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Lipid-Polyamide–Polyethyleneimine Microcapsules for Immobilization of Free Cofactors and Multienzyme System

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ABSTRACT

Lipid-polyamide membrane microcapsules containing multienzyme system convert ammonia into glutamate. L-Glutamic dehydrogenase, alcohol dehydrogenase, NAD^+, and alpha-ketoglutarate retained within the microcapsules do not leak out. This way the multienzyme system can recycle the free cofactor NAD^+ and synthesize glutamate from external lipophilic ammonia diffusing into the microcapsules. By using polyethyleneimine instead of hemoglobin as microcapsule filler it is possible to obtain higher conversion rates and to increase the storage stability of the recycling activity.

Index Entries: Ammonia removal; glutamic acid formation; lipid-polyamide microcapsules; immobilized multienzyme system; cofactor recycling; artificial cells.

INTRODUCTION

The feasibility of using microencapsulated multienzyme systems for the conversion of urea and ammonia into amino acids has been demonstrated (*1*). The earlier systems are not able to retain the cofactor within the microcapsules (*2,3*). The NAD^+ linked to polyethyleneimine or dextran (*4,5*) can be retained and recycled inside collodion and polyamide microcapsules (*6–8*). Lipid-polyamide microcapsules, containing L-glutamic dehydrogenase, alcohol dehydrogenase, NAD^+, and alpha-

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ketoglutarate can convert ammonia into glutamate (*8,9*). These microcapsules with lipid-polyamide membranes can retain free NAD^+ and the substrate alpha-ketoglutarate, in addition to the multienzyme system. In this way, external lipophilic ammonia diffusing into the microcapsules is converted into glutamate. The NAD^+ is recycled into NADH in the microcapsules by alcohol dehydrogenase in the presence of ethanol.

In these studies (*9,10*), hemoglobin alone or hemoglobin together with polyethyleneimine was used as filler for the microcapsules. Hemoglobin preparations contain enzymes that deactivate NAD^+ (*8*). Purification of hemoglobin removes this contaminant (*8*). However, this is a slow and laborious process. To solve this problem, we have now successfully used polyethyleneimine as filler for this multienzyme system.

MATERIALS AND METHODS

Materials

Enzymes

Alcohol dehydrogenase (EC 1.1.1.1) from yeast (400 U/mg) was obtained from Boehringer Mannheim, Canada. L-Glutamic dehydrogenase (EC 1.4.1.3) from bovine liver, type III (36 U/mg), was obtained from Sigma Chemical Co., St. Louis, MO.

Substrates and Cofactors

Grade III NADH and NAD^+ from yeast were obtained from Sigma Chemical Co. Alpha-Ketoglutarate was obtained from Boehringer Mannheim, Canada.

Chemicals

Chemicals used were as follows: ADP (disodium salt) (Boehringer Mannheim); polyethyleneimine, 50% solution (ICN K&K Inc.); 1,6-hexanediamine (Fisher Scientific Co., Fair Lawn, NJ); terephthaloyl chloride (ICN K&K Inc.); cholesterol (Fisher Scientific Co.); egg lecithin (Nutritional Biochemical Corp., Cleveland, OH); HPLC grade acetonitrile (Fisher Scientific Co.); ethanethiol (Pierce Chemical Co.); and orthophthalaldehyde (Pierce Chemical Co., Rockford, IL). Other reagents and solvents were of the highest available analytical grades.

Methods

Preparation of Lipid-Polyamide Microcapsules Containing Multienzyme System, NAD^+, and alpha-Ketoglutarate

The microcapsules were prepared according to the procedure of Yu and Chang (*10*), with the following modifications. The major modification was the omission of hemoglobin as microcapsule filler. For a typical microcapsule preparation, the following procedure was used:

1. An organic solvent of 200 mL was prepared by mixing 40 mL of chloroform with 160 mL of cyclohexane, to give a chloroform:cyclohexane (1:4) solution; 1 mL of Span 85 was added to the organic solvent before use.
2. Terephthaloyl chloride, 220 mg, was added to 60 mL of the organic solvent. The mixture was stirred in an ice bath for 4 h, using a magnetic stirrer, and then filtered through Whatman #1 paper.
3. Lipid–organic liquid: 0.35 g lecithin and 0.43 g cholesterol were added to 50 mL of tetradecane and stirred for 4 h at room temperature.
4. Diamine solution: 0.378 g of sodium bicarbonate and 0.464 g of 1,6-hexadiamine were dissolved in 5 mL of distilled water. The pH of the solution was adjusted to 9.0 with 6*N* HCl, and 2 mL of 50% polyethyleneimine were added. The solution was mixed and the pH was readjusted to 9.0. The final volume of 10 mL was achieved by adding distilled water.
5. A solution of the materials to be encapsulated was prepared by dissolving 12.5 mg L-glutamic dehydrogenase, 6.25 mg alcohol dehydrogenase, 1.0 mg ADP, 60 mg alpha-ketoglutarate, 2.5 mg $MgCl_2 \cdot 6H_2O$, 1.0 mg KCl, and 0.74 mg NAD^+ in 2.5 mL of Tris-HCl buffer, pH 8 (0.01*M* in Tris) (low NAD^+ microcapsules with cofactor recycling). In some encapsulations, no NAD^+ was used (control microcapsules). In others, no alcohol dehydrogenase was added (low NAD^+ microcapsules with no cofactor recycling). In still another type, 40.43 mg of NADH were used instead of NAD^+, and the alcohol dehydrogenase was omitted (high NADH microcapsules with no cofactor recycling).
6. 2.5 mL of the above enzyme solution and 2.5 mL of diamine solution were mixed in a 150-mL beaker, kept in an ice bath, using a jumbo magnetic stirrer (Fisher Scientific Co.), with a 4-cm stirring bar and speed setting of 4 (~1000 rpm). After 10 s, 25 mL of cold organic solvent were added and the stirring continued for another 60 s. Then, 25 mL of cold (4°C) terephthaloyl chloride solution were added, and the reaction was allowed to proceed for another 3 min at the same stirring speed. After the microcapsules had settled (about 2 min), the supernatant was discarded and another 25 mL of cold terephthaloyl chloride solution were added. The stirring was continued for another 3 min at a speed setting of 1 (~100 rpm). After the settling of the microcapsules, the supernatant was discarded again. The stirring bar was removed and 50 mL of cold organic solvent were added. The suspension was gently mixed with a glass rod, allowed to settle, and the supernatant siphoned off. The washing with 50 mL of or-

ganic solvent was repeated, and the microcapsules were rinsed twice with 10 mL of the lipid–organic liquid. Finally, 10 mL of the lipid–organic liquid were added and the suspension was slowly rotated for 30 min at 4°C on a multipurpose rotator. After this stage, the supernatant was decanted and the resultant lipid-polyamide membrane microcapsules were stored at 4°C until used.

A typical batch of microcapsules was 5.0 mL in vol and 4.8 g in weight.

Assay of Ammonia Conversion Into Glutamate: Rate of Decrease of Ammonia Concentration

In each experiment, 2.4 g of lipid-polyamide membrane microcapsules were added to 5.0 mL of ammonia–ethanol substrate solution in a 10-mL glass-stoppered flask. The substrate solution contained ammonium acetate (20 m*M*), ethanol (200 m*M*), and Tris (5 m*M*), with the pH adjusted to 8.8 with HCl. The reaction was carried out in a rotary shaker (Lab-Line Orbit Environ Shaker 18) at 30°C and 140 rpm. As control, 7.5 mL of substrate solution were shaken in another 10-mL flask at the same time and under the same conditions. At 15, 30, 60, 90, 120, 150, and 180 min after starting the reaction, 75-μL aliquots of the reaction mixture were taken, put in 0.5-mL closed, conical, plastic tubes, and centrifuged for 3 min at 10,000*g*. Samples of 50 μL of the clear supernatant were diluted 5.0 times with water and immediately assayed for ammonia content with a Technicon autoanalyzer.

