«Research Note»

A Simple Method for Evaluating Whole Body Protein Synthesis in Chick Embryos *in vivo*

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The measurement of whole body protein synthesis *in vivo* in chick embryos has been attempted in our laboratory after a pulse injection of [^{15}N] phenylalanine followed by sampling at various time points¹. However, this method requires many embryos to be sacrificed to obtain the entire time course of isotope abundance of free and protein-bound [^{15}N] phenylalanine. In the present study, therefore, three different methods for evaluating whole body protein synthesis *in vivo* in chick embryos were compared. The results indicated that a simple method of area calculation only at two time points would give reasonably accurate estimates.

Materials and Methods

Fertilized eggs from White Leghorn hens maintained in our laboratory were incubated for 12 days in an incubator, the temperature and humidity of which were controlled at 38°C and about 70%, respectively. On 12th day of incubation, 16 eggs were taken and the embryos were injected intraperitoneally with L-[^{15}N] phenylalanine of 94.635 atom % excess at 7.56 μmole per egg, which was more than twice the total pool size of free phenylalanine in the whole body. The eggs were, then, returned to the incubator. At 10, 30, 60, and 90 minutes after the injection, 4 eggs were taken from the incubator and each embryo was removed, frozen in liquid N_2 and stored at -20°C until analyzed. The enrichment (atom % excess), i.e. an isotopic abundance above the natural occurrence of a stable isotope, of free and protein-bound [^{15}N] phenylalanine was determined with a selected-ion quadrupole gas-chromatograph mass-spectrometer (Shimadzu QP-1000, Shimadzu Co. Ltd., Kyoto, Japan).

Frozen embryos were homogenized by adding 3 volumes of ice-cold water. The homogenate of whole embryos was deproteinized with 5 volumes of 2% perchloric acid followed by extensive washing of the pellet, which was then subjected to acid hydrolysis with 6 N HCl at 110°C for 24 hours. The resultant amino acids in free and protein-bound fractions were derivatized to form n-trifluoro-acetyl-n-butyl esters prior to the gas-chromatograph mass-spectrometric measurement. Details of analytical method were given elsewhere^{1,2}. For the determination of the pool size of phenylalanine in free and protein fractions, the method of Garlick *et al.*³ was employed with slight modifications. Instead of tyrosine decarboxylase (EC 4.1.1.25) in the original method, phenylalanine decarboxylase (EC 4.1.1.53) was used for enzymatic reaction in order to increase the conversion rate of phenylalanine to phenylethylamine. The recovery of the reaction product, phenylethylamine, was ranging from 50 to 55%.

The estimation of whole body protein synthesis was made by three different methods. In the first method, which was assumed to be most valid on a theoretical basis, simultaneous differential equations for free and protein-bound phenylalanine pools were solved by using the Runge-Kutta procedure⁴⁾ on a microcomputer and all the parameters were determined by a least square procedure. The differential equations were as follows:

for the free phenylalanine pool,

$$dE_f/dt = -(K + K_o) \cdot E_f + K_d \cdot R \cdot E_b \dots\dots\dots(1)$$

and for the protein-bound phenylalanine pool,

$$dE_b/dt = K/R \cdot E_f - K_d \cdot E_b \dots\dots\dots(2)$$

where E_f , E_b , K , K_o , K_d and R stand for enrichment of free fraction (atom % excess), that of protein fraction (atom % excess), the first order rate constants from free to protein fractions (/day), free fractions to be oxidized (/day) and protein to free fractions (/day), and the relative pool size of protein-bound to free phenylalanine. The fractional rate of protein synthesis (%/day) was defined as $K/R \times 100$ from the parameter in the above equations.

The second method was based on all the measurements of enrichment to give the area under the enrichment curve⁵⁾. In the third method, the area calculation was made by using only the two time points at 10 and 90 minutes. The analogous method was described elsewhere³⁾.

Analysis of variance was done on the data and significance of differences between means was tested by the protected least significant difference method⁶⁾.

Results and Discussion

The content of phenylalanine in the free and protein fractions of chick embryos is shown in Table 1, indicating that the value for R was approximately 40 ($=19.0/0.57$). The R value of 40 was considerably low compared with that of more than 200 found in chick muscle⁷⁾, indicating that relatively large amounts of phenylalanine were not incorporated into protein but still present in the free form. This may be in line with the finding that protein content

Table 1. Concentration of phenylalanine and changes in isotopic abundance of [¹⁵N]phenylalanine in free and protein fractions of chick embryos

	Time (min)	Sampling site	
		Free	Protein
Phenylalanine conc. ¹ (μ mole/g embryo)	90	0.57 $\pm$ 0.08 ²	19.0 $\pm$ 1.1
Isotope abundance (atom % excess)	10	83.4 $\pm$ 2.3	ND ³
	30	83.3 $\pm$ 2.3	ND
	60	61.0 $\pm$ 4.5*	ND
	90	57.6 $\pm$ 2.6*	12.0 $\pm$ 1.4

1; Administered L-[¹⁵N]phenylalanine was not included.

