

# Enzymatic Degradation of Uric Acid by Uricase-Loaded Human Erythrocytes

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**ABSTRACT** Erythrocytes containing pig liver uricase have been prepared by hypotonic hemolysis in the presence of the enzyme. Uricase is shown to be active within the erythrocytes and to degrade uric acid as rapidly as it enters the cells when high intracellular enzyme concentrations are employed. The kinetics and characteristics of uric acid entry are shown to be the same for hemolysed and normal erythrocytes. At physiological concentrations of uric acid, loaded erythrocytes can degrade a maximum of about 21  $\mu$ mol uric acid/liter erythrocytes per min. The possible application of enzyme-loaded erythrocytes to medicine is discussed.

## INTRODUCTION

The possibility of enzyme replacement therapy for various diseases has attracted wide interest in recent years. Ideas currently being explored included direct injection of the enzyme, enzyme modification by drug-like agents to increase or alter pre-existing catalytic activity, enzymes immobilized on insoluble matrices or gels, and enzymes encapsulated in some sort of semi-permeable container. (For the proceedings of a recent conference, see reference 1).

Ihler, Glew, and Schnure (2) have shown that enzymes can enter erythrocytes through holes opened during reversible hypotonic hemolysis and become permanently entrapped after addition of isotonic saline. The resulting enzyme-loaded erythrocytes might be useful in two types of clinical situations. First, in diseases involving the accumulation of intracellular substrates in phagocytic cells, the enzyme could be introduced into the phagocytic cell during phagocytosis of the enzyme-containing erythrocyte. Depending on how the erythrocyte was pretreated, it can be directed to a unique organ, notably the spleen or the liver (3). Enzyme ther-

apy of sphingolipidoses, such as Gaucher's disease, which involve these visceral organs, for example, might thus be feasible.

Secondly, it should be possible to degrade circulating small molecules by circulating enzyme-loaded erythrocytes, provided (a) the substrate diffuses or is transported into the erythrocyte sufficiently rapidly, (b) the enzyme is active inside the erythrocyte, and (c) the enzyme-loaded erythrocyte continues to circulate more or less normally. As a model system we have chosen initially to begin with the enzyme uricase and its substrate uric acid, since this system can be easily studied both in vitro and in vivo. This reaction is commonly employed by many living organisms (but not humans) to dispose of uric acid. The products of the reaction are not toxic, and even in humans as much as 25% of the uric acid is metabolized in the intestines by bacteria to allantoin and further degradation products, which are reabsorbed and excreted by the kidney (4). Since uric acid is a normal end product with no known function in vivo, the possibility of degrading too much of the substrate is not an important consideration. Animals or birds could be used to demonstrate whether uricase-loaded erythrocytes can in fact lower the plasma urate concentration.

In this work, we show that uric acid is transported into hemolysed erythrocytes at the same rate and with the same characteristics as normal erythrocytes. When the hemolyzed erythrocytes are loaded with excess uricase, the uric acid is degraded as fast as it enters the cell. The reaction is shown to be catalyzed by intracellular enzyme and not by enzyme released by lysed cells or enzyme bound to the cell surface.

## METHODS

### Materials

Pig liver uricase (2 mg/ml) was purchased from Boehringer Mannheim Corp., New York. [2-<sup>14</sup>C]Uric acid (56.7 mCi/mmol) was purchased from Amersham/Searle Corp., Arlington Heights, Ill.

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## Experimental procedure

**Enzyme assay.** We define 1 U of activity as the amount of enzyme required to degrade 1  $\mu\text{mol}$  of uric acid/min at pH 7.4. At this pH, the enzyme has a specific activity of 1.3 U/mg. When assayed under optimal conditions (pH 8.5, borate buffer), the enzyme has a specific activity of 4.5  $\mu\text{mol}/\text{mg}$ . The optical assay for activity consisted of following the decrease in absorption at 290 nm in a Gilford recording spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) (5). The radioisotope assays were generally carried out in a volume of 0.2 ml in a buffer consisting of 0.9% NaCl; 500 mg/liter glucose; 4 mM KCl; and 0.02 M sodium phosphate buffer, pH 7.4. Loaded erythrocytes, if present, were usually used at hematocrit of 25%.

Uric acid concentrations, unless otherwise noted, were 0.3 mM. Radioactive uric acid was usually present at about  $10^9$  cpm/ $\mu\text{mol}$ .

10- $\mu\text{l}$  samples of the reaction mixture were withdrawn and placed in 0.5 ml of ice-cold distilled water (this dilution lyses the erythrocytes if present and reduces the ionic strength of the assay mixture). The sample (0.5 ml) was then passed through a small TEAE column (0.25 ml packed volume) in an eye dropper. The column was washed with an additional 0.5 ml of distilled water and the entire eluate was counted in Triton X-100 counting medium in a Packard scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Unreacted uric acid can be recovered from the column by washing with 1 M NaCl solution, and the sum of the low-salt and high-salt eluates accounts for all the added radioisotope.

