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FIBRIN MEMBRANE ENDOWED WITH BIOLOGICAL FUNCTION VII. AN APPROACH TO AN ARTIFICIAL LIVER; CONVERSION OF AMMONIA TO UREA *in vitro*

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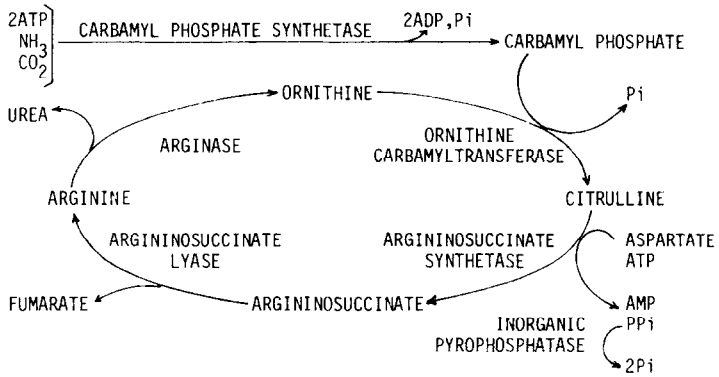
As an "artificial liver" for the conversion of ammonia to urea, a group of enzymes in ornithine cycle together with carbamyl phosphate synthetase I and inorganic pyrophosphatase were embedded in a single fibrin membrane. The immobilized enzyme system thus prepared had an ability to convert ammonia to urea not only in a buffer solution but also in human plasma.

INTRODUCTION

The final obligatory step for metabolism of nitrogeneous compounds in human body is the conversion of ammonia to urea through carbamyl phosphate and the ornithine cycle with the aid of ATP. Accumulation of ammonia in human blood is observed in patients with liver failure and hepatic coma and finally gives rise to a serious damage to the brain. The metabolic pathway of urea synthesis from ammonia is shown below;



which is mainly conducted in liver. Immobilization of these enzymes mentioned above into a matrix may make an "artificial liver" for the patients with ammoniemia. We have taken advantage of fibrinogen-fibrin conversion process to immobilize enzymes. Attempts of using fibrin membrane as a matrix for

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embedding biological materials has been carried out in our laboratory, and we have successfully immobilized living cells such as Chlorella cells and sea urchin eggs(1), as well as enzymes, such as asparaginase(2), chloroplast ATPase (3), lysozyme and ribonuclease(4) and multienzymes including uricase, catalase, allantoinase and allantoicase(5) which have an ability of degradation of uric acid to glyoxylic acid. Since this reaction proceeds under mild conditions (at neutral pH, room temperature and in aqueous solution), embedding of enzymes into fibrin polymer can be performed without imparing their functions even if the enzymes have subunit structures and are rather unstable. Another advantage of the properties of the matrix is that this may scarcely cause the excitation of immune system; 1) fibrinogen is obtained from human blood. 2) immobilized enzymes can be separated physically from immune system.

In the present paper, it was reported that conversion of ammonia to urea proceeds effectively by using a single fibrin membrane containing urea cycle system in human plasma.

MATERIALS AND METHODS

Fibrinogen(93% clottable) and thrombin(12.5 units/mg) from human blood were supplied by Green Cross Co. The fibrinogen preparation contains a small amount of blood coagulation factor XIII. The enzymes embedded in the fibrin polymer are as follows; carbamyl phosphate synthetase I(EC 6.3.4.16), ornithine carbamyl transferase(EC 2.1.3.3), argininosuccinate synthetase(EC 3.5.3.1), argininosuccinate lyase(EC 4.3.2.1), arginase(EC 3.5.3.1) and inorganic pyrophosphatase (EC 3.6.1.1). Inorganic pyrophosphatase was used to remove pyrophosphate formed by argininosuccinate synthetase, which strongly inhibits the latter enzymes(6). Carbamyl phosphate synthetase I and ornithine carbamyltransferase were partially purified from rat liver by the method of Raijman and Jones(7). Argininosuccinate synthetase and argininosuccinate lyase were also partially purified from beef liver by the methods of Rochovansky and Ratner(6) and Havir et al.(8), respectively. Arginase(55 units/mg protein) from bovine liver was purchased from Sigma Chemical Co. Inorganic pyrophosphatase(200 units/mg protein) from yeast was obtained from Boehringer Co.

The immobilized enzymes were prepared as follows; Ten milliliter of the enzyme solution in 2 mM cysteine and 50 mM $CaCl_2$ was mixed well with 10 ml of 5% fibrinogen in 0.4 M glucose and 0.1 M Na-citrate buffer(pH 6.9). Then, thrombin(40 units) was added to the mixture and the solution was poured in a flat plastic box(6 x 5.5 cm^2). After incubation of the sample solution for 2 hr at 30°C, fibrin clot was taken out from the box and pressed between filter papers(300 g/cm^2) for 12 hr at 4°C to make a thin membrane. The membrane was washed with water containing 0.5 mM dithiothreitol for 4 hr to remove free enzymes.

