sensitivity of detection). Moreover, occurrence of a futile cycle between FdUMP and FdUrd (thus involving a FdUrd kinase activity) was excluded because no labeling whatsoever of FdUMP was found upon incubation of hemolysates supplemented with 2 mM FdUMP and 4 mM [$\gamma-^{32}$P]-ATP for up to 4 hr at 37°C and at pH 8.0 (not shown).

Three additional findings show that nucleoside triphosphates only are direct inhibitors of the FdUMP phosphatase system. (*i*) ADP decreased the rate of FdUMP dephosphorylation in hemolysates, but this effect was clearly related to rapid equilibration, through adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3) activity, to AMP and ATP, the latter nucleotide being ultimately responsible for the inhibition (Table 3). (*ii*) The concentrations of both GTP and

Table 2. Metabolic properties of FdUMP-loaded human erythrocytes

| | Unloaded | | Loaded | |
|---|---|---|---|---|
| Erythrocyte suspension | 0 min | 60 min | 0 min | 60 min |
| ATP, μmol/ml of RBC | 1.11 | 1.00 | 0.72 | 0.80 |
| ADP, μmol/ml of RBC | 0.10 | 0.16 | 0.37 | 0.29 |
| AMP, μmol/ml of RBC | 0.05 | 0.06 | 0.18 | 0.11 |
| GSH, μmol/g of Hb | 4.22 | 4.23 | 4.08 | 4.21 |
| Lactate production, μmol/ml of RBC per hr | | 2.5 | | 2.6 |
| HMS activity, nmol/ml of RBC per hr | | 51 | | 44 |
| Osmotic fragility* | 0.38 | 0.40 | 0.40 | 0.41 |

Incubations of native, unloaded, and FdUMP-loaded erythrocytes (RBC) (5.2 μmol per ml of packed erythrocytes) were performed at a 15% hematocrit in isotonic saline buffered with 5 mM Tris·HCl (pH 7.4) containing 5 mM glucose at 37°C in a shaking water bath. Each value is the average of three experiments yielding closely comparable results. Lactate production, as a measure of glycolytic activity, and hexose monophosphate shunt (HMS) activity were estimated after preincubation of the three erythrocyte suspensions for 60 min at 37°C. GSH, reduced glutathione.

*NaCl concentration (g/100 ml) producing 50% hemolysis.

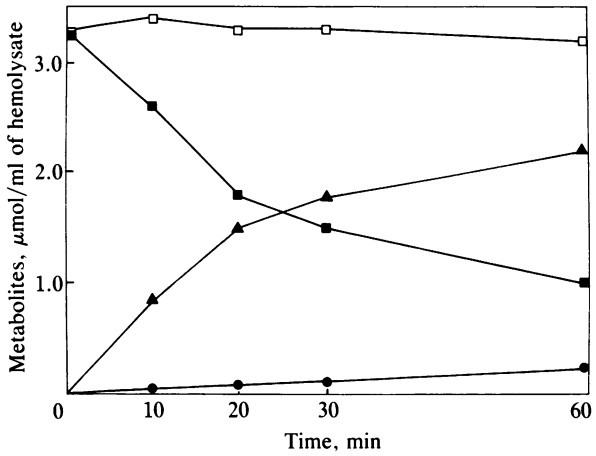


FIG. 2. Conversion of FdUMP to FdUrd in erythrocyte lysates. Hemolysates were obtained by dilution of washed and packed erythrocytes in 10 mM Tris·HCl buffer (pH 7.4) to a final hematocrit of 50% and subsequent sonication for 5 sec. For the experiments at pH 8.2, packed erythrocytes were diluted in 10 mM Tris·HCl buffer (pH 8.2) and sonicated, and the resulting pH was adjusted with 0.1 M NaOH. Aliquots of FdUMP were added to the hemolysates to achieve a final concentration of 5 mM, and samples were incubated at 37°C in a shaking water bath. At the time intervals indicated, aliquots were removed, and HPLC analysis of metabolites was performed on neutralized perchloric acid extracts. ●, Formation of FdUrd at pH 8.2; ▲, formation of FdUrd at pH 7.4; ■, consumption of FdUMP at pH 7.4; □, sum of residual FdUMP and FdUrd formed at pH 7.4. Each value is the average of three experiments, with different hemolysates yielding closely comparable results.

