# Conversion of Ammonia or Urea into Essential Amino Acids, L-Leucine, L-Valine, and L-Isoleucine Using Artificial Cells Containing an Immobilized Multienzyme System and Dextran-NAD$^+$

## L-Lactic Dehydrogenase for Coenzyme Recycling

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Received August 10, 1989; Accepted October 3, 1989

## ABSTRACT

A multienzyme system consisting of leucine dehydrogenase (EC 1.4.1.9), L-lactic dehydrogenase (EC 1.1.1.27), urease (EC 3.5.1.5), and dextran-NAD$^+$ was microencapsulated within artificial cells. This system could convert ammonia and urea into essential amino acids, L-leucine, L-valine, and L-isoleucine. L-lactate acted as a cosubstrate for the regeneration of dextran-NADH. Greater concentrations of L-lactate favored the higher conversion ratios. The effects of ammonium salts and urea on reaction rate were also studied. The relative reaction rates in ammonium salts solutions were 44.6–78.8% of those in urea solutions. More than 90% of the original activity was retained when artificial cells were kept at 4°C for 6 wk.

**Index Entries:** Artificial cells; microencapsulation; immobilization; multienzyme system; leucine dehydrogenase; L-lactic dehydrogenase; coenzyme recycling; dextran-NAD$^+$; essential amino acids; L-leucine; L-valine; L-isoleucine; ammonia; urea.

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## INTRODUCTION

Most metabolic functions are carried out in cells by complex multienzyme systems with coenzyme requirements. Multienzyme systems with coenzyme recycling immobilized by microencapsulation can carry out some of these functions (*1,2*). Since 1977 microencapsulated multienzyme systems with coenzyme recycling have been studied for the conversion of urea and ammonia into L-amino acids. Artificial cells containing urease, L-glutamic dehydrogenase, and glucose-6-phosphate dehydrogenase converted urea into ammonia, which joined with α-ketoglutarate in the presence of NADPH to form L-glutamic acid (*3*). Glucose-6-phosphate acted as a cosubstrate for the regeneration of NADPH. To allow for the use of D-glucose instead of glucose-6-phosphate, another system consisting of urease, L-glutamic dehydrogenase, and glucose dehydrogenase was used (*4*). This way, D-glucose functioned as an energy source to recycle the coenzyme (*4*). Optimization of this system has allowed the use of D-glucose at concentrations normally existing in the blood (*5*). Further development of this approach was to convert L-glutamate formed from urea or ammonia into L-alanine (*6*). In these systems, a very low external concentration of coenzymes was required for continuous reaction. However, it would be much more desirable to retain coenzymes within the artificial cells. One approach would be to use lipid-polymer membrane (*7, 8*). Multienzyme systems and coenzyme retained within lipid-polymer membrane artificial cells could continuously catalyze permeant external substrates (*7,8*). Another approach would be to use polymer membrane articifial cells to microencapsulate multienzyme systems together with a coenzyme linked to soluble macromolecules, such as dextran, polyethylenimine, or proteins (*9–12*). In this case, artificial cells containing urease, L-glutamic dehydrogenase, alcohol dehydrogenase, and dextran-$NAD^+$ could continuously convert urea into L-glutamic acid with simultaneous dextran-NADH regeneration (*13*).

Although these are good experimental model systems, L-glutamic acid and L-alanine are nonessential amino acids. It would be much more useful to convert waste metabolites like ammonia and urea into essential amino acids, which the body cannot synthesize. A multienzyme system consisting of urease, leucine dehydrogenase, glucose dehydrogenase, and dextran-$NAD^+$ has been microencapsulated in artificial cells. This system could effectively convert ammonia or urea into essential amino acids, L-leucine, L-valine, and L-isoleucine. In this case, D-glucose acted as a cosubstrate for the regeneration of dextran-NADH (*14*). The use of alcohol dehydrogenase instead of glucose dehydrogenase to regenerate dextran-NADH was also investigated (*15*). The commercial availability and high specific activity of L-lactic dehydrogenase make it attractive for use in multienzyme systems (*16,17*). The L-lactic dehydrogenase regeneration system also has possible applications in vivo, because L-lactate exists

within the body and could function as a cosubstrate for coenzyme regeneration. Therefore, the present report describes the use of L-lactic dehydrogenase for the regeneration of dextran-NADH. A multienzyme system consisting of leucine dehydrogenase, L-lactic dehydrogenase, urease, and dextran-$NAD^+$ was used to convert ammonia and urea into L-leucine, L-valine, and L-isoleucine.