The following test was used to test for leakage: 200-μL samples of the reaction mixture were taken 30 min after starting the reaction. After centrifugation, 50 μL of the supernatant were diluted and immediately assayed for ammonia concentration, as usual. The rest of the supernatant was put in a 0.5-mL, closed, conical, plastic tube and placed in the rotary shaker. The ammonia concentrations were then analyzed at different intervals. Leakage of enzymes, cofactor, and substrate occurred during the first 30 min of the reaction would be shown by a continuing decrease of ammonia in the supernatant.

Assay of Ammonia Conversion Into Glutamate by Direct Measurements of Glutamic Acid Formed Inside the Microcapsules

In each experiment, six 2.4-g portions of freshly prepared microcapsules were added to 5.0-mL batches of ammonia–ethanol substrate solution in 10-mL glass-stoppered flasks. The substrate contained ammonium acetate (20 m*M*), ethanol (200 m*M*), and Tris (5 m*M*), with the final pH adjusted to 8.8 with HCl. Each flash was shaken for a specific period of time, ranging from 15 to 180 min, in a rotary shaker at 30°C and 140 rpm. In some control experiments, microcapsules with no alpha-ketoglutarate were used. In other control experiments, the substrate solution did not contain any ammonium acetate. Immediately after

shaking for the specific period of time, each batch of microcapsules was filtered through Whatman #1 filter paper, washed with 5 mL of water, and homogenized in a 2-mL glass homogenizer. The filtering, washing, and homogenizing took about 5 min. The homogenates were transferred into small test tubes and heated for 2 min in boiling water to deactivate the enzymes and stop the glutamic acid production. Immediately after cooling, the homogenates were centrifuged for 15 min at 25,000*g*. The relatively clear aqueous phases were transferred into 2-mL test tubes and cooled for 5 min in an ice bath. Then, all six samples were centrifuged together for another 15 min at 25,000*g*. the supernatant was filtered through Ultipor 0.45-μm filters. Aliquots (0.1 mL) of the filtrates were diluted 20.0 times with water, vortexed for 3 s, and passed through Centriflo CF 50A (Amicon Corp., Boston, MA) cones for 15 min at 800*g*.

The determination of glutamic acid in the resulting ultrafiltrates was carried out by high-performance liquid chromatography (HPLC), based on Hill et al. (*11*).

RESULTS AND DISCUSSION

Conversion of Ammonia Into Glutamate by Four Different Types of Lipid-Polyamide Membrane Microcapsules

The conversion of ammonia into glutamate is shown schematically in Fig. 1. The rate of this conversion by four different types of lipid-polyamide membrane microcapsules was investigated by measuring the decrease in substrate ammonia concentrations. The results are shown in Fig. 2.

1. When 2.5 mL of control microcapsules with no NAD^+ were added to 5.0 mL of ammonia–alcohol substrate solution (pH 8.8), the ammonia concentration decreased to 16.5 m*M*. This corresponds to the equilibration of lipophilic ammonia into the lipid-polymer membrane microcapsules along a concentration gradient.
2. When 2.5 mL of microcapsules, containing 0.50 μmol NAD^+ and no alcohol dehydrogenase, were added to 5.0 mL of substrate solution, the change in ammonia concentrations was not significantly different from the control microcapsules. This result showed that, without recycling, the 0.50 μmol NAD^+ in the microcapsules did not induce any significant conversion of ammonia to glutamate (Fig. 2).
3. 2.5 mL of microcapsules containing 25.0 μmol NADH and no alcohol dehydrogenase were used. When these microcapsules were added to 5.0 mL of substrate solution, the ammonia concentration fell rapidly during the first 60 min and then leveled off at 11.5 m*M* (Fig. 2). The change of ammonia con-