The optical and radioisotope assays were compared by removing samples for radioactivity determination from a reaction that was also monitored optically. The results of optical and isotopic assays corresponded closely. If the reaction is followed to completion, greater than 99% of the isotopically labeled uric acid can be converted to product.

**Loading procedure.** Human erythrocytes were washed several times with 0.9% saline or other buffer to remove plasma, and the buffy coat was removed by aspiration. To keep volumes to a minimum, the hemolysing solution (usually distilled water containing a variable amount of enzyme in 0.01 M Tris, pH 7.4) was added to loosely packed erythrocytes at room temperature. The ratio of cell volume to hemolysing solution varied from 1:3 to 1:6 in different experiments. After a period of time, usually 30 or 60 s, hemolysis was terminated by addition of sufficient 9% NaCl to bring the final concentration to 0.9%. A longer hemolysis period will allow better equilibration of enzyme, but we feel that short hemolysis times may result in less damage to the cell.

The loaded cells were then washed more than four times by centrifugation to remove excess enzyme. Erythrocyte concentrations were determined as the hematocrit with capillary tubes and an Adams Autocrit centrifuge (Clay Adams, Parsippany, N. J.).

**Density fractionation on ficoll.** In experiments used to determine the kinetics of uric acid entry and exit, it was necessary to ensure that no unhemolysed cells were present. Normal cells readily centrifuge through 30% Ficoll. Hemolysed cells band in a 30-0% Ficoll gradient (Pharmacia Fine Chemicals, Piscataway, N. J.) with a broad distribution, but at densities approximating 10% Ficoll. Preparative removal of unhemolysed cells was accomplished by layering

the cells on 10 or 20% Ficoll and removing the top layer of cells after centrifugation.

**Rate of uric acid uptake.** Hemolysed and normal erythrocytes were suspended at a hematocrit of 5-15% in the desired medium. The rate of uptake of radioactive uric acid was determined by diluting the cells 0.1 ml/5 ml into cold buffer and filtering on glass fiber filter papers. The papers were then washed with an additional 5 ml of buffer. In some experiments involving centrifuging the erythrocytes, similar results were obtained. The filters were counted in Triton X-100 counting fluid containing 5% H<sub>2</sub>O. The erythrocytes remain on the filter while the uric acid dissolves in the counting medium, so that there is little quenching by hemoglobin.

For exit experiments, the same erythrocytes used for the entry experiments were diluted 1/50 into buffer after the cells had come to equilibrium. The rate of exit was then determined in the same manner.

**Sodium transport.** Cells were hemolysed with <sup>22</sup>Na ( $2.85 \times 10^8$  cpm/ml) present in hemolysis mixture. After 60 s, KCl was added to 155 mM and the cells were incubated at 37°C for 30 min. After centrifugation four times and removal of unhemolysed cells in 20% Ficoll, the cells were resuspended in 130 mM NaCl, 5 mM KCl, 1 mM phosphate buffer, pH 7.4, 10 mM Tris buffer, pH 7.4. Where added, glucose was 2.8 mM, inosine was 10 mM, ouabain was  $5 \times 10^{-5}$  M, and hexokinase (Sigma Chemical Co., [St. Louis, Mo.] yeast hexokinase with an activity of 14 nmol glucose/min per mg at pH 8.5, 25°C) was 200  $\mu\text{g}/\text{ml}$  in the hemolysis solution. The cells contained about  $1.8 \times 10^8$  cpm/ml of erythrocytes at the beginning of the efflux experiment. At various times, 0.1-ml samples were pipetted into 2 ml of buffer and the cells were removed by centrifugation. Samples of the supernate were counted to determine extracellular radioactivity.

**Lactate production.** Cells were incubated in the same buffer used for the <sup>22</sup>Na efflux experiments except that glucose where present was 5.6 mM, hexokinase at 40  $\mu\text{g}/\text{ml}$ , adenine at 5 mM, and guanine at 2.5 mM. At zero time and 60 min, samples were precipitated with an equal volume of 8% perchloric acid and the precipitate was removed by centrifugation. The solution was adjusted to pH 7-8 with K<sub>2</sub>CO<sub>3</sub> and the precipitate was removed by centrifugation. Samples were assayed for lactate with lactic dehydrogenase and hydrazine buffer (6).