## MATERIALS AND METHODS

### Enzymes and Coenzyme

L-Lactic dehydrogenase (1400 U/mg protein) from rabbit muscle, leucine dehydrogenase (97 U/mg protein) from Bacillus species, glucose dehydrogenase (26 U/g solid) from calf liver, alcohol dehydrogenase (340 U/mg protein) from yeast, and $NAD^+$ (grade III) from yeast were purchased from Sigma Chemical Co. Urease (49 U/mg protein) was obtained from Millipore Co.

### Substrates and Chemicals

α-ketoisocaproate, α-ketoisovalerate, DL-α-keto-β-methyl-*n*-valerate, L-(+)-lactic acid, ADP (grade I), and polyethylenimine (50% aqueous solution) came from Sigma Chemical Co.; ammonium acetate from J. T. Baker Chemical Co.; urea from Fisher Scientific Co.; dextran T-70 (M.W. 70,000) from Pharmacia Co.; 1,6-hexanediamine from Eastman Kodak Co.; terephthaloyl chloride from ICN Pharmaceuticals, Inc.; and Tween 20 and Span 85 from Atlas Powder Co. All other solvents and chemicals were of the highest available analytical grade.

### Preparation of Water Soluble Dextran-$NAD^+$

$NAD^+$-$N^6$-[*N*-(6-aminohexyl)-acetamide] was prepared as described (*18,19*). This derivative was covalently linked to soluble dextran T-70 molecules using the described procedures (*10,20*).

### Preparation of Semipermeable Nylon-Polyethylenimine Artificial Cells

Microencapsulation was carried out using the updated procedures (*2,11*). In a typical preparation, 25 U of leucine dehydrogenase, 8200 U of L-lactic dehydrogenase, 20 mg of albumin, and 25 U of urease were dissolved in 0.3 mL of phosphate buffer (0.1 *M*, pH 8.0) in a 30-mL beaker kept in an ice bath. Then 0.2 mL of dextran-$NAD^+$ (2 μmol of $NAD^+$ linked to 35–45 mg of dextran T-70) was added. The resulting solution was mixed with 0.35 mL of the (0.4 *M*) 1,6-hexanediamine and (10%) polyethylenimine solution. Five mL of the organic solvent (chloroform/cyclo-

hexane, 1:4) containing 2.5% Span 85 was added. The aqueous phase was emulsified in the organic phase for 1 min using a magnetic stirrer at 1600 rpm. Then, 5 mL of the organic solvent with saturated terephthaloyl chloride was added. Interfacial polymerization occurred while stirring. After 3 min, the polymerization was terminated by adding 10 mL of the organic solvent. The artificial cells formed were washed with a 50% Tween 20 aqeuous solution. Approximately 1.5 mL of artificial cells were made with a mean diameter of 90 μm. The resulting artificial cells were resuspended in the same phosphate buffer and stored at 4°C. Artificial cells containing glucose dehydrogenase multienzyme system or alcohol dehydrogenase multienzyme system were also prepared by the same way (*14,15*).

### Conversion and Analysis

For bath conversion, 0.2 mL of artificial cells was mixed with 0.8 mL of the corresponding substrate solutions. The final reaction solution contained 20 m*M* ammonia (or 20 m*M* urea), 20 m*M* α-ketoisocaproate (or α-ketoisovalerate, or DL-α-keto-β-methyl-*n*-valerate), 200 m*M* L-lactate, 5 m*M* KCl, and 0.1 m*M* ADP. The artificial cells were kept in suspension during reaction by shaking in a Lab-Line Orbit Environ-Shaker at 140 rpm and 30°C. The concentrations of resulting L-leucine, L-valine, and L-isoleucine were measured by High-Performance Liquid Chromatography (HPLC).

