Phenylalanine ammonia-lyase immobilized in semipermeable microcapsules for enzyme replacement in phenylketonuria

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Phenylalanine ammonia-lyase immobilized within semipermeable microcapsules has an assayed enzyme activity which is 20% ± 4% of the enzyme in free solution The K_m for the immobilized enzyme remained the same as that of the free enzyme The pH optimum also remained unchanged at pH 8 5 ± 1 0 At the lower pH range, enzyme activity is higher for the immobilized enzyme Daily oral administration of microencapsulated phenylalanine ammonia-lyase to phenylketonuric rats decreased the systemic phenylalanine level by 35 ± 8% in 2 days ($P < 0$ 05) and by 75 ± 8% in 7 days ($P < 0$ 001)

Phenylketonuria *Artificial cell* *Microencapsulation* *Phenylalanine ammonia-lyase* *Enzyme immobilization* *Inborn errors of metabolism*

1. INTRODUCTION

Artificial cells are semipermeable microcapsules of cellular dimensions with an ultrathin (200 Å) membrane [1–3]. Enzymes immobilized within artificial cells can act effectively on permeant external substrates. Cells, antibodies, and proteolytic enzymes from the external environment are unable to come in direct contact with the microencapsulated enzymes. Artificial cells have been successfully used for experimental enzyme replacement in acatalasemia, an inborn error of metabolism [3–6]. In this case, the enzyme catalase, immobilized in artificial cells, was administered to acatalasemic rats via intraperitoneal injections or extracorporeal shunts. Intravenous injection of enzymes immobilized in liposomes has also been investigated for other types of experimental enzyme replacement therapy [7]. In both approaches the need for parenteral injections has delayed clinical applications because of the potential accumulation of the injected material. The use of extracorporeal circulation or oral administration has been used to avoid these problems [3,6]. The special case of phenylketonuria (PKU) is ideal for investigating the oral administration approach. Artificial cells can be administered orally for substrate reduction in the gastrointestinal tract. Once immobilized within the artificial cells, the enzyme is protected from proteolytic enzymes in the intestinal tract [3,6]. The artificial cells can act on phenylalanine from ingested food, as well as phenylalanine diffusing from the blood into the gastrointestinal tract. This approach has potential advantages in enzyme therapy when compared to earlier approaches in experimental enzyme therapy, based on parenteral injections or extracorporeal circulation of blood.

PKU is an inborn error of metabolism caused by a deficiency of the enzyme phenylalanine hydroxylase. This results in a deficiency in the conversion of phenylalanine to tyrosine, and in an increased level of phenylalanine and other metabolites [8,9]. The high phenylalanine levels can be reduced by the use of a low phenylalanine diet. This diet is dif-

Abbreviation. PAL, phenylalanine ammonia-lyase, EC 4 3 1 5

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ficult to follow, however, and does not prevent rises in blood phenylalanine during episodes of fever and infection [10]. A possible alternative to this diet is the use of enzyme therapy [11]. However, there are problems related to the large scale isolation and purification of the enzyme phenylalanine hydroxylase from mammalian tissues. Furthermore, this enzyme requires co-factors [12]. PAL is another enzyme which converts phenylalanine, by deamination, into *trans*-cinnamic acid. This enzyme requires no co-factors and can be readily obtained from microbial sources [13]. PAL was therefore chosen for the present study.

PAL was previously immobilized within semipermeable microcapsules by the standard method [3,6]. Previous in vitro studies carried out here have shown that microencapsulated PAL acts effectively in the conversion of phenylalanine into *trans*-cinnamic acid [14]. Microencapsulated PAL has an apparent enzyme activity which is 20 ± 4% of the activity of the enzyme in free solution. The K_m of the immobilized enzyme is not changed, whereas the V_m is decreased. In order to test the feasibility of oral administration, further in vitro studies were carried out to study the pH optimum of the immobilized enzyme at pH values corresponding to those along the gastrointestinal tract. These artificial cells were then tested in vivo on phenylketonuria-induced rats.