## RESULTS

**Rate of transport of uric acid.** Uric acid is believed to enter erythrocytes by a passive carrier mechanism (7, 8). Hemolysis might have little effect on this carrier system, or might instead result in loss or inactivation of the carrier protein, or create pores in the membrane through which uric acid could freely enter or exit. We have compared the rates of entry and exit of uric acid into normal and hemolysed erythrocytes and find them to be very similar (Fig. 1, top). In studies on urate entry, the erythrocytes were suspended at a hematocrit of 5-15% in buffer or plasma containing radioactive uric acid. After various intervals the intracellular urate was determined by filtration of the cells on glass fiber filters (similar results are obtained by centrifuging the cells). For exit, cells preequilibrated

with urate were diluted 1/50 into buffer and the rate of exit of the radioactive uric acid was determined.

Since the concentration of uric acid in the intracellular water phase is equal to the extracellular concentration at equilibrium (7), we have used the following equations to describe the entry and exit of uric acid.

$$\begin{array}{ll} \text{Entry (Eq. 1)} & \text{Exit (Eq. 2)} \\ \frac{dc_i}{dt} = k(c_o - c_i) & -\frac{dc_i}{dt} = k(c_o - c_i) \\ \ln \frac{c_o}{c_o - c_i} = kt & \ln \frac{c_e - c_o}{c_i - c_o} = kt \end{array}$$

where  $c_o$  is the concentration outside the cell,  $c_i$  is the intracellular concentration, and  $c_e$  is the initial equilibrium intracellular concentration for the exit equation.

The data from Fig. 1, top, are replotted according to these equations in Fig. 1, below. Comparison of entry and exit data shows that hemolysed and normal cells have the same rate constants ( $0.07 \text{ min}^{-1}$ ) for both entry and exit, indicating that uric acid is transported similarly by both types of erythrocytes. The rate constant for entry in each case is equal to the rate constant for exit, as would be expected for a system with an equilibrium constant of unity.

Plasma has no effect on the rate of urate transport when compared with buffer (exp. 2, Table I). Thus plasma does not contain significant concentrations of a competitive inhibitor for uptake.

The rate of entry is temperature dependent. At  $26^\circ\text{C}$  the rate is about one-half the rate at  $37^\circ\text{C}$ . The rate of uric acid transport at  $0^\circ\text{C}$  is extremely slow, less than 1% of the rate at  $37^\circ\text{C}$  (exps. 3 and 4, Table I).

Over the range of uric acid concentrations we have examined (up to 1.2 mM), there is no significant change in the rate constant, indicating that the carrier is not saturated in this concentration range (exps. 5 and 6, Table I). Lassen (7) has shown for normal cells that at uric acid concentrations above 2 mM (almost 10-fold higher than the physiological concentration), the rate constant decreases, implying the existence of a saturable carrier system.

Hypoxanthine is known to inhibit the uptake of uric acid by a competitive process (8). Entry into both hemolysed and normal erythrocytes is markedly inhibited by 5 mM hypoxanthine (exps. 7 and 8, Table I). Since this inhibition is probably due to competition for a common carrier, this observation supports the idea that uric acid transport into hemolyzed cells, as in normal cells, is carrier-mediated.

After hemolysis, much of the intracellular contents are lost and as a result it would be expected that the aqueous space of the cell should increase. In agreement

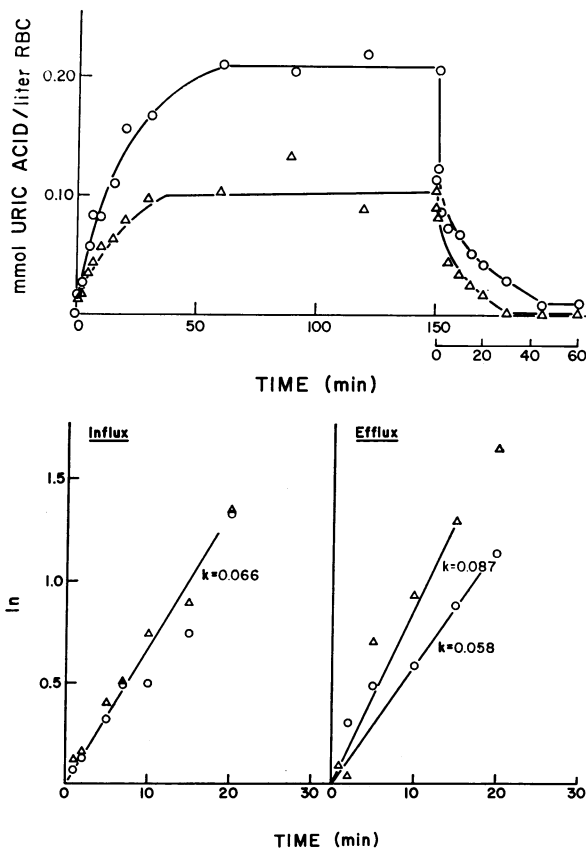


FIGURE 1 Kinetics of uptake of uric acid. Above: Washed red cells were resuspended in buffer at a hematocrit of 15% and incubated with uric acid (0.3 mM). At various times samples were diluted 0.1 ml/5 ml into cold buffer, filtered, and washed. The rate of exit was determined in the same manner after the cells were diluted 1/50 into fresh medium. The cells were those incubated for 150 min during the entry experiment. Below: The data from above replotted according to the equations given in the text.  $\circ$ — $\circ$ , hemolysed cells;  $\triangle$ — $\triangle$ , normal cells.