## RESULTS AND DISCUSSION

Figure 1 shows the schematic representation of conversion of ammonia or urea into L-leucine, L-valine, and L-isoleucine in artificial cells. Artificial cells retain leucine dehydrogenase (LEUDH), L-lactic dehydrogenase (LDH), urease, and dextran-$NAD^+$. Ammonia (or urea) and the α-keto acids diffusing into the artificial cells are converted into L-leucine, L-valine, and L-isoleucine. L-lactate acts as a cosubstrate for the regeneration of dextran-NADH.

### Conversion of Ammonia of Urea into L-Leucine, L-Valine and L-Isoleucine

Figures 2–4 show the results of batch studies in 20 m*M* ammonium acetate or 20 m*M* urea substrate solutions. In 20 m*M* ammonium acetate solutions, 0.2 mL of artificial cells could produce 4.92 μmol of L-leucine, 8.72 μmol of L-valine, or 6.18 μmol of L-isoleucine, respectively, within 8 h. The production ratios were 24.6, 43.6, and 30.9%. In 20 m*M* urea substrate solutions, 13.24 μmol of L-leucine, 14.92 μmol of L-valine, or 12.54 μmol of L-isoleucine were produced. The corresponding production ratios were 66.2, 74.6, and 62.7% The conversion ratios in urea solutions were much higher than those in ammonium acetate solutions. These results

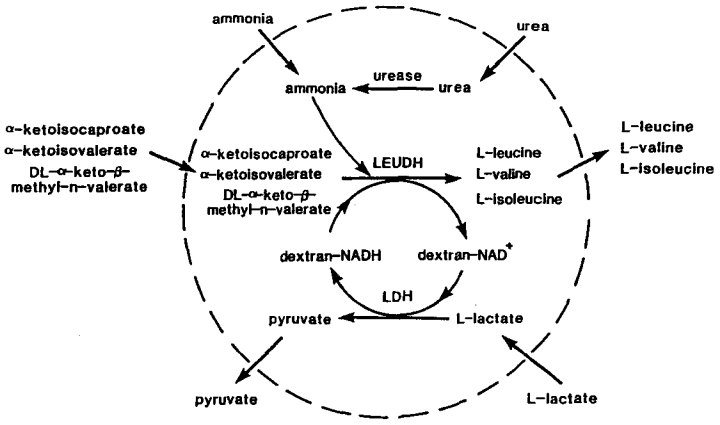


Fig. 1. Schematic representation of an artificial cell containing a multienzyme system of L-lactic dehydrogenase, leucine dehydrogenase, urease, and dextran-$NAD^+$ for the continuous conversion of ammonia or urea into L-leucine, L-valine, and L-isoleucine.

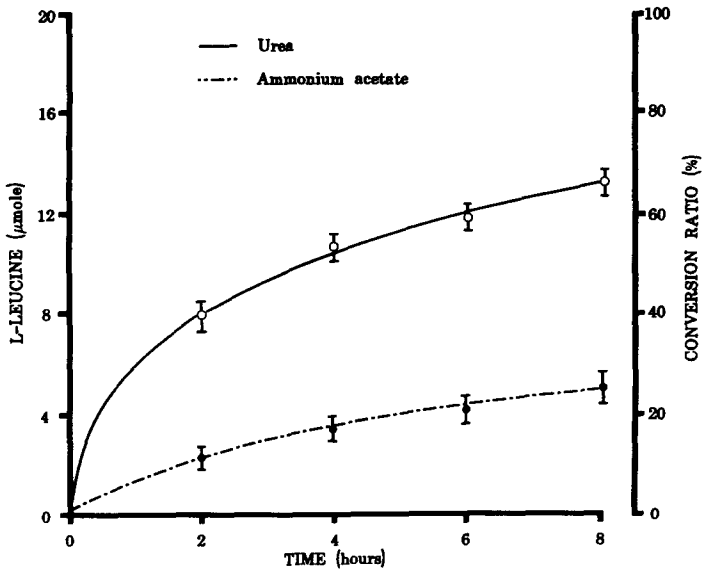


Fig. 2. Time course of conversion of α-ketoisocaproate into L-leucine with ammonium acetate or urea as ammonium sources.