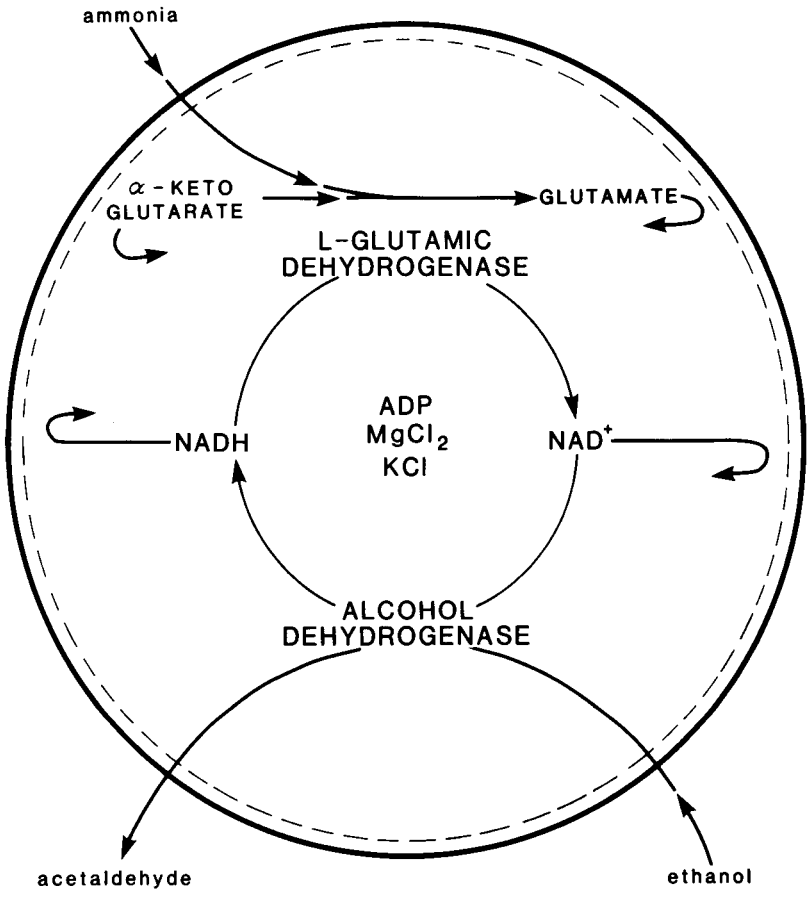

Fig. 1. Schematic representation of lipid-polyamide membrane microcapsules: Dotted line, polyamide component of membrane; Solid line, lipid component of membrane. L-glutamic dehydrogenase, alcohol dehydrogenase, NAD^+, NADH, and alpha-ketoglutarate are all retained within the microcapsules. External ammonia and ethanol entering the microcapsules are converted into, respectively, glutamate and acetaldehyde.

centration in the 5.0 mL substrate solution, from 20 to 11.5 m*M*, indicated that ammonia entering the microcapsules had been converted into glutamate.

4. When 2.5 mL of microcapsules containing 0.50 μmol NAD^+ and both enzymes were added to 5.0 mL of substrate solution, there was a continuous decrease in substrate ammonia concentration (Fig. 2). Ammonia was converted into glutamate within the first 3 h of the reaction.

The leakage test described earlier showed that there was no leakage of enzymes and cofactor. Other tests in which enzymes were added to the supernatant showed that there was no leakage of cofactors or alpha-ketoglutarate.