with this idea, we find that the concentration of uric acid inside hemolysed cells (based on hematocrit as a measure of total cell volume) is about 70–80% of the external concentration, whereas in normal cells it is about 40–50%. This effect can be seen in Fig. 1a, where the final uptake of uric acid into hemolysed cells is greater than uptake into normal cells.

*Kinetics of uric acid degradation.* The concentration of enzyme within the hemolysed erythrocytes can readily be varied by changing the concentration of the enzyme in the hypotonic lysing medium. At high concentrations of intracellular enzyme, enzyme will be in excess, so that the reaction rate will be limited by the rate of uric acid influx into the cell. At low concentrations enzyme will be limiting, so that the velocity of the reaction will be proportional to the enzyme concentration in the cell.

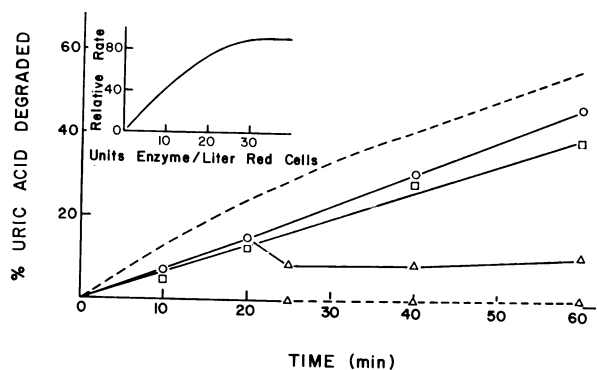


FIGURE 2 Kinetics of intracellular enzyme. 1 ml of washed packed red cells was hemolyzed with 4 ml of distilled water containing 0.21 U uricase. The activity of the loaded red cells is 9.6 U/liter. O—O, kinetics of intracellular enzyme;  $\Delta$ — $\Delta$ , cells removed by centrifugation at 25 min. Uric acid present from time 0;  $\Delta$ -- $\Delta$ , cells removed by centrifugation at 25 min and uric acid then added;  $\square$ — $\square$ , kinetics of sonicated loaded cells sonicated before addition of uric acid; -----, maximum rate of reaction at infinite intracellular enzyme concentration (Eq. 4). Inset, reaction rate as percentage of the maximum rate versus intracellular enzyme concentration as calculated from Eq. 3.

The maximum effective enzyme concentration occurs when the enzyme is neither limiting nor in excess. This concentration of enzyme can be estimated

TABLE I  
Kinetic Constants for Uric Acid Transport

Experiment no.	Cell type	Medium	$k_{influx}$ <i>min</i> <sup>-1</sup>	$k_{efflux}$ <i>min</i> <sup>-1</sup>
1	Hemolysed	Buffer	0.066	0.058
	Normal	Buffer	0.066	0.087
2	Hemolysed	Plasma	0.050	0.070
	Normal	Plasma	0.050	0.050
3	Hemolysed	Buffer 37°	0.047	
	Hemolysed	Buffer 26°	0.027	
	Hemolysed	Buffer 0°	0.001	
4	Normal	Buffer 37°	0.070	
	Normal	Buffer 26°	0.025	
	Normal	Buffer 0°	0.0005	
5	Normal	Buffer 1.2 mM urate	0.028	
	Normal	Buffer 0.6 mM urate	0.042	
	Normal	Buffer 0.3 mM urate	0.035	
	Normal	Buffer	0.035	
6	Hemolysed	Buffer 1.2 mM urate	0.038	
	Hemolysed	Buffer 0.6 mM urate	0.042	
	Hemolysed	Buffer 0.3 mM urate	0.042	
	Hemolysed	Buffer 0.02 mM urate	0.036	
7	Normal	Buffer	0.070	
	Normal	Buffer + 5 mM Hypoxanthine	0.010	
	Hemolysed	Buffer	0.068	
	Hemolysed	Buffer + 5 mM Hypoxanthine	0.007	

The rate constants were determined as discussed in Fig. 1 and in the text.

with the formula below. At equilibrium, the rate of influx of uric acid will be matched by the rate of its enzymatic degradation.

$$\frac{dc_i}{dt} = 0 = k(c_o - c_i) - \frac{(\text{units enzyme}) c_i}{K_m + c_i} \quad (3)$$

We take the extracellular uric acid concentration,  $c_o$ , to be a constant ( $3 \times 10^{-4}$  M for these experiments),  $K_m$  to be  $2 \times 10^{-5}$  M<sup>-1</sup> (9), and  $k = 0.07$  min<sup>-1</sup> as determined in the previous section. A plot of the reaction rate relative to the maximum possible (maximal uric acid influx at zero intracellular uric acid concentration) versus enzyme concentration is shown in the inset to Fig. 2. It can be seen that values in the range of 15–20 U/liter of erythrocytes have reaction rates only slightly slower than the maximum possible and thus there is no reason to load the cells with more than this amount of enzyme.

