FORMATION OF AMINO ACID FROM UREA AND AMMONIA BY SEQUENTIAL ENZYME REACTION USING A MICROENCAPSULATED MULTI-ENZYME SYSTEM

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SUMMARY

A microencapsulated multi-enzyme system has been used for the conversion of urea and ammonia into an amino acid, glutamate. The microencapsulated multi-enzyme system contains urease (E.C.3.5.1.5), glutamate dehydrogenase (E.C.1.4.1.3), and glucose-6-phosphate dehydrogenase (E.C.1.1.1.49). The conversion of urea into glutamate is achieved by the sequential reaction of urease and glutamate dehydrogenase; while glutamate dehydrogenase and glucose -6-phosphate dehydrogenase allow for the cyclic regeneration of $NADP^+$:NADPH required for the reaction. The rate of production of glutamate is 1.3 µmole per min per ml of microcapsules. The encapsulated multi-enzyme system thus allows for the sequential enzyme reaction for the conversion of urea and ammonia into an amino acid.

INTRODUCTION

Enzymes have been immobilized by adsorption, covalent-linkage, gel-entrapment, and microencapsulation (1). The use of these different types of immobilized enzymes in basic and applied research has been extensively reviewed (2-9). Of particular basic research interest are studies of immobilized multi-enzyme systems for sequential reaction (5,10). Microencapsulation of enzymes (11) allows for the immobilization of an unlimited number of enzyme systems in high concentrations (4,6). Microencapsulated multi-enzyme systems have been used for the cyclic regeneration of cofactors (12,13). We now report studies using a microencapsulated multi-enzyme system for a complex sequential reaction in the conversion of urea or ammonia into an amino acid, glutamate. The cofactor requirement in this reaction is fulfilled by the simultaneous microencapsulation of a cofactor regenerating enzyme system.

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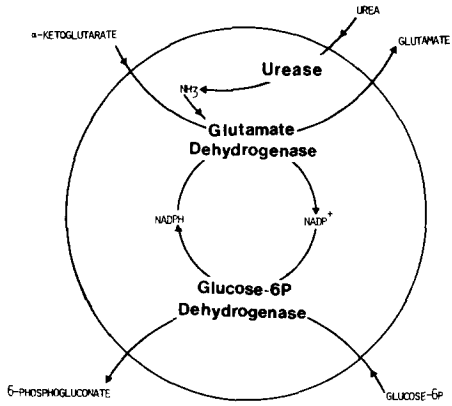

Figure 1

The schematic representation for the conversion of urea into an amino acid. Urease converts urea into ammonia which is then incorporated to α-ketoglutarate by glutamate dehydrogenase. This is coupled to the recycling of $NADP^+$:NADPH by glucose-6-phosphate dehydrogenase which converts glucose-6-P into 6-phosphogluconate in the recycling process.

MATERIALS AND METHODS

Enzymes and Substrates

The following enzymes are used: glucose-6-phosphate dehydrogenase (E.C.1.1.49) from yeast (Sigma Chemical Co., 385 units/mg); glutamate dehydrogenase (E.C.1.4.1.3) from bovine liver (Sigma Chemical Co., 50 units/mg); 6-phosphogluconate dehydrogenase (E.C.1.1.1.44) from yeast (Sigma Chemical Co., 54 units/mg); and urease (E.C.3.5.1.5) from jack bean (Worthington Biochemical Co., 60 units/mg). Other chemicals included: glucose-6-phosphate (monosodium salt, Sigma Chemical Co.); α-ketoglutaric acid (Sigma Chemical Co.); 6-phosphogluconic acid (trisodium salt, Sigma Chemical Co.); $NADP^+$ from yeast (sodium salt, Sigma Chemical Co.); type I NADPH (Sigma Chemical Co.); grade I ADP from equine muscle (Sigma Chemical Co.); and urea (Baker Chemical Co.).