were consistent with those obtained by other multienzyme systems using glucose dehydrogenase or alcohol dehydrogenase. This is mainly the result of pH change in urea substrate solutions and has been explained in detail in previous studies (*15*). Of the 3 α-keto acids, the conversion ratios of α-ketoisovalerate were the highest in both the ammonium acetate and the urea substrate solutions. This is because of the substrate specificity of leucine dehydrogenase (*15*).

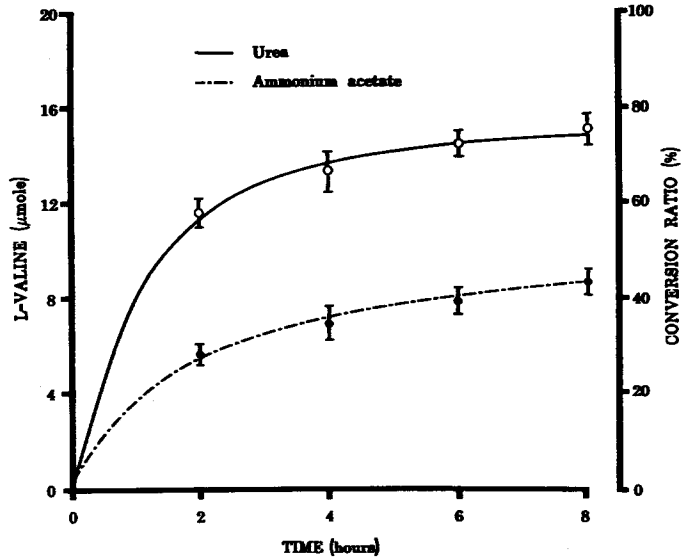


Fig. 3. Time course of conversion of α-ketoisovalerate into L-valine with ammonium acetate or urea as ammonium sources.

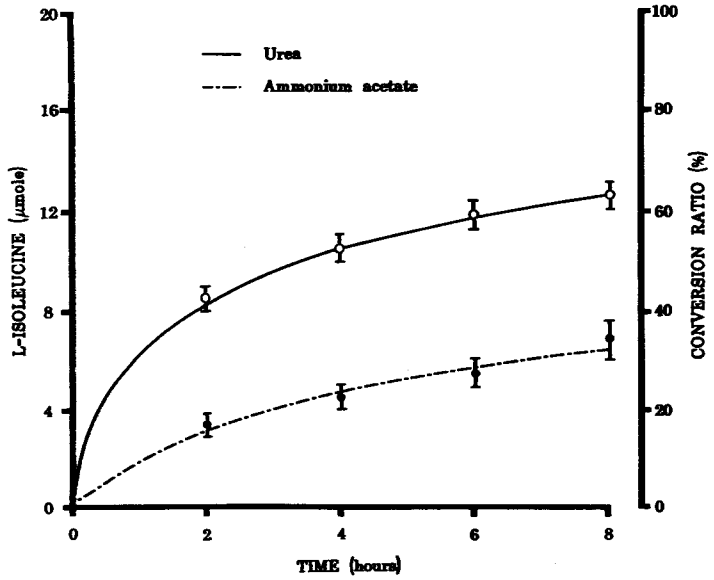


Fig. 4. Time course of conversion of DL-α-keto-β-methyl-*n*-valerate into L-isoleucine with ammonium acetate or urea as ammonium sources.