If an excess of enzyme is present, the maximum rate of urate degradation will be determined by the rate of influx into the erythrocytes when the intracellular uric acid concentration is zero. This rate is given by the following equations:

$$\frac{-dc_o}{dt} = (k)(\text{Hct})c_o \quad (4)$$

$$\ln \frac{c_o^{\text{initial}}}{c_o} = (k)(\text{Hct})t$$

where Hct represents hematocrit. As is shown later, the experimental maximum rate observed with enzyme excess follows this equation closely.

Fig. 2 shows the kinetics of the reaction by cells loaded with 9.6 U of enzyme/liter of erythrocytes. The rate is nearly linear for 60 min. The dotted line represents the kinetics expected at the maximum rate of reaction (influx limited) for the hematocrit used (25%). The experimental rate is slightly less than the maximum possible rate suggesting that the enzyme concentration in the cells is only slightly less than that needed to produce maximal rates.

From the insert in Fig. 2, 9.6 U/liter erythrocytes would be expected to produce a rate 40% of maximum. The initial relative rate shown in Fig. 2 is 55% of the calculated maximum, which is in reasonable agreement with the expected value.

The kinetics of the reaction catalyzed by enzyme released from sonicated cells is also shown in Fig. 2, where it is seen that the rate of reaction by free enzyme is approximately the same as that for the encapsulated enzyme. It is concluded, therefore, that the enzyme is not present in great excess within the cell. Since the enzyme in this experiment is not present in excess in the cell and also is not significantly limiting,

it must be approximately the maximum effective concentration.

Fig. 3 shows the kinetics of the reaction in cells loaded with 130 U of uricase/liter of erythrocytes. The rate follows closely the kinetics predicted by the entry-limiting model. The presence of excess enzyme is shown by the rate of urate hydrolysis observed with the sonicated erythrocytes, which is approximately sevenfold greater than intact cells. Thus these cells contain about seven times as much enzyme as is actually necessary to achieve maximal rates. These data suggest that only about 20 U/liter of erythrocytes are necessary to achieve maximal rates of urate degradation.

Although we have no direct demonstration that enzyme is contained in 100% of the hemolyzed erythrocytes, the observed rate of degradation closely follows the calculated maximum rate. This calculation assumes that 100% of the cells contain enzyme and suggests that most of the cells do contain enzyme.

Several observations eliminate the possibility that the observed reaction is actually being catalyzed by extracellular enzyme derived from lysed cells or incomplete washing. After the cells were incubated for 25 min, as in the experiment shown in Fig. 2, the cells were centrifuged and removed, and the incubation was continued with the supernatant fraction. Degradation of uric acid ceases when the cells are removed, indicating that the enzyme was removed with the erythrocytes. When labeled uric acid was added to the supernate *after* the cells were removed by centrifugation, none of the added uric acid was degraded. We conclude therefore that all the enzyme was intracellular (or at least bound to the cells), that the cells did not lyse during the incubation, and that uric acid hydrolysis occurred within the cell.

To eliminate the remote possibility that the enzyme is adsorbed to the surface of the cell, rather than being intracellular, we have used hypoxanthine to inhibit the rate of uptake of uric acid into the cell. We showed previously that 5 mM hypoxanthine decreases the rate of uric acid transport about sevenfold (Table I). However, this concentration also inhibits uricase activity about twofold (data not shown). Uricase is not inhibited by 2 mM hypoxanthine (Fig. 4), but the rate of transport is decreased to only one-half the rate obtained in the absence of hypoxanthine. Fig. 4 shows that 2 mM hypoxanthine decreases the rate of reaction by enzyme-loaded erythrocytes to about one-half the value observed in the absence of hypoxanthine, a value consistent with the decrease in the rate of entry of uric acid. This result indicates that the enzyme is probably not bound to the surface of the cell but rather that the reaction actually proceeds intracellularly, catalyzed by intracellular enzyme.