Preparation of Semipermeable Microcapsules Containing Urease, Glutamate Dehydrogenase, Glucose-6-Phosphate Dehydrogenase

Urease (10 mg), glutamate dehydrogenase (8 mg), and glucose-6-phosphate dehydrogenase (2 mg) are dissolved in 1.5 ml of 7% hemoglobin solution. This is then microencapsulated using the procedure of interfacial polymerization (4,6,14) with modifications (15). The contents of the microcapsules are then cross-linked with a bi-functional reagent, glutaraldehyde, according to a procedure previously described by Chang (16) and modified as follows: The microcapsules suspended in a final volume of 15 ml in 0.1M phosphate, pH 8.0, containing 15 mg $NADP^+$ are reacted with 30 µl of 50% (W/W) glutaraldehyde at 4°C for 45 minutes and the reaction stopped by the addition of 5 volumes of 0.1M phosphate, pH 8.0. The microcapsules are collected by centrifugation,

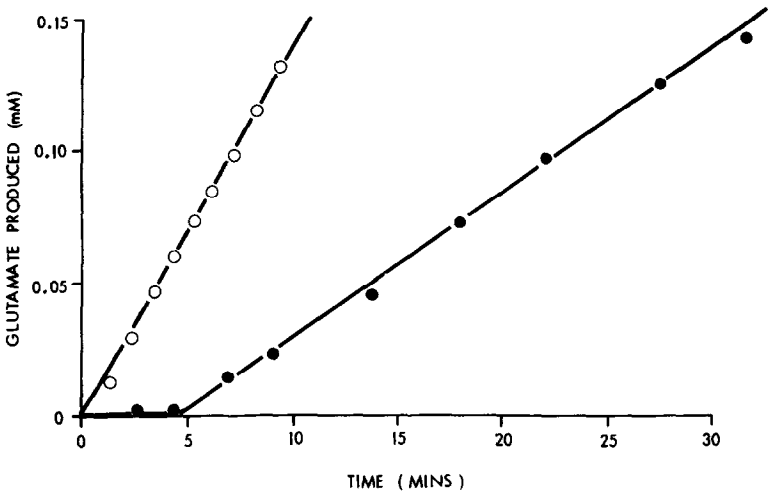

Figure 2

The production of glutamate. The production of glutamate is determined by the incubation of 100 µl microcapsules at 37°C in 3.0 ml of 0.1M Na^+-K^+ phosphate, pH 7.5, containing 0.1mM ADP; 4mM α-ketoglutarate; 0.21mM NADPH; 40mM ammonium acetate (○) or 10mM urea (●). Aliquots of the suspension were withdrawn with time and the change of the adsorbance of the filtrate at 340nm recorded. (●—●) urease acting in sequence with glutamate dehydrogenase. (○—○) glutamate dehydrogenase activity alone.

washed three times with 0.1M phosphate, pH 7.5, suspended in the same buffer (2.3 ml of packed microcapsules in 10.0 ml) and stored at 4°C.

Measurement of Activity for Each Individual Enzyme Inside the Microcapsules

The activity of individual enzymes inside the microcapsules is determined by the incubation of 100 µl of suspended microcapsules in 3.0 ml of 0.1M phosphate, pH 7.5, containing appropriate substrates and cofactors. Glucose-6-phosphate dehydrogenase activity is measured in the presence of 5mM glucose-6-P and 0.21mM $NADP^+$. Glutamate dehydrogenase activity is measured in the presence of 40mM ammonium acetate, 4mM α-ketoglutarate, 0.1mM ADP and 0.21mM NADPH. The activity of urease is measured in the coupled system with glutamate dehydrogenase by the addition of 10mM urea instead of ammonium acetate. The reaction is performed in a Lab-Line Orbit Environ-Shaker 18 at 37°C and 200 RPM. At predetermined intervals, aliquots are taken and filtered through a Millipore filter to separate the microcapsules from the reaction mixture. The absorbance of the filtrate is measured on a DBGT Beckman Spectrophotometer at 340nm.

