# INFLUENCE OF PHENYLALANINE IN THE MEDIUM ON PROTEIN SYNTHESIS OF CHICKEN EMBRYO FIBROBLASTS

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## Summary

The influence of phenylalanine (Phe) in the medium on protein synthesis of chicken embryo fibroblasts (CEF) was examined. CEF was derived from 9-d-old embryos by trypsin-EDTA digestion. To examine the deficiency of Phe in the medium, CEF was cultured in Dulbecco's modified Eagle's medium (DMEM) with or without Phe. CEF was also cultured in Dulbecco's phosphate buffered saline (PBS ($Ca^{2+}$, $Mg^{2+}$)) with or without 400 $\mu$M Phe in order to examine the effect of Phe supplementation. All media were supplemented with 10% (v/v) fetal calf serum. After incubating for 6, 30 and 54 h, protein synthesis was measured by the incorporation of L-[2, 6-$^3$H] Phe into CEF for further 18 h. Protein synthesis of CEF cultured in DMEM was higher than that in PBS ($Ca^{2+}$, $Mg^{2+}$). High specific radioactivity of Phe due to the low concentration of Phe in the medium resulted in the apparent increase in protein synthesis of CEF. Protein synthesis cultured in PBS ($Ca^{2+}$, $Mg^{2+}$) with Phe did not increase during 72 h of cell culture.
(**Key Words** : Protein Synthesis, Phenylalanine, Chicken Embryo Fibroblast)

## Introduction

One of the most important factors which affect the rate of protein synthesis in the whole-body and various tissues is the alteration in nutritional conditions. When dietary protein intakes were decreased from the requirement level to protein deficiency, the rate of protein synthesis in the whole-body (Kita et al., 1993; Muramatsu et al., 1987) and livers of young chickens (Kita et al., 1996) decreased, and excess protein intakes also decreased whole-body and liver protein synthesis (Kita et al., 1989, 1996; Kita and Okumura, 1993).

Kino and Okumura (1986) reported that single amino acid deprivation reduced food intake, body weight and nitrogen balance of chicks. However, the influence of single amino acid deprivation in the medium on protein synthesis of chicken cells cultured *in vitro* has not been clarified. In the present study, therefore, we examined the influence of phenylalanine (Phe) in the medium on protein synthesis of chicken embryo fibroblasts (CEF).

## Materials and Methods

### Cell culture of CEF

Ten fertilized eggs from single comb White Leghorn hens maintained in our laboratory were incubated for 9 days. At this time six embryos were taken from eggs, decapitated, rinsed in Dulbecco's phosphate buffered saline (PBS {$Ca^{2+}$, $Mg^{2+}$)} including 0.25 mg/ml Fungizone (GIBCO LABORATORIES Life Technologies Inc., NY, U.S.A.) and 50 $\mu$g/ml gentamycin (Wako Pure Chemical Industries, Ltd., Osaka, Japan). After removing wings and feet, embryos were eviscerated, rinsed in PBS ($Ca^{2+}$, $Mg^{2+}$) including Fungizone and gentamycin and then minced finely with scissors. The minced tissues were mixed with 15 ml of phosphate buffered saline (PBS) including 0.25% (w/v) trypsin (TRYPSIN 1:250, DIFCO LABORATORIES, MI, U.S.A.) and 0.025% (w/v) EDTA, and incubated at 37℃ for 15 min. After centrifuge (500 × g, 1 min), the supernatant was taken and mixed with 15 ml of Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Company, MO, U.S.A.) including 10% (w/v) tryptose phosphate broth (TPB, DIFCO LABORATORIES, MI, U.S.A.) and 5% (v/v) fetal calf serum (FCS, GIBCO LABORATORIES Life Technologies Inc., NY, U.S.A.). The cells were settled down by centrifuge for 5 min at 1,000 × g, and then the supernatant was removed. After rinsing cell pellet with DMEM including 10% TPB and 5% FCS, the cells were

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resuspended in 20 ml of DMEM with 10% FCS, and then seeded in an 80 ml flask at $3 \times 10^7$ cells, and incubated at 37℃ in 5% $CO_2$/95% air (v/v). Approximately after 1 h of seeding cells, the medium was changed. Just before cells were confluent, attached cells were resuspended in PBS including 0.25% trypsin and 0.025% EDTA. The suspended cells were seeded into 24-well plate with $1 \times 10^7$ cells/well (2 $cm^2$), and incubated in DMEM with 10% FCS at 37℃. When expanded to approximate 50% of a well, the effect of serum on protein synthesis was examined.