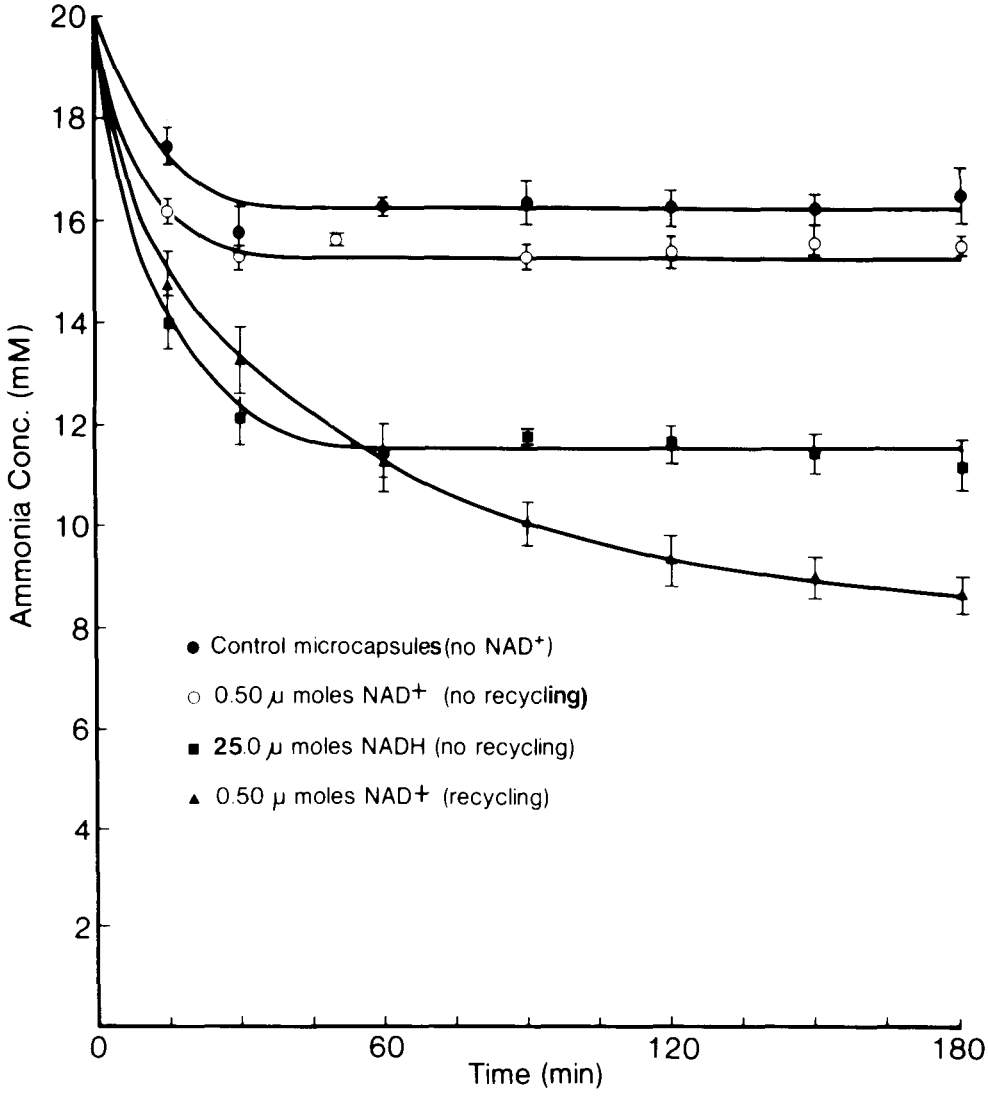

Fig. 2. Changes in ammonia concentrations after the addition of different types of lipid-polyamide membrane microcapsules.

Assay of Ammonia Conversion Into Glutamate by Measurement of Glutamic Acid Formation Inside the Microcapsules

When "low NAD^+ microcapsules with recycling" were used, the amount of glutamic acid produced after 90 min was 31.5 μmol (Fig. 3). In a control experiment using the same microcapsules, but with no ammonium acetate in the substrate solution, no glutamic acid was detected in the microcapsules after 90 min. Therefore, the ammonia necessary for glutamic acid production came from the ammonia outside the artificial cells. The actual amounts of ammonia converted into glutamic acid can be calculated from Fig. 3. The results corresponded to the actual amounts