Conversion of Urea into Glutamate by the Sequential Reaction of the Microencapsulated Multi-Enzyme System Including Cofactor Recycling (Figure 1)

The conversion of urea into glutamate by the sequential reaction of urease, glutamate dehydrogenase, and glucose-6-phosphate dehydrogenase is

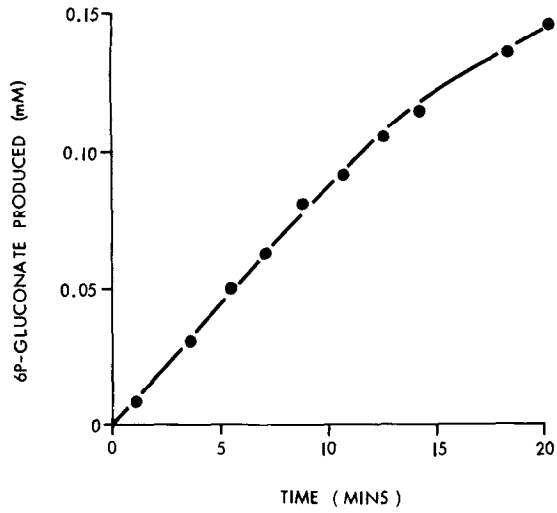

Figure 3

The production of 6-phosphogluconate. The 6-phosphogluconate formed is determined from the increase of the absorption at 340nm for 100 µl microcapsules incubated at 37°C in 3.0 ml of 0.1M Na^+-K^+ phosphate, pH 7.5, containing 5mM glucose-6-P and 0.21mM $NADP^+$. (●—●) glucose-6-phosphate dehydrogenase activity alone.

studied as follows: The microcapsules containing the multi-enzyme system are suspended in 0.1M phosphate, pH 7.5, containing urea, α-ketoglutarate, ADP, and glucose-6-P, followed one minute later by the addition of the cofactor NADPH to initiate the cyclic process. Aliquots of the suspension are withdrawn at various intervals and heated at 100°C for 2 minutes. The microcapsules are then removed by Millipore filtration and the filtrate analyzed for 6-phosphogluconate.

As controls, the same procedure as described above are repeated except that the microcapsules are boiled for 2 minutes before use.

The rate for the production of glutamate is estimated from the rate of production of 6-phosphogluconate. The quantity of 6-phosphogluconate is determined as follows: After termination of the cyclic process, the filtrate (0.5 ml) is added to 1.0 ml of 0.1M phosphate, pH 7.5, containing 6-phosphogluconate dehydrogenase (0.15 units) and 2mM $NADP^+$ at 37°C. After 30 minutes of incubation, the observed increase of the absorbance at 340nm is noted and read on a standard curve.

RESULTS AND DISCUSSION

Conversion of Urea and Ammonia into Glutamate in the Presence of a High Concentration of Cofactor Without Cofactor Recycling