### Measurement of protein synthesis

When CEF occupied approximately 50% of a well, the medium was drawn off from the well. CEF was rinsed with DMEM without FCS twice and was then incubated in DMEM excluding FCS under 5% $CO_2$/95% air atmosphere at 37℃. After 2 h of incubation, the medium was removed and added one of experimental media which were DMEM with or without Phe, and PBS ($Ca^{2+}$, $Mg^{2+}$) with or without 400 $\mu$M Phe. All media included 10% (v/v) fetal calf serum. After incubating for either 6, 30 or 54 h, L-[2, 6-$^3$H] phenylalanine (2.11 TBq/m mol, 37 MBq/ml) Amersham LIFE SCIENCE, Ltd., Tokyo, Japan) was added into the medium, in which the radioactivity was 1 $\mu$Ci/ml. Thereafter, CEF was incubated for further 18 h, and then the medium was drawn away and cells were rinsed with ice-cold PBS ($Ca^{2+}$, $Mg^{2+}$) twice. The intracellular free amino acids were removed by rinsing with ice-cold 5% (w/v) trichloroacetic acid twice. After rinsing with ice-cold water, 1 ml of 0.5 M NaOH/0.1% Triton X-100 was added and incubated at room temperature for 30 min. After dissolving protein by pipetting, the radioactivity in NaOH/Triton X-100 solution was measured using a scintillation counter as an index of protein synthesis.

### Statistical analysis

Statistical analysis of data was performed by three-way ANOVA (Cochran and Cox, 1992) followed by a protected LSD to assess the difference between means using the General Linear Model Procedures of SAS (SAS/STAT Version 6, SAS Institute, Cary, NC U.S.A).

## Results and Discussion

Protein synthesis of CEF cultured either in DMEM with or without Phe or in PBS ($Ca^{2+}$, $Mg^{2+}$) with or