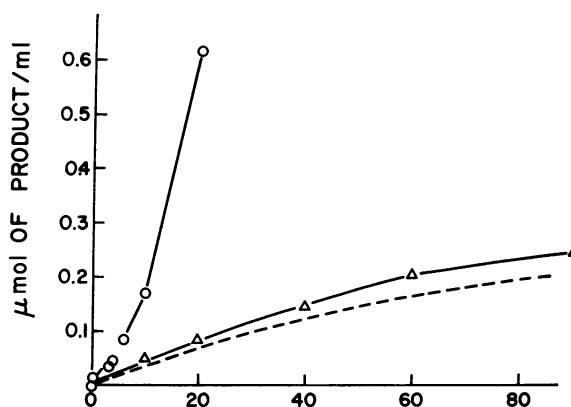


FIGURE 3 Kinetics of cells loaded with excess enzyme. 0.2 ml of washed packed red cells were hemolyzed with 0.8 ml of distilled water containing 0.42 U of enzyme. The loaded cells have an activity of 19 U/liter. The enzyme released by sonication was assayed at a 1:5 dilution relative to the loaded cells due to the large amount of enzyme present. The data are presented as an undiluted mixture for direct comparison with the hemolyzed cells. The cells contained 130 U of enzyme/liter.  $\Delta$ — $\Delta$ , kinetics of intracellular enzyme;  $\circ$ — $\circ$ , kinetics of free enzyme released from cells by sonication; - - - - , maximum rate of reaction at infinite enzyme concentration (Eq. 4). The abscissa represents time in minutes.

We have not examined in detail the rate of transport of the product across the membrane. However, the rate must be relatively rapid since most of the product remains in the supernate when the cells are removed (Fig. 1 and other data not shown). The removal of some of the product with the erythrocytes, however, is also consistent with the idea that the reaction proceeds within the cell.

Fig. 2 shows that the sonicate of the erythrocytes was slightly less active than the enzyme within the erythrocytes. However, in most other experiments we have found the sonicate to be slightly more active (120-140%) than intracellular enzyme. We believe that uricase activity may be inhibited slightly within erythrocytes. When increasing amounts of erythrocyte sonicate are added to a constant amount of extracellular enzyme, the relative rate of the reaction decreases somewhat (Fig. 5). Possibly we should extrapolate this curve to 100% hematocrit, and suppose that the intracellular enzyme is only about 50% as active as extracellular enzyme in the absence of erythrocyte extract. We do not know the reason for this inhibition or whether this extrapolation is appropriate, but in any case, the inhibition is relatively small and can be easily overcome by adding slightly more enzyme.

In control experiments we have shown that plasma does not inhibit the reaction relative to the rate observed

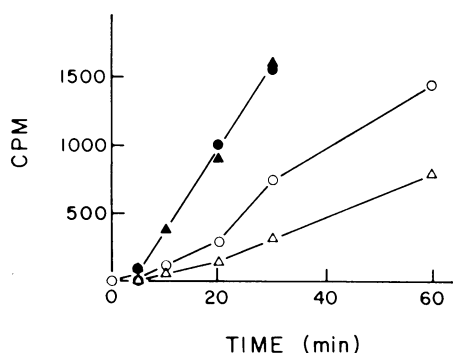


FIGURE 4 Effect of hypoxanthine on reaction rate. 1 ml of washed packed cells was hemolyzed with 3 ml of distilled water containing 0.15 U of enzymes. The uric acid concentration used was  $3 \times 10^{-5}$  M ( $9 \times 10^5$  cpm/ $\mu$ mol). Similar results were obtained when the nonradioactive uric acid was omitted entirely.  $\circ$ — $\circ$ , intracellular enzyme, no hypoxanthine;  $\triangle$ — $\triangle$ , intracellular enzyme, 2 mM hypoxanthine;  $\bullet$ — $\bullet$ , sonicated cells, no hypoxanthine;  $\blacktriangle$ — $\blacktriangle$ , sonicated cells, 2 mM hypoxanthine.

in buffers, demonstrating that the plasma does not contain an inhibitor of uricase (data not shown). We have assayed plasma and erythrocytes (normal and hemolyzed, sonicated and unsonicated) for endogenous uricase activity and found none. Thus, the enzyme activities discussed above are those of added enzyme and do not represent endogenous activity.

*Ion transport and glycolysis in hemolyzed cells.* Hemolyzed erythrocytes are known to transport sodium and potassium via the ouabain-sensitive, ATP-requiring transport system (10). Our enzyme-loaded erythrocytes also transport sodium normally. In these experiments,  $^{22}\text{Na}$  (with or without variable amounts of nonradioactive sodium) was introduced into the cell at the time of hemolysis. Subsequent exit of the trapped sodium was determined by centrifugation of the cells and determination of the amount of radioisotope in the supernate. Efflux is time-dependent and about 80% of the sodium efflux can be prevented by the addition of ouabain (Table II).