2; Mean $\pm$ SEM of 4 embryos.

3; Not determined.

Single intraperitoneal injection of L-[¹⁵N]phenylalanine with 94.6 atom % excess was done at a dose of 7.56 μ mole on 12 days of egg incubation.

*; Significantly different from the 10 min value at $P < 0.01$.

Fig. 1. Time course of isotope abundance of $[^{15}\text{N}]$ phenylalanine in free and protein fractions of 12-day chick embryos after a single intraperitoneal injection of the isotope with 94.6 atom % excess at a dose of 1.25 mg. The two fitted curves were obtained by solving simultaneous differential equations representing a two-pool model. The values are means $\pm$ SEM of 4 embryos.

Table 2. Fractional rate of whole body protein synthesis calculated by different methods in chick embryos

Method of calculation	(%/day)
Differential equation ¹	297 $\pm$ 44 ⁴ (100)
Area calculation ²	291 $\pm$ 43 (98)
Simplified area calculation ³	272 $\pm$ 40 (92)

1; Simultaneous differential equations were solved by using the Runge-Kutta method, and all the parameters were determined by a least square procedure.

2; The area under the enrichment curve of free $[^{15}\text{N}]$ phenylalanine was calculated by using the values at each measurement point.

3; The area under the enrichment curve of free $[^{15}\text{N}]$ phenylalanine was calculated by using only the two measurement points at 10 and 90 min.

4; Means $\pm$ SEM of 4 embryos.

None of the difference was significant at $P < 0.05$.

Figures in parentheses were the percentage of the value obtained by the differential equation method.

of chick embryos on day 12 was only 4.8% compared with 13.8% on day 19¹⁾. Table 1 also gives the values for isotopic abundance in free and protein-bound $[^{15}\text{N}]$ phenylalanine at various time intervals. During the measurement period the enrichment of free $[^{15}\text{N}]$ phenylalanine decreased almost linearly, although there were some departures from the linearity.

By solving the simultaneous differential equations that gave a set of parameters resulting in the minimum error mean square, the best enrichment curves of $[^{15}\text{N}]$ phenylalanine for the free and protein fractions were obtained (Fig. 1). Judging from the extent of deviation from the curves, whole body protein synthesis and degradation processes in chick embryos seemed to be satisfactorily described by the two-pool model.

Fractional rates of protein synthesis in the whole chick embryo calculated by the three different methods are given in Table 2. The two estimates determined by area calculation tended to be slightly lower than that obtained by solving the differential equations, but none of the difference in the estimates was significant. Consequently, under the present experimental conditions the three calculation methods were found to be equally good.

Theoretically, the estimate obtained by solving the differential equations should be most reliable and valid, provided that the metabolism of phenylalanine in chick embryos *in vivo* could well be described by the two-pool model. However, this method requires the measurement of R, the relative pool size, and enrichment values at various time points. Similarly in the method of area calculation based on all the measurement points, many embryos would have to be sacrificed to obtain the entire time course of isotope abundance of free and protein-bound [^{15}N] phenylalanine. Thus, for a routine work the differential equation method and that based on all the measurement points may not be practical. In conclusion, therefore, the simple method of area calculation only at two time points would give reasonably accurate estimates, and hence can be useful for evaluating whole body protein synthesis rate in chick embryos. With this simple method, values for fractional synthesis rates of whole body protein in chick embryos, which showed a marked developmental fall while incubating from 12 to 19 days of embryonic age, were found to be almost identical to those based on all the measurement points¹⁾.

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鶏胚の蛋白質合成を *in vivo* で測定するための簡便法

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孵卵 12 日目の鶏胚について、蛋白質合成を *in vivo* で測定するに際し、3 種類の計算法を用いてその精粗を比較した。これら 3 種類の方法は次のごとくして蛋白質合成率 (%/day) を求めるものである。1) 遊離および蛋白質中のフェニルアラニンプールについてその同位体濃度を経時的に測定し、その変化に関する連立微分方程式を解く、2) 遊離フェニルアラニンプール中の同位体濃度

の各測定時間における全ての測定値を用いて面積計算をする、3) 面積計算に際し、特定の 2 測定時間における測定値のみを用いる。

計算の結果によると、これら 3 種の方法ではほぼ同様な合成率の値が示されたので、2 測定時間の測定値を用いて面積計算をする簡便法が実用的であると考えられた。

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