UTP added to hemolysates were unmodified over the time required to measure the FdUMP-to-FdUrd conversion (5 min at 37°C). (*iii*) The FdUMP-dephosphorylating activity of two partially purified preparations of erythrocyte deoxyribonucleotidase was 75–80% inhibited by ATP at a FdUMP/ATP ratio of 1 (see below).

Removal of ghosts from the sonicated hemolysates did not modify the observed rate of FdUMP dephosphorylation, thus indicating the cytosolic localization of the enzyme activity responsible for this reaction. The following lines of evidence seem to identify this phosphatase activity with the isozyme of PyrNase specific for pyrimidine deoxyribonucleotides, recently described by Paglia *et al.* (22): (*i*) the similarity of $K_m$ values recorded for FdUMP conversion to FdUrd to those reported for activity with dUMP and dTMP as substrates (22); (*ii*) the pH dependency (22) of FdUMP

Table 3. Inhibition of the FdUMP-to-FdUrd dephosphorylation in hemolysates by various metabolites

| | Ratio* | | | |
|---|---|---|---|---|
| Inhibitor(s) | 0.1 | 1 | 2 | 3 |
| ATP | 0 | 76 | 80 | 91 |
| ATP/ADP/AMP (1:1:1)† | 0 | ND | ND | 69 |
| GTP | ND | 97 | 99 | ND |
| UTP | ND | 89 | 92 | ND |
| β-Glycerophosphate | 0 | 0 | 22 | 25 |
| 2,3-Bisphosphoglycerate | 0 | 0 | 19 | 24 |
| Phospho*enol*pyruvate | 0 | 0 | 0 | ND |

Results are expressed as percentages of inhibition relative to control (without inhibitor). FdUMP and FdUrd concentrations were determined after a 5-min incubation at 37°C. ND, not determined.

*Ratio between the concentrations of inhibitor and FdUMP (5 mM) in the hemolysate at zero time.

†ADP was actually added at an [ADP]/[FdUMP] ratio of 3, but nearly equimolar amounts of ATP, ADP, and AMP were present within less than 1 min because of adenylate kinase activity (see text).

hydrolysis (Table 4); (*iii*) the incomplete extent of inhibition (50%) of this reaction at 5 mM $Pb^{2+}$ (22); (*iv*) the normal rate of FdUMP hydrolysis catalyzed by hemolysates from patients with either genetic or acquired deficiency of the ribonucleotide-specific isozyme of PyrNase (Table 4); and (*v*) the criterion of inhibition by ATP on two different preparations of PyrNase partially purified from human erythrocytes. Specifically, the less-purified preparation (a mixture of the two isozymes—i.e., ribonucleotidase and deoxyribonucleotidase) was 47% inhibited by ATP with UMP as substrate and 75% inhibited with FdUMP as substrate, at a [substrate]/[ATP] ratio of 1. The more-purified PyrNase preparation, enriched in deoxyribonucleotidase as shown by substrate specificities and by a shift of the pH optimum to 6.2 (22), was 80% inhibited by ATP at a [FdUMP]/[ATP] ratio of 1, thus reproducing with remarkable approximation the extent of susceptibility to inhibition observed in hemolysates (see Table 3).

**Intraerythrocytic Formation and Release of FdUrd.** When the erythrocytes were loaded with FdUMP and then incubated in saline, two parallel events were observed—i.e., progressive decrease of intracellular FdUMP and appearance of FdUrd in the medium (Fig. 3). Coencapsulation of sufficiently high amounts of ATP to reach an intraerythrocytic [FdUMP]/[ATP] ratio comparable to one of those established in hemolysates (Table 3) resulted in 70% inhibition of intracellular FdUMP consumption and of FdUrd release in the medium. A major factor influencing the rate of FdUMP dephosphorylation proved to be the starting intracellular concentration of FdUMP (which is not surprising given the high $K_m$ value of the enzyme), with a poor record of reaching saturation over the range of actual-encapsulatable amounts of this deoxynucleotide. Thus, for instance, with the same sample of erythrocytes (and at identical concentrations of ATP and ADP), the observed rates of FdUrd appearance in the medium at initial intraerythrocytic levels of 5.0, 6.0, and 9.5 mM FdUMP were 0.9, 1.2, and 2.2 μmol, respectively, of the deoxynucleoside released per ml of erythrocytes per 10 min at 37°C.