To lend further support to the idea that enzyme-loaded cells transport sodium, enzyme-loaded cells were fractionated on linear Ficoll sucrose density gradients and the enzyme levels and the sodium transport were determined on the various fractions. Most of the enzyme activity was found in the area of 10% Ficoll and near the top of the 65-40% sucrose gradient. The cells at these densities also transported sodium (data not shown). This, of course, does not prove that the enzyme-containing cells transport sodium, but it is consistent with this idea.

The energy for ion transport by hemolyzed cells can be derived either from ATP itself (introduced during

hemolysis) or from inosine (10). If enzyme-loaded cells are to survive *in vivo*, however, they must be able to use glucose as the energy source and have an intact glycolytic pathway. Schrier (11) has shown that in extensively hemolyzed erythrocyte ghosts, the level of all the glycolytic enzymes is extremely low. However, we would expect that if the cells are kept concentrated during hemolysis by the addition, for example, of only 4 vol water to 1 vol cells, a substantial residual level ( $\geq 20\%$  of normal) of the glycolytic enzymes should remain in the cells. These cells might retain sufficient capacity for glycolysis despite the lower levels of these enzymes. Even if they do not retain such a capacity, it should be possible to restore enzyme levels by using as the hemolysis medium a dialyzed extract of sonicated or freeze-thawed cells, which should contain the appropriate proportions of each enzyme in the same concentrations as they normally occur in the cell. As an alternative, glycolysis might be enhanced by simply including hexokinase in the hemolysis medium, since hexokinase is the rate-limiting enzyme under ordinary circumstances.

We have examined lactate production by hemolyzed cells and their capacity to transport  $^{22}\text{Na}$  by using glucose as energy source. The data in Table III shows that ghosts are capable of transporting intracellular sodium at comparable rates, with either inosine or glucose as the energy source. The data in Table IV shows that ghosts are somewhat deficient in lactate production relative to normal cells, synthesizing about 40% as much lactate as normal cells from glucose. Hemolyzed cells do utilize inosine more effectively than glucose, since they generate about three times as much lactate from inosine as from glucose. Supplementation of the ghosts with hexokinase in the hemolysis medium results in a modest increase in the rate of lactate production from glucose.

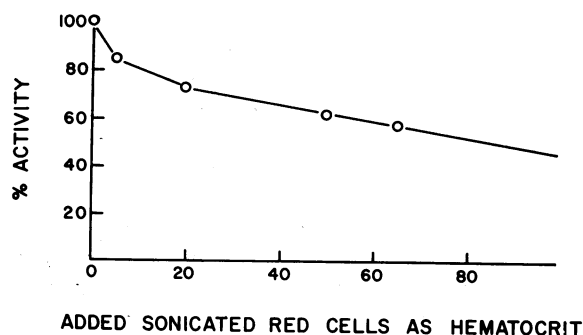


FIGURE 5 Effect of red cell sonicate on uricase. Uricase (0.005 U) was assayed in a volume of 0.2 ml and 0.3 mM uric acid for 30 min. Sonicated red cells were added in increasing amounts to different reaction mixtures. The amount added is expressed as the hematocrit that would be present had the cells not been sonicated.

TABLE II  
Sodium Transport by Hemolysed Cells:  
Time Course of <sup>22</sup>Na Efflux

Time	Without ouabain		With ouabain	
	min	cpm	cpm	%
20	705	6.6	209	2.0
30	1,161	10.7	391	3.8
60	2,071	19.2	555	5.4
90	2,768	25.6	811	7.9

Cells were loaded with <sup>22</sup>Na during hemolysis and the rate of <sup>22</sup>Na efflux was determined. The results are expressed as percent of the initial intracellular radioactivity.

Addition of adenine and guanine also has a stimulatory effect on lactate production.

These results demonstrate that the ghosts are capable of substantial lactate synthesis and ATP generation. However, the actual values should be taken only as preliminary results, because the cells contain unknown amounts of adenine and pyridine nucleotides, the work load of the cell is undefined, and the actual residual levels of the enzymes have not been determined.

#### DISCUSSION

In these experiments we have characterized some of the *in vitro* properties of enzyme-loaded erythrocytes. An important parameter is the rate at which the substrate enters the erythrocytes. As we have shown here and previously (2), the concentration of the enzyme in the erythrocyte can be made arbitrarily high. The final concentration of enzyme is limited only by the solubility of the enzyme, since at the time of loading the intracellular and extracellular substances are approximately equilibrated. If the enzyme concentration is high enough that any substrate molecules entering the cell are immediately degraded, then the rate-limiting step becomes the rate of substrate entry.