2. MATERIALS AND METHODS

2.1. *Materials*

PAL from microbial source was purchased from P.L. Biochemicals. Collodion, bovine hemoglobin, L-phenylalanine, *para*-chlorophenylalanine methyl ester from Sigma, St. Louis, MO, tris-(hydroxymethyl)aminomethane, sodium acetate, succinic acid, potassium phosphate from Fisher, and 150–200 g male Sprague Dawley rats from Charles River Co. Distilled-deionized water was used throughout. 28.44 mg/ml PAL were suspended in buffered 3.0 mM L-phenylalanine for assaying the enzyme activity.

2.2. *Instrumentation*

2.2.1. Spectrophotometer

A Cary UV spectrophotometer (Cary 219) with a deuterium lamp was employed to monitor the absorption of *trans*-cinnamic acid in vitro at 290 nm and phenylalanine plasma level at 290 and 315 nm [15]

2.3. *Methods*

2.3.1. PAL-loaded artificial cells

5 units of PAL (spec. act. = 0.84 U/mg) were dissolved in 2.3 ml of a 10 g/dl hemoglobin solution. This was emulsified as described [3,6], then an ultrathin cellulose nitrate membrane (200 Å thick) was formed around each microdroplet (mean diameter of 150 μm).

2.3.2. pH activity profile

The enzyme activity of artificial cells was studied using a stir-batch technique at various pH levels. The buffers used in the different pH ranges were as follows: pH 2–3 (0.1 M Tris–HCl), pH 4–5 (0.1 M acetate), pH 6.0 (0.1 M succinate), pH 7.0 (0.05 M phosphate) and pH 8–13 (0.1 M Tris–HCl). Aliquots were collected at timed intervals and levels of *trans*-cinnamic acid analyzed by a standard technique [15].

2.3.3. In vivo experiments

In vivo experiments were performed on 150 g phenylketonuric male Sprague-Dawley rats. This phenylketonuric rat model was derived using slight modifications of methods previously described [16–18]. Briefly, rats were given daily intraperitoneal injections of L-phenylalanine (100 mg/kg body wt) and DL-*p*-chlorophenylalanine methyl ester (300 mg/kg body wt). The following 3 groups of rats were studied: (i) 3 normal control rats; (ii) 10 control phenylketonuric rats were given orally 0.5 ml of control artificial cells containing hemoglobin but no enzyme, and (iii) 10 phenylketonuric rats were given 0.5 ml of artificial cells containing 5 units (3.2 mg) of PAL. In both cases, artificial cells were administered orally at 9.00 a.m. every day for 7 days. Daily blood samples, taken 6 h after the oral administration of the artificial cells, were collected from the tail vein. Blood phenylalanine levels were analysed using the established method of Shen and Abell [15] and used by workers in the field (Ambrus et al. [16]).

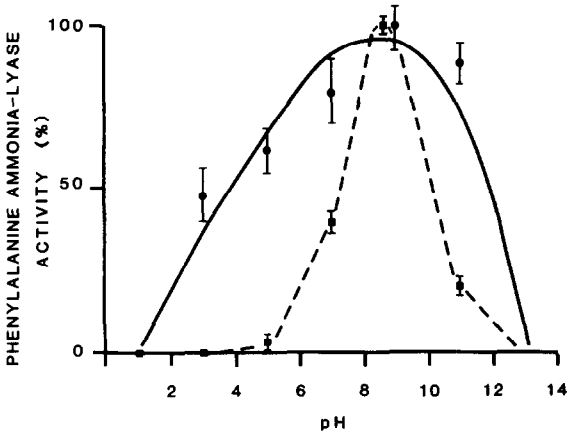

Fig 1 pH vs activity profile with all values expressed as means ± SD. Activity of free enzyme in solution (—■—), and immobilized PAL in artificial cells (—●—) at various pH values

20-fold with all values being significant to the $P < 0.001$ level. Daily oral administration of microencapsulated PAL effectively reduced the systemic blood phenylalanine level of the treated phenylketonuric rats. Compared to control PKU rats, the phenylalanine blood level was decreased to 65 ± 8% on day 2 ($P < 0.05$), and to 25 ± 8% on day 7 ($P < 0.001$) (table 1). After 7 days of this form of enzyme therapy, systemic blood phenylalanine levels of the phenylketonuric rats had been lowered to a level which was not significantly different from those of the normal rats ($P < 0.10$). Ambrus et al. [16] have reported a smaller decrease of blood phenylalanine using PAL covalently linked to an extracorporeal shunt. Furthermore, their approach involves the use of extracorporeal blood circulation.