Under particular encapsulation conditions (see *Materials and Methods*), the intraerythrocytic pH value of FdUMP-loaded erythrocytes could be raised to 7.8–8.0. These elevated pH values were found to revert to normal ones within 30–60 min of incubation at 37°C in buffered saline at pH 7.4. In these conditions, the observed rates of FdUrd release in the medium were consistently lower at the initial times of incubation than after verified normalization of intraerythrocytic pH (not shown).

Table 4. Dephosphorylation of FdUMP and UMP in hemolysates from normal and PyrNase-deficient subjects

| Subject | *n* | pH | Nucleoside formation,* μmol/ml of hemolysate per 10 min | |
|---|---|---|---|---|
| | | | FdUrd | Uridine |
| Normal controls | 4 | 7.4 | 1.05 ± 0.15 | 1.42 ± 0.17 |
| | | 6.5 | 1.34 ± 0.18 | ND |
| | | 8.0 | 0.38 ± 0.09 | 1.28 ± 0.08 |
| Homozygous PyrNase deficient | 1 | | | |
| A.A.† | | 7.4 | 1.11 | 0.04 |
| Lead intoxicated | 2 | | | |
| T.S.‡ | | 7.4 | 0.92 | 0.64 |
| M.S.‡ | | 7.4 | 1.20 | 0.51 |

*Estimated with 5 mM FdUMP or UMP, respectively, as reported in the legend to Fig. 2.

†PyrNase activity of this patient, assayed with UMP as the substrate under the conditions described by Paglia *et al.* (22), was 0.81 μmol of $P_i$ released per hr/g of hemoglobin, indicating homozygosity of PyrNase deficiency. Blood from this patient was provided by A. Zanella (Ospedale Maggiore, Milan, Italy).

‡Both subjects, chronically exposed to lead, had almost undetectable levels of erythrocyte δ-aminolaevulinate dehydratase (porphobilinogen synthase, EC 4.2.1.24) activity as assayed by the method of Seelig and Wüst (23). Blood from these patients was provided by F. Ottenga and F. Copello (University of Genoa, Italy).

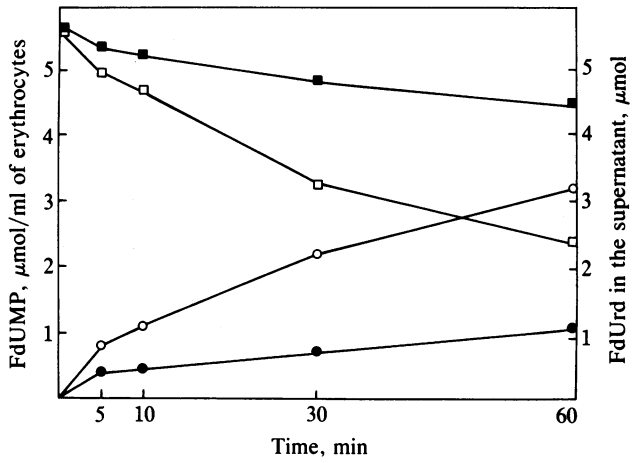


FIG. 3. Intraerythrocytic conversion of FdUMP to FdUrd and release of FdUrd in the presence and absence of coencapsulated ATP. One milliliter of packed erythrocytes containing 5.6 mM FdUMP was incubated with gentle stirring at 37°C in 6.66 ml of isotonic NaCl buffered with 5 mM Tris·HCl (pH 7.4) and containing 5 mM glucose. Values shown on the ordinate, referring to a representative experiment, indicate the amounts of intraerythrocytic FdUMP or of FdUrd present in the whole supernatant (6.66 ml). □ and ○, Consumption of FdUMP (□) and formation of FdUrd (○) in the absence of ATP; ■ and ●, consumption of FdUMP (■) and formation of FdUrd (●) in the presence of coencapsulated ATP (at an [ATP]/[FdUMP] ratio of 1).

## DISCUSSION

The strategy of encapsulating prodrugs within erythrocytes with the aim of obtaining their conversion to active drugs is not without precedent. Thus, attempts were made to obtain intraerythrocytic dephosphorylation of encapsulated cytosine arabinoside monophosphate to the antineoplastic drug cytosine arabinoside (24, 25). However, presence in human erythrocytes of an active cytosine arabinoside deaminase resulted so far in a useless side reaction—i.e., substantial formation of the pharmacologically inactive compound uracil arabinoside (25).