A direct comparison of the entry and exit of uric acid into hemolysed and normal erythrocytes shows that the rate of entry and exit are the same for both kind of cells. Hemolysis has apparently not inactivated the carrier protein for uric acid, nor has it created large holes in the membrane through which uric acid or other molecules might directly pass. The kinetic constants for entry and exit are identical, as in fact they should be if the concentrations of uric acid outside and inside (in the aqueous compartment of the cell) are equal.

Transport of uric acid into hemolyzed cells is inhibited by hypoxanthine, shown to be a competitive inhibitor of uric acid transport (8). We interpret this to indicate that uric acid is transported into hemolyzed cells by the same mechanism responsible for transport

TABLE III  
Sodium Transport by Hemolysed Cells: Effect of Inosine,  
Glucose, and Hexokinase on <sup>22</sup>Na Efflux

Inosine	Glucose	Hexokinase	Without	With
			ouabain	ouabain
			%	%
+	+	-	41 (32)	15 (15)
+	+	+	42 (32)	22 (20)
-	+	-	33 (38)	18 (18)
-	+	+	40 (40)	21 (22)
+	-	-	40	20
+	-	+	41	19
-	-	-	23	18
-	-	+	25	24

Cells were loaded with <sup>22</sup>Na during hemolysis and the rate of <sup>22</sup>Na efflux was determined. The results are expressed as percent of the initial intracellular radioactivity. Only the results for the 90-min time point are given. The numbers in parentheses refer to a separate experiment in which <sup>22</sup>Na efflux from hemolysed cells is high and not increased by hexokinase.

into normal erythrocytes. This conclusion is supported by other experiments that show that the rates of uptake at various temperatures are the same for both kinds of cells.

When the intracellular uricase concentration is limiting, the rate of reaction is comparable to the rate observed with extracellular enzyme. Thus, encapsulated uricase seems to function normally. While this might not be true of every enzyme, it should be possible in many cases to find from some appropriate source a variant of the desired enzyme that is stable and active within erythrocytes. When excess enzyme is present within the erythrocyte, the reaction rate is equal to the rate of entry. Uric acid is degraded intracellularly, since the reaction terminates when the erythrocytes are re-

TABLE IV  
Lactate Production by Hemolysed Cells: Effect of  
Adenine, Guanine, and Glucose

Cells	Substrate	Hexokinase	Adenine and guanine	Lactate production
				%
Normal	Glucose	-	-	100
Normal	Inosine	-	-	130
Normal	Glucose	-	+	133
Normal	-	-	-	25
Hemolysed	Glucose	-	-	42
Hemolysed	Glucose	+	-	58
Hemolysed	Glucose	-	+	69
Hemolysed	Inosine	-	-	125
Hemolysed	-	-	-	2

Lactate production is expressed as percent of that observed with normal cells with glucose (1.78 mmol lactate/liter cells per h).

moved by centrifugation and since hypoxanthine inhibits both the rate of entry and the rate of reaction to the same extent.

Uricase-loaded erythrocytes are potentially capable of removing as much uric acid as the human kidney. When loaded with an excess of enzyme, the rate of transport and the rate of degradation of uric acid are both about 21  $\mu$ mol uric acid/liter per min at a serum urate concentration of 0.3 mM (5 mg/100 ml). At higher or lower serum urate concentrations, the rate would be proportionately higher or lower. The average daily production of uric acid is about 4.4 mmol/day (12, 13), so that it would require about 150 ml of enzyme-loaded erythrocytes to degrade this amount of uric acid.

It is important to note that enzyme-loaded erythrocytes can be useful only if the rate of enzymatic reaction is sufficiently rapid to achieve the desired effect. This in turn depends largely on the serum substrate concentration and the kinetic constant for transport. It can readily be calculated, from known values for the concentrations and kinetic constants, that some substrates cannot be degraded rapidly enough to obtain a significant lowering of serum concentrations.

Enzyme-loaded erythrocytes are capable of ion transport and glycolysis, both probably necessary for in vivo survival. Ion transport is nearly normal, but the capacity for glycolysis is somewhat reduced. We assume this is due either to a decrease in the levels of intracellular glycolytic enzymes or to depletion of adenine or pyridine nucleotides. If so, it should be relatively easy to restore normal rates of glycolysis by supplementing the erythrocytes with these nucleotides and enzymes. The simplest way to ensure normal concentrations of enzymes would be to use a dialyzed extract of freeze-thawed or sonicated erythrocytes as the hypotonic hemolysis medium. Nucleotides could be added as desired. We are only beginning to explore the question of cell survival, but our preliminary results in birds indicate survival times up to 70% of normal.

If the remaining problems can be solved, erythrocytes loaded with any of several dozen enzymes or proteins could play a useful therapeutic role in a number of different diseases. One such possibility, closely related

to uric acid metabolism, would be the use of human erythrocyte hypoxanthine-guanine phosphoribosyl transferase (HGPRT) for human HGPRTase deficiency.

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