After an initial delay of about 4 minutes, the production of glutamate

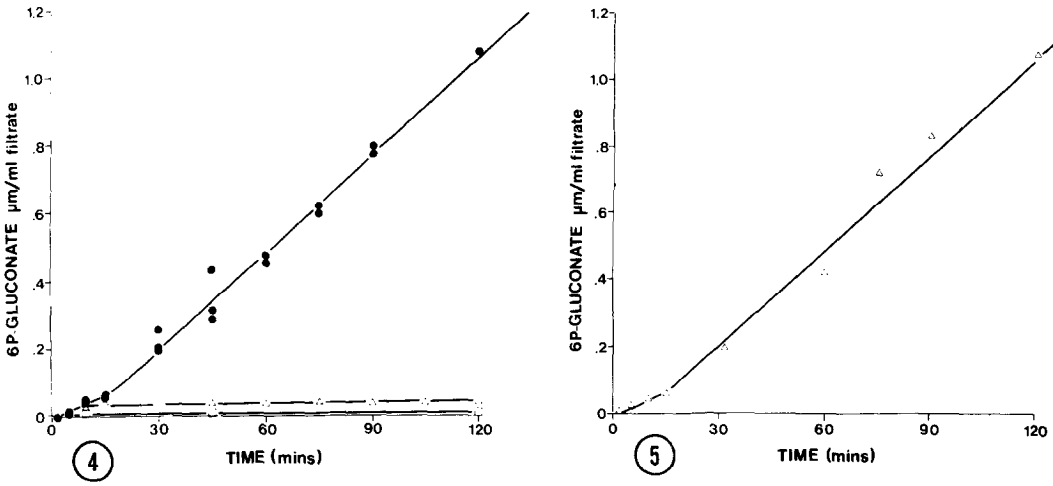

Figure 4

Sequential reaction of glutamate dehydrogenase and glucose-6-P dehydrogenase in the conversion of ammonium into glutamate. In testing the complete cycle, 0.6 ml of microcapsules are suspended in 18.0 ml of 0.1M Na^+-K^+ phosphate, pH 7.5, containing 40mM ammonium acetate; 4mM α-ketoglutarate; 5mM glucose-6-P; 0.1mM ADP; 0.042mM NADPH. At various time intervals, the amount of 6-phosphogluconate produced was determined as described in Materials and Methods, and expressed as µmole of 6-P-gluconate in 1 ml of the filtrate. (●—●) complete cycling; (□—□) recycling of cofactors blocked by the omission of glucose-6-P; (△—△) recycling blocked by the omission of α-ketoglutarate, and the use of $NADP^+$ instead of NADPH.

Figure 5

Sequential reaction of urease, glutamate dehydrogenase, and glucose-6-P dehydrogenase in the conversion of urea into glutamate. 0.6 ml of microcapsules are suspended in 18.0 ml of 0.1M Na^+-K^+ phosphate, pH 7.5, containing 10mM urea; 4mM α-ketoglutarate; 5mM glucose-6-P; 0.1mM ADP; 0.042mM NADPH. At various time intervals, the amount of 6-phosphogluconate produced is determined as described in Materials and Methods, and expressed as µmole in 1 ml of the filtrate (△—△).

from urea reaches a maximum and continues at this rate for 30 minutes (Figure 2). When ammonium acetate instead of urea is used as substrate, the activity of glutamate dehydrogenase alone can be measured and the rate for the formation of glutamate is doubled that of when urea was used (Figure 2). This higher rate in the case of ammonium acetate is most likely due to the omission of the extra urease sequence (Figure 1).

Cyclic Regeneration of Free $NADP^+$: NADPH Inside the Microcapsules

The activity of glucose-6-phosphate dehydrogenase is shown in Figure 3. Glutamate dehydrogenase and glucose-6-phosphate dehydrogenase give activities in a ratio of about 2:1 under the conditions of the assay. The schematic representation of the sequential enzyme reaction is illustrated in Figure 1. The rate of formation of 6-phosphogluconate is used to measure the cyclic regeneration of $NADP^+$:NADPH. As shown in Figure 4, 6-phosphogluconate is produced at a linear rate for 120 minutes corresponding to the formation of 1.3 μmole of 6-phosphogluconate per min per ml of microcapsules. This is equivalent to the recycling of 1.3 μmole of cofactor per min per ml of microcapsules. When glucose-6-P was omitted from the reaction mixture, the recycling of cofactors was blocked and no 6-phosphogluconate was produced. When glucose-6-P was added with $NADP^+$ in the absence of α-ketoglutarate, $NADP^+$ could not be recycled, and only a small quantity of 6-phosphogluconate was formed. No leakage of enzyme from these glutaraldehyde cross-linked microcapsules was detected throughout the two hour experiment.