Here, the oral administration of PAL im-

Table 1

Phenylalanine plasma levels of normal, PKU control and PKU treated rats (mg/100 ml)

Days	Normal	PKU treated	PKU control	Significance Student's *t*-test treated vs normal	Significance Student's *t*-test control vs treated
0 (initial)	16 4 ± 8.0	25 0 ± 7 0	33 0 ± 10.0	$P < 0$ 50	$P < 0$ 60
2	41.3 ± 20.6	339 7 ± 28.8	522.1 ± 70 2	$P < 0.001$	$P < 0$ 05
4	41.3 ± 28 9	251.2 ± 30.2	468 2 ± 44.0	$P < 0$ 001	$P < 0$ 001
7	33 6 ± 29.3	82 7 ± 7.0	331.4 ± 26.4	$P < 0$ 10	$P < 0$ 001

Values are means ± SE

3. RESULTS AND DISCUSSION

Results of the pH activity profile are shown in fig.1. The microencapsulated enzyme was more active than the free enzyme at lower pH values. This is probably due to the buffering capacity of hemoglobin within the artificial cells, as well as the effect of the high concentration of hemoglobin in stabilizing the enzyme. Results showed that the optimal pH for free enzyme in solution and enzyme immobilized in artificial cells were both at pH 8.5 ± 1.0 (fig.1). The pH optimum corresponds to the average pH range of the small intestine. The in vivo results indicate that the phenylalanine blood levels were significantly increased in control phenylketonuric rats compared to normal control rats. This sustained increase varied from 15- to

mobilized within artificial cells was significantly more effective in lowering systemic phenylalanine level without the need for extracorporeal blood circulation or parenteral injections.

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REFERENCES

[1] Chang, T.M.S (1964) Science 146, 524–532
[2] Chang, T.M.S , Mason, S.G. and MacIntosh, F.C (1966) Can J. Physiol. Pharm 44, 115–128

[3] Chang, T.M.S. (1972) in: Artificial Cells (Chang, T M.S. ed.) pp.1–250, Charles C Thomas, Springfield.
[4] Chang, T.M.S and Poznansky, M.J (1960) Nature 146, 243–245.
[5] Poznansky, M.J. and Chang, T M.S (1974) Biochim. Biophys. Acta 334, 103–115.
[6] Chang, T.M.S. (1977) in: Biomedical Applications of Immobilized Enzymes and Proteins (Chang, T.M S. ed.) vols I & II, pp.169–202, Plenum, New York
[7] Gregoriadis, G. (1979) in: Drug Carriers in Biology and Medicine (Gregoriadis, G. ed.) pp.50–175, Academic Press, New York
[8] Nylan, W.L. (1974) in: Heritable Disorders of Amino Acids Metabolism: Patterns of Clinical Expression and Genetic Variations (Nylan, W L. ed.) pp.109–159, John Wiley and Son.
[9] Bickel, H., Hudson, F.P. and Woolf, L.I (1971) in: Phenylketonuria (Woolf, L.I. ed.) pp.1–47, Thiere, Stuttgart
[10] Wannemacher, R W., Klainer, A.S , Dintermann, R.E and Beisel, W.R (1976) Am J Clin. Nutr 29, 997–1004.
[11] Chang, T.M.S (1984) Appl Biochem. Biotech 10, 5–23.
[12] Kaufman, S. (1983) Adv. Hum. Genet. 13, 217–297
[13] Hodgins, D S. (1971) J Biol. Chem 9, 2977–2985.
[14] Bourget, L. and Chang, T M.S (1984) Appl Biotech Biochem. 10, 57–60.
[15] Shen, R S. and Abell, C.W. (1977) Science 197, 665–667
[16] Ambrus, C.M., Ambrus, J L., Horvath, C., Pedersen, H., Sharma, S., Kant, C., Mirand, E., Guthrie, R. and Paul, T. (1978) Science 201, 837–840.
[17] Lipton, M.A , Gordon, R., Guroff, G and Udenfriend, S (1967) Science 156, 248–252
[18] Koe, B K. and Weissman, A. (1966) J Pharmacol Exp Ther. 154, 499–506.