## Effects of Ammonium Salts and Urea on Reaction Rate

In this study, the same equivalent ammonium concentration (20 m*M*) was used in both the urea and the ammonium salts solutions. Conversion reactions were carried out for 2 h using 0.2 mL of artificial cells. As shown in Table 1, the relative reaction rates in the ammonium salts solutions

Table 1
Comparison of Production Rate with Different Ammonium Sources

| Product* | Urea (10 mM) | $NH_4Cl$ (20 mM) | $CH_3COONH_4$ (20 mM) | $(NH_4)_2SO_4$ (10 mM) | $(NH_4)_2CO_3$ (10 mM) |
|---|---|---|---|---|---|
| L-leucine | 3.91±0.60 | 2.23±0.33 | 2.20±0.34 | 2.26±0.44 | 2.84±0.41 |
| L-valine | 8.57±0.69 | 5.50±0.41 | 5.45±0.44 | 5.56±0.46 | 6.75±0.49 |
| L-isoleucine | 7.20±0.70 | 3.24±0.24 | 3.21±0.31 | 3.36±0.31 | 4.31±0.33 |

*Products with the unit of μmol.

were 44.6–78.8% of those in the urea solutions. The difference was mainly the result of the change of pH in the reaction solutions. In the urea substrate solutions, urea was converted into ammonia by urease, and the pH of the solutions rose from 8.0 to 8.6 within 20 min. While in the ammonium salts solutions, there was no significant difference in pH during the reaction. Leucine dehydrogenase multienzyme system had a optimum pH of 8.5–9.0 for the reductive amination (*15*). The increase in pH range during the conversion of urea into ammonia was close to the optimum pH range for the reductive amination. This resulted in higher reaction rates in urea solutions when compared to ammonium salts solutions.

## Effects of L-Lactate Concentration on Amination Reaction

The effects of L-lactate concentrations on the amination reaction were also studied. With the regeneration system consisting of L-lactate and L-lactic dehydrogenase (LDH) (*21*):

$$\text{L-Lactate} + \text{NAD}^+ \xleftrightarrow{\text{LDH}} \text{pyruvate} + \text{NADH}$$

$$\frac{[\text{pyruvate}][\text{NADH}]}{[\text{L-lactate}][\text{NAD}^+]} = 2.76 \times 10^{-6} \text{ (pH 7.0, P.B., 25°C)}$$

Higher concentrations of L-lactate favor the regeneration of dextran-NADH. In multienzyme systems with dextran-$NAD^+$ recycling, the rate of formation of L-amino acids is dependent on the regeneration rate of dextran-NADH (*13*). Thus, increase in L-lactate concentrations (30–200 m*M*) increased the conversion ratios (Fig. 5). The greater concentrations of L-lactate, the higher conversion ratios.

A 15 m*M* urea substrate solution was used to correspond to the systemic urea level in renal failure. Urea at this concentration was converted into L-leucine, L-valine, and L-isoleucine in the presence of 30 m*M* α-keto-acids and 30 m*M* L-lactate. The low concentration of L-lactate resulted in lower conversion ratios. However, 0.2 mL of artificial cells could still produce 8.01 μmol of L-leucine, 9.53 μmol of L-valine, or 7.68 μmol of L-isoleucine, respectively, within 8 h. The production ratios of the 3 L-amino acids were 25.6–31.8% (Table 2).

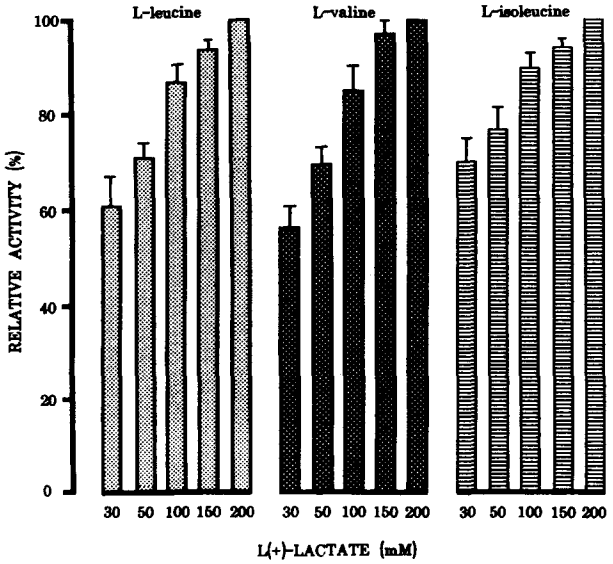


Fig. 5. Effects of L-lactate concentrations on conversion ratio (100% for 200 m*M* L-lactate).