Formation of Glutamate from Urea or Ammonia Coupled with the Cyclic Regeneration of a Low Concentration of Cofactor

A much lower concentration of NADPH is required when the cofactor is recycled by the sequential reaction of the microencapsulated enzymes. In this case, the rate of conversion of urea into glutamate can be followed by the rate of formation of 6-phosphogluconate (Figure 1). The results in Figure 5 indicate that 6-phosphogluconate is produced at a linear rate throughout the 120 minutes. This is equivalent to the formation of 1.3 μmole of glutamate per min per ml of microcapsules. A similar rate is observed when ammonium acetate is used instead of urea (Figure 4). Glutamate is formed at the same rate irrespective of whether urea or ammonium acetate is used as substrate. This suggests that the rate limiting step in this sequential reaction is most likely in the regeneration of the cofactor. At this low cofactor concentra-

tion, the conversion of urea and ammonia into glutamate ceases after 10 minutes if cofactor recycling is blocked (Figure 4).

This microencapsulated multi-enzyme system converts urea into glutamate at a rate of 0.65 μmole per min per ml of microcapsules. This corresponds to the conversion of 2 mg urea into glutamate per hour per ml of microcapsules. The activity ratio of individual enzymes inside the microcapsules could be further optimized to increase the rate of conversion of urea and ammonia. However, at present, this work is mainly concerned with the basic biochemical mechanism of a model microencapsulated multi-enzyme system for the conversion of urea or ammonia into amino acids. In this respect, the results in this paper have demonstrated the feasibility of using a microencapsulated multi-enzyme system consisting of urease, glutamate dehydrogenase, and glucose-6-phosphate dehydrogenase for the conversion of urea and ammonia into an amino acid, glutamate.

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REFERENCES

1. Sundarum, P.V., Pye, E.K., Chang, T.M.S., Edwards, V.H., Humphrey, A.E., Kaplan, N.O., Katchalski, E., Levin, Y., Lilly, M.D., Manecke, G., Mosbach, K., Porath, J., Weetall, H., and Wingard, W.B., Jr. (1972) Biotech. Bioeng. 14, 15-18.

2. Goldman, R., Goldstein, L., and Katchalski, E. (1971) in "Methods in Enzymology" (Mosbach, K., ed.) Vol. 44, pp. 1-78, Academic Press, New York-London.

3. Zaborsky, O. (1973) "Immobilized Enzymes", monograph, p. 175, CRC Press, Cleveland, Ohio.

4. Chang, T.M.S. (1972) "Artificial Cells", monograph, p.207, Charles C. Thomas Publisher, Springfield, Illinois.

5. Mosbach, K. (1977) "Methods in Enzymology", Vol. 44, p. 1040, Academic Press, New York-London.

6. Chang, T.M.S. (1977) "Biomedical Applications of Immobilized Enzymes and Proteins", p. 560, Plenum Publishing Corporation, New York, New York.

7. Weetall, H.H. (1975) "Immobilized Enzymes, Antigens, Antibodies, and Peptides", p. 226, Raven Press, New York, New York.

8. Salmona, M., Saronia, C., and Garattini, S. (1974). "Immobilized Enzymes", p. 226, Raven Press, New York, New York.

9. Pye, E.K. and Wingard, L.B., Jr. (1975) "Enzyme Engineering", p. 470 Plenum Press, New York, New York.

10. Mattiasson, B.O. and Mosbach, K. (1971) Biochim. Biophys. Acta 235, 253-257.

11. Chang, T.M.S. (1964) Science 146, 524-525.

12. Campbell, J. and Chang, T.M.S. (1975) Biochim. Biophys. Acta 397, 101-109.

13. Campbell, J. and Chang, T.M.S. (1976) Biochem. Biophys. Res. Commun. 69, 562-569.

14. Chang, T.M.S. (1976) in "Methods in Enzymology" (Mosbach, K., ed.) Vol. 44, pp. 201-217, Academic Press, New York-London.

15. Ostergaard, J.C.W. and Martiny, S.C. (1973) Biotech. Bioeng. 15, 561-563.

16. Chang, T.M.S. (1971) Biochem. Biophys. Res. Commun. 44, 1531-1536.