The case of fluoropyrimidine compounds reported in this paper seems to be uncomplicated by further metabolic transformation of both the precursor FdUMP and the diffusible product FdUrd. Much attention was paid in this investigation to establish the possible occurrence in the erythrocyte of competing metabolic pathways for both compounds, since the apparent inhibition exerted by ATP (and by GTP and UTP as well) could suggest a phosphorylation of FdUrd back to FdUMP or of FdUMP to the corresponding di- and triphosphates (FdUDP or to FdUTP). Self-feeding mechanisms of this type could in fact be useful to slow down the final step—i.e., production of FdUrd and its leakage from erythrocytes—over a reasonably prolonged time interval (see below). However, careful experiments using radioactive compounds and assays of enzyme activities in the hemolysates led us to exclude any kinase acting on FdUrd and FdUMP as substrates. Possible steady mechanisms of salvage of 5-fluorouracil (not detectable in our conditions) to yield the corresponding ribo(deoxy)nucleosides 5-fluorouridine and FdUrd and the ribo(deoxy)nucleotides 5-fluoro-

uridine 5'-monophosphate and FdUMP were also ruled out. Accordingly, the human erythrocyte behaves as a single-step bioreactor for the production and delivery of FdUrd, and the only effective ways available at present for inhibiting this metabolic function seem to be coentrapment of nucleoside triphosphates (ATP, GTP, or UTP) (see Table 3 and Fig. 3) or elevation of intraerythrocytic pH values.

Identification of the FdUMP-dephosphorylating enzyme with the deoxyribonucleotidase described by Paglia *et al.* (22) may suggest additional maneuvers for its programmed regulation within the erythrocyte as soon as it is purified and its properties are elucidated. Down-regulation of FdUMP dephosphorylation seems to be necessary in view of current trends concerning use of fluoropyrimidine drugs in cancer therapy (11, 26), which advocate prolonged release of FdUrd into the liver microcirculation rather than systemic bolus injection. With respect to this, unrestrained[§] performance of 10 ml of autologous FdUMP-loaded erythrocytes (at starting levels of 6.0 μmol/ml) would ensure the production and release in circulation of 7–9 mg of FdUrd approximately within the first hour, followed by smaller amounts being released subsequently. These figures, although still nonoptimal and subject to improvement by intraerythrocytic regulation (see above), are close to recommended daily doses of FdUrd in the treatment of solid tumors by combination chemotherapy (27) and also of liver metastases from colorectal carcinomas (28–30).

The issue of hepatic targeting of FdUrd-releasing erythrocytes is of particular importance because of the efficacy of this fluoropyrimidine drug in chemotherapy of liver metastases, as also shown by improvement of the therapeutic index of FdUrd through hepatic arterial infusion compared with systemic intravenous administration (26). However, the technical complexity and high cost of the arterial drug delivery system (not to mention the potential morbidity involved) stimulate attempts at achieving regional hepatic chemotherapy by alternative biological procedures. To this purpose, animal erythrocytes loaded with antineoplastic drugs (31, 32) can be manipulated to be selectively targeted to the reticuloendothelial system of the liver. These treatments, resulting in the hepatic localization of carrier erythrocytes, include cross-linking of membrane proteins with glutaraldehyde (31, 32), energy depletion of erythrocytes by incubation at 42°C (33), and removal of the membrane sialic acid with neuraminidase (33). Attempts at extending these observations to humans seem to be justified to couple the requirement of time-programmed production of FdUrd with its delivery in the proximity of liver tumors.

[§]Average ATP concentrations in human erythrocytes are 1.438 ± 0.099 μmol/ml of red cells (13). Accordingly, the [ATP]/[FdUMP] ratio is approximately 0.2 (at 6 mM FdUMP)—i.e., too low to exert inhibitory effects.

We are indebted to Dr. M. Chiappara (Galliera Hospital, Genoa, Italy) for kindly performing the SEM analyses. This work was supported in part by the Special Project "Biotecnology" of Consiglio Nazionale delle Ricerche (Rome), by the Biotechnology Action Programme of the Commission of the European Communities (Grant BAP-0056-I), and by the Associazione Italiana per la Ricerca sul Cancro.

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