Table 2
Production of L-Amino Acids in 30.0 m*M* L-Lactate Solutions

| Product ($\mu$mol) | 2 hours | 4 hours | 6 hours | 8 hours |
|---|---|---|---|---|
| L-leucine | 5.10±0.33 | 6.52±0.37 | 7.34±0.32 | 8.01±0.16 |
| L-valine | 6.43±0.38 | 8.00±0.23 | 8.92±0.20 | 9.53±0.17 |
| L-isoleucine | 4.54±0.31 | 6.06±0.25 | 6.88±0.38 | 7.68±0.28 |

## Storage Stability

The leucine dehydrogenase and L-lactic dehydrogenase multienzyme system encapsulated within artificial cells showed good storage stability. These artificial cells kept in 0.1 *M* phosphate buffer (pH 8.0) at 4°C retained more than 90% of the original activity after 6 wk (Fig. 6). This multienzyme system was much more stable than the 2 other multienzyme systems. At 4°C in the glucose dehydrogenase and leucine dehydrogenase system, 65.5% of the original activity was retained after 5 wk. In the yeast alcohol dehydrogenase and leucine dehydrogenase system, 72.0% of the original activity was retained when artificial cells were kept at 4°C for 6 wk. The only difference among the 3 multienzyme systems was the type of the regeneration enzymes used in the artificial cells. Therefore, the different storage stabilities among these multienzyme systems were attributed mainly to different storage stabilities of the regeneration enzymes in the multienzyme systems.

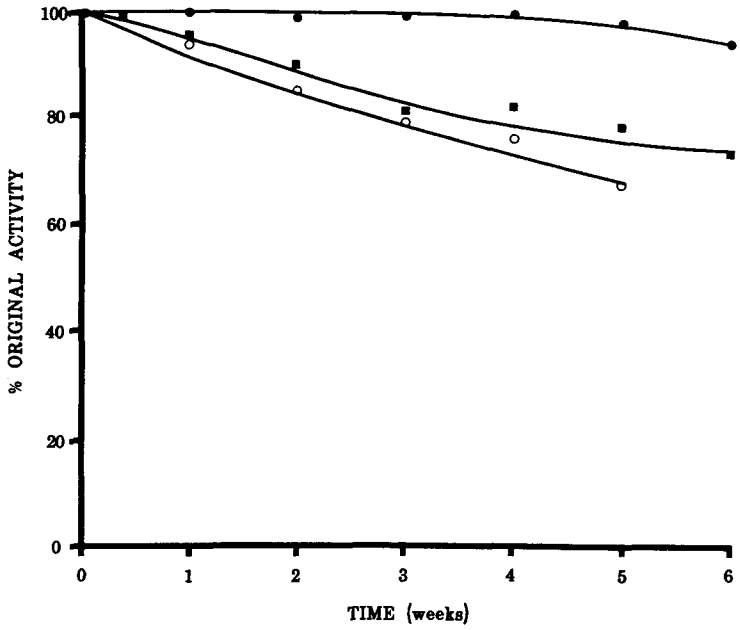


Fig. 6. Storage stability at 4°C of leucine dehydrogenase multienzyme systems within artificial cells. (●---●), L-lactic dehydrogenase and leucine dehydrogenase multienzyme system; (○---○), glucose dehydrogenase and leucine dehydrogenase multienzyme system; (■---■), alcohol dehydrogenase and leucine dehydrogenase multienzyme system.

## ACKNOWLEDGMENTS

The support of the MRC term grant (MT 9100) and the MESST Virage "Centre of Excellence in Biotechnology" grant to TMSC is gratefully acknowledged. The technical assistance of Colin Lister is much appreciated.

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