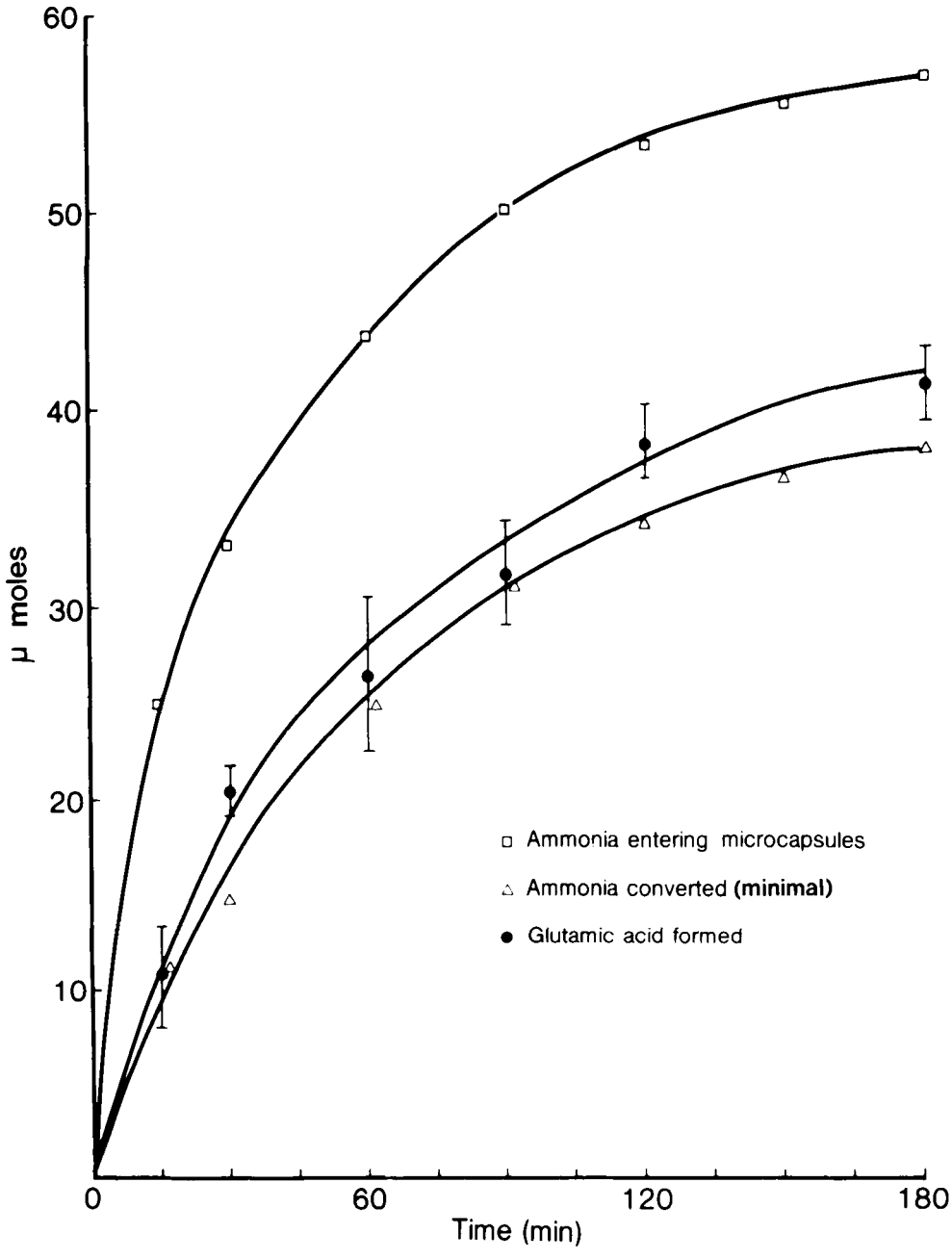

Fig. 3. Glutamic acid formed in the microcapsules compared to the amount of ammonia that has entered the microcapsules.

of glutamic acid formed inside the microcapsules at different time intervals during the reaction.

The result in Fig. 2 showed that 2.5 mL of "low NAD^+ microcapsules with recycling" converted 40 μmol of ammonia into glutamate within the first 3 h of the reaction. Thus, the 0.50 μmol of NAD^+ retained in the 2.5-mL microcapsules could be recycled 80 times to convert 40 μmol of ammonia.

Storage Stability

The storage stability of 2.5 mL of "low NAD^+ microcapsules with recycling" is shown in Fig. 4. At intervals throughout a 21-d storage period (4°C), 2.5-mL, aliquots of microcapsules were tested. The amounts of ammonia converted into glutamic acid after 3 h of reaction were as follows: 40 μmol after 1 d storage; 32.5 μmol after 7 d of storage, 22.5 μmol after 16 d of storage; and 15 μmol after 21 d of storage. Taking the activity of the freshly prepared (1 d) microcapsules in converting ammonia to glutamate during the first 3 h of the reaction as 100%, 81% of this activity was retained after 7 d, 56% after 16, and 37% was still retained after 21 d.