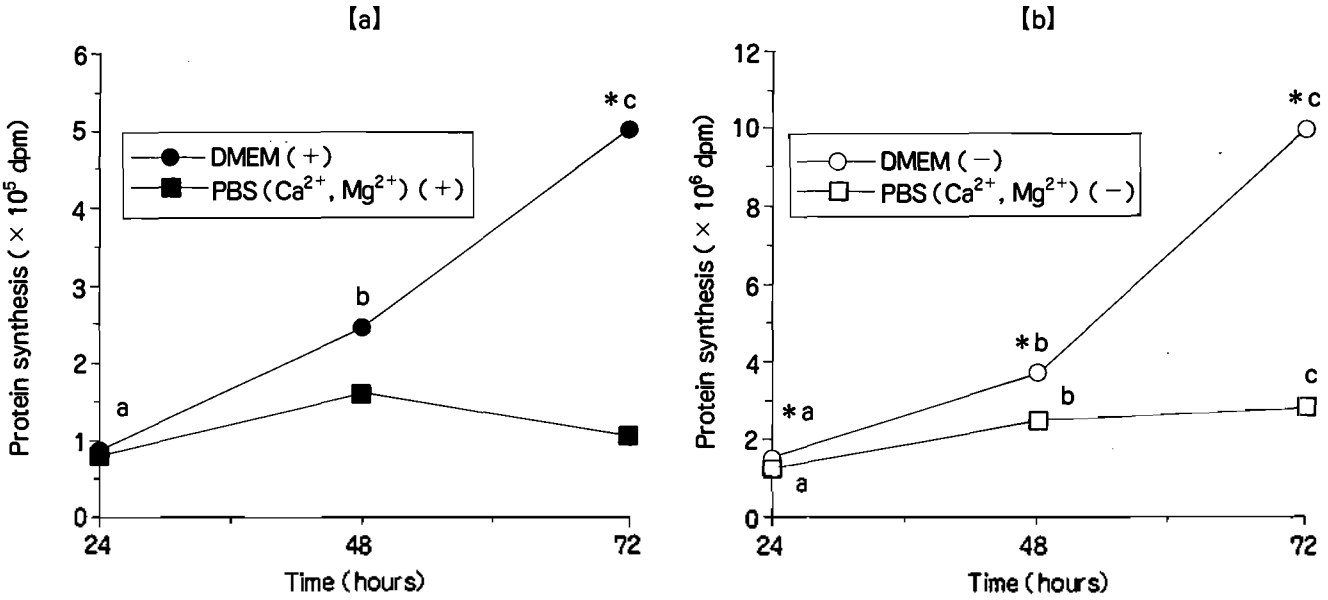


Figure 1. Time course change in protein synthesis of chicken embryo fibroblasts cultured in Dulbecco's modified Eagle's medium (DMEM) ( ● , ○ ) and Dulbecco's phosphate buffered saline (PBS ($Ca^{2+}$, $Mg^{2+}$) ( ■ , □ ) with [a] ( ● , ■ ) or without [b] ( ○ , □ ) phenylalanine. All media included 10% (v /v) fetal calf serum. The incorporation of L-[2, 6-$^3$H] phenylalanine into cells after 24, 48 and 72 h of incubation was measured as an index of protein synthesis. Means not sharing a common superscript on the same line are significantly different at $p < 0.05$ (a, b, c). The asterisk indicates the significant difference between DMEM and PBS ($Ca^{2+}$, $Mg^{2+}$).

without Phe is shown in figure 1. Regardless of the existence of Phe in the medium, protein synthesis of CEF cultured in DMEM was higher than that in PBS ($Ca^{2+}$, $Mg^{2+}$). Since the composition of DMEM was shown by Dulbecco and Freeman (1959), DMEM has been widely used to culture animal cells. In the present study, normal cell growth indicated that all kinds of nutrients which are necessary for cell growth were supplied from DMEM. Therefore the decrease in protein synthesis of CEF cultured in PBS ($Ca^{2+}$, $Mg^{2+}$) was due to the lack in the supply of various nutrients from the medium. When we measured the rate of *in vivo* liver protein synthesis, food-deprivation brought about similar decrease in protein synthesis (Kita et al., 1996). These results concluded that in both *in vivo* and *in vitro* conditions, the lack of nutrients in the supply decreased protein synthesis.

It was surprising that in absence of Phe in DMEM a marked increase in protein synthesis of CEF was observed, which was inconsistent with the *in vivo* study reported by Kino and Okumura (1987) who reported that single amino acid deficiency brought about a fall in whole-body protein synthesis of chicks. This discrepancy might be due to the high specific radioactivity of Phe due to the low concentration of Phe because Phe was supplied only from FCS and tracer solution.

Elevated protein synthesis of CEF cultured in PBS ($Ca^{2+}$, $Mg^{2+}$) without Phe along with incubation time was observed, as might be resulted by the nutrient supply from FCS to CEF. However, protein synthesis cultured in PBS ($Ca^{2+}$, $Mg^{2+}$) supplemented with Phe did not increase during 72 h of cell culture. As Okumura et al. (1980) and Okumura and Yamaguchi (1980) found that graded level of Phe from the requirement to excess level brought about an obvious fall in protein retention in the body, excess level of Phe in the medium may inhibit the process of protein synthesis due to the toxicity of excess Phe.

As stated above, it was concluded that Phe deficiency in the medium does not induce a fall in protein synthesis of CEF as long as other nutrients and FCS were fully supplied in the culture medium and that the excess Phe concentration inhibits protein synthesis in the case of malnutritional conditions in the medium.

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