The amount of citrulline and urea formed was spectrophotometrically determined by the modified methods of Nakamura and Jones(9) and Schimke(10), respectively. Scince urea interferes the determination of citrulline and vise urea, it was necessary to set up simultaneous equations to get true values. The formation of other reaction products and intermediates were not able to be determined due to the lack of appropriate procedures.

RESULTS AND DISCUSSION

In a previous paper, it was found that the immobilized ornithine cycle system was functioning well in a buffer solution and had an improved efficiency over enzymes in solution(11). In that experiment we used carbamyl phosphate as starting substrate. However, to make better use of this immobilized enzymes we have included carbamyl phosphate synthetase I as well, which should enable us to convert ammonia to urea.

We have set the pH of the reaction mixture at 7.4 to 7.5, because it is the pH of human blood. As far as those enzymes of which optimal pH deviated greatly from 7.5 were concerned, we were compelled to use rather larger amounts of enzymes. The optimal pH for arginase, for example, is reported to be 9,7 (10). The reaction was carried out at 37°C with gentle stirring.

The fibrin membrane was introduced to a buffer solution(PIPES buffer pH 7.5) containing substrates and cofactors and the amounts of urea and citrulline formed were measured with time. The result is shown in Fig. 1. The amount of urea increased linerly with incubation time(curve A). On the other hand, the accumulation of citrulline, an intermediate of urea cycle, preceded that of urea. The amount of citrulline increased for the first 40 min of incubation and tended to approach a constant level(curve C). The sum of urea and citrulline formed after 2 hr was greater than the amount of ornithine introduced initially, 20 μmoles. These results indicate that urea cycle is running to synthesize urea from ammonia through citrulline, and the production and degradation of citrulline are in equilibrium after 40 min of incubation. Since two of the enzymic reaction are endothermic, production of citrulline or urea was not observed at all in the absence of ATP(curve B).

As a more practical approach to an artificial liver, a similar experiment was tried in human plasma instead of buffer solution. As is seen in Table 1, immobilized enzymes had also an ability to synthesize urea from ammonia through urea cycle in plasma. The original amount of urea and citrulline in plasma are 13.2 μmoles and 3.7 μmoles, respectively. Neither urea nor citrulline was formed without the immobilized enzymes. The potential problem of lysis of

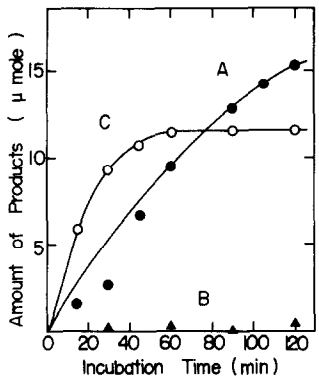

Fig. 1. Urea synthesis from ammonia through carbamyl phosphate and the ornithine cycle with immobilized enzymes.
The amount of each enzyme added to the fibrinogen solution was as follows; 16.2 units of carbamyl phosphate synthetase I and ornithine carbamyltransferase, 9.0 units of argininosuccinate synthetase, 72 units of argininosuccinate lyase, 600 units of arginase and 150 units of inorganic pyrophosphatase. One-third of the fibrin membrane prepared was used in the experiments. The reaction medium contains 50 mM NH_4HCO_3, 25 mM ATP, 10 mM N-acetyl-L-glutamate, 2 mM ornithine, 10 mM aspartate, 30 mM KCl, 20 mM $MgCl_2$ and 1 mM $MnCl_2$ in 10 ml of 20 mM PIPES buffer(pH 7.5).
Curve A, B; The amount of urea formed in the presence or absence of ATP, respectively. Curve C; The amount of citrulline accumulated.

the fibrin matrix by the fibrinolytic activity of plasma was obviated by adding approtinine(Trasylol 100 U/ml of plasma) to human plasma anticoagulated with 0.4% Na-citrate buffer.

In the present paper, we have successfully synthesized urea from ammonia with the fibrin membrane containing enzymes. As far as we know, appropriate

Table 1.

Incubation Time (hour)	Amount Produced(μmoles) Citrulline	Urea
0	0	0
1	7.9	10.0
2	7.7	15.5
3	6.9	19.7

Urea synthesis from ammonia by immobilized enzymes in human plasma. The amount of each enzyme added to the fibrinogen solution was as follows; 14.4 units of carbamyl phosphate synthetase I and ornithine carbamyltransferase, 33 units of argininosuccinate synthetase, 78 units of argininosuccinate lyase, 600 units of arginase and 150 units of inorganic pyrophosphatase. One-third of fibrin membrane prepared was introduced to the mixture of 5 ml of human plasma(0.4% Na-citrate pH 7.4) contained Trasylol(100 U/ml of plasma) and 5 ml of a solution containing substrates and cofactors.(see Fig. 1) At a given time, 0.5 ml of plasma was taken out and the amounts of urea and citrulline were determined after removing plasma proteins with 0.55 N $HClO_4$. The value shown in Table 1 are the amounts of urea and citrulline formed after incubation.

treatment to remove toxic ammonia in plasma of patients with ammoniemia has not been found. Under the circumstances, the present study was designed so as to give a clue to the development of an "artificial liver" for patients having trouble with metabolism of ammonia.

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