From these results, it is demonstrated that the activity of the multienzyme system in ammonia removal is high, even after 2–3 wk of

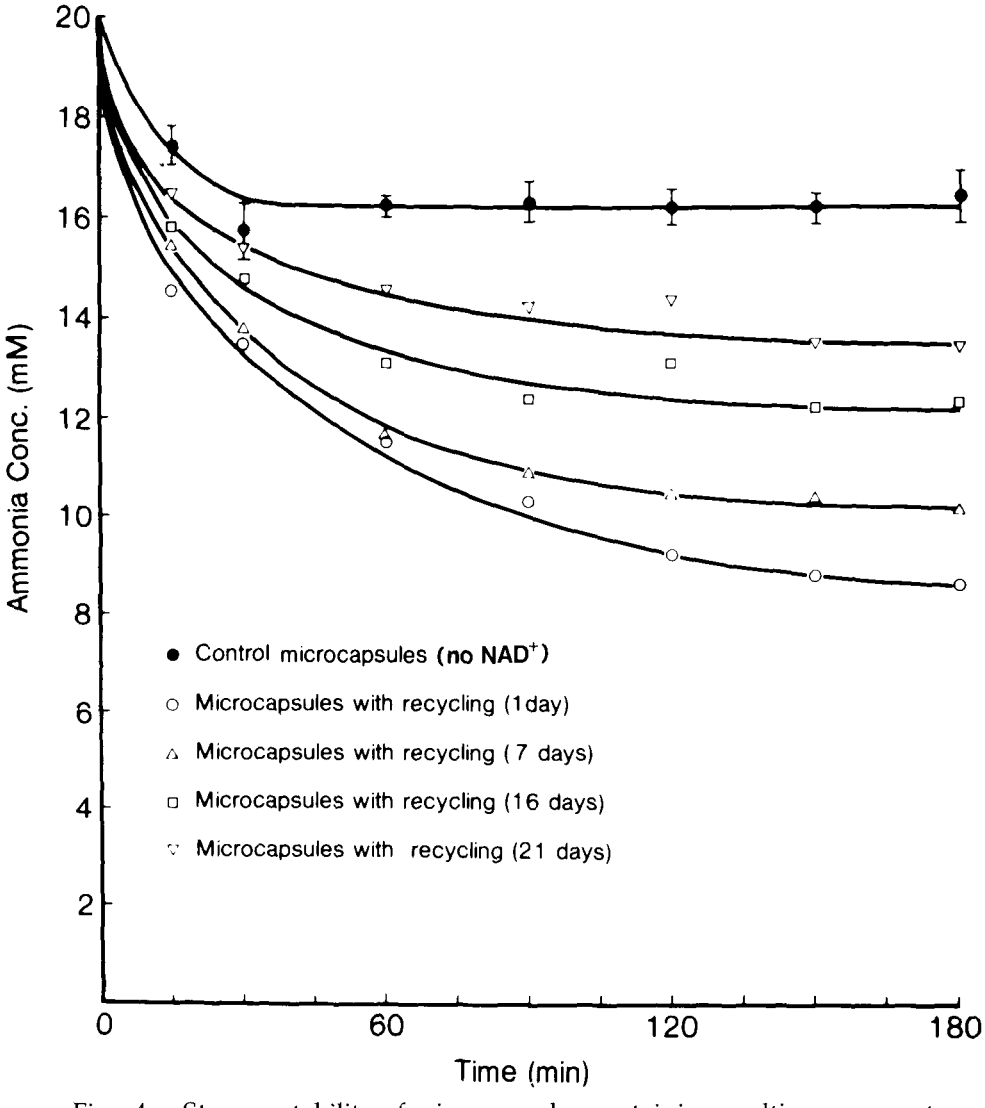

Fig. 4. Storage stability of microcapsules containing multienzyme system NAD^+ and alpha-ketoglutarate.

storage of the microcapsules. This improvement will contribute to the applications of a microencapsulated multienzyme system and cofactors in biotechnology and medicine (*12,13*).

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