Incorporation of ^{14}C -Tyrosine into Soluble Ribonucleic Acid from Baker's Yeast

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The incorporation of ^{14}C -tyrosine into S-RNA catalyzed by a partially purified tyrosine activating enzyme from baker's yeast was observed. The maximum incorporation was shown in the presence of 5 μmoles of ATP, 10 μmoles of MgCl_2 and 10~100 μmoles of KCl in the reaction mixture of total volume of 1 ml, at pH 7.8 when 1.2 mg of S-RNA, 0.1 μmole of ^{14}C -tyrosine and 400 μg of enzyme protein were used. Beyond the concentration of ATP, MgCl_2 and KCl described above, the tendency of inhibition was observed. The incorporation was strongly inhibited by pCMB and reactivated by cysteine. Manganese and calcium ions were effective as substitutes for magnesium. S-RNA used was prepared from whole baker's yeast cell with phenol, but S-RNA obtained from the supernatant of the ground yeast had lost its incorporating activity.

INTRODUCTION

It has been considered in protein synthesis that the carboxyl group of free amino acid is first activated by the amino acid activating enzymes specific for certain amino acids (Equation 1). These enzymes are known to catalyze also in the second reaction (Equation 2) including the transfer of the amino acyl moiety to a specific soluble ribonucleic acid (S-RNA) ^{1,2)}.

The reaction of amino acyl S-RNA formation has been studied extensively by a number of workers in different systems³⁾. And some

studies using yeast^{4~11)} have been performed, but the accumulation of data in this field is considered necessary.

In the preceeding paper¹²⁾, we described the properties of a tyrosine activating enzyme from baker's yeast which was investigated by the hydroxamate formation. This paper deals with the several properties of the same enzyme in relation to the incorporation of ^{14}C -tyrosine into S-RNA including the over all reaction (Equation 1 and 2), and some data are added here.

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MATERIALS AND METHODS

Preparation of the Enzyme.

A partially purified tyrosine activating enzyme, free of RNA, was prepared from baker's yeast according to a method with a slight modification described in the preceding paper¹²⁾.

Dried baker's yeast (300 g) was mixed with 900 ml of 0.1 M potassium bicarbonate and incubated at 37°C for 4 hours. The autolyzed mixture was centrifuged at 10,000×g for 20 minutes. All the following procedures were performed under 5°C. The supernatant fluid (480 ml) was diluted to 2 fold volumes with cold water, and to 40 per cent concentration with addition of cold absolute ethanol. The solution was centrifuged at 10,000×g for 10 minutes and the supernatant was discarded. The precipitate was extracted with 600 ml of 0.05 M Tris buffer, pH 8.0, and the insoluble material was removed by centrifugation. One tenth volume of 1 M BaCl₂¹³⁾ was added to the supernatant, and most of RNA in it was precipitated. The forming precipitate was removed. The small amounts of RNA remaining in the supernatant were precipitated by the addition of streptomycin sulfate, and streptomycin-RNA complex was removed by the centrifugation. After the supernatant solution was dialyzed against distilled water, ammonium sulfate was added to 50 per cent saturation. The precipitate was removed and to the supernatant solution was added ammonium sulfate till it became 70 per cent. The solution was centrifuged and the precipitate was dissolved in Tris buffer and stored in -20°C. In the experiment for the enzyme reactions, an aliquot of the solution was withdrawn, and dialyzed against distilled water for 24 hours. The amount of protein was determined by the Lowry method¹⁴⁾.

Preparation of S-RNA.

S-RNA was isolated as described by Monier et al.¹⁵⁾ from baker's yeast. To 450 g of fresh baker's yeast, were added 800 ml of water and 1,200 ml of 90 per cent aqueous phenol. After 2 hours of constant stirring at room temperature, the mixture was centrifuged and the aqueous layer was obtained. Two volumes of cold absolute ethanol were added to the turbid aqueous layer in the presence of 2 per cent potassium acetate, and RNA was precipitated at -20°C. The

precipitate was then dissolved in water and any insoluble material was removed by the centrifugation at 40,000×g for 30 minutes. With the clear supernatant fluid, alcohol precipitation was repeated twice. The precipitated RNA was dissolved in water and dialyzed against cold water. The dialyzed solution was then centrifuged if any insoluble material appeared, and the supernatant was concentrated by lyophilization. By treatment with 2-methoxy-ethanol¹⁶⁾ or the chromatography on DEAE-cellulose column¹⁷⁾ polysaccharide material was removed. But in some cases, these treatments were omitted because polysaccharide material had little effect on the ¹⁴C-amino acid incorporation into S-RNA¹⁸⁾. The amount of RNA was determined spectrophotometrically¹⁹⁾. The yield was about 200~300 mg RNA from 450 g of baker's yeast.

Micellaneous.

DL-Tyrosine-2-¹⁴C (1.0 mc per mmole) was obtained from Daiichi Pure Chemicals Co. Ltd. (Tokyo). The Na-salts of ATP (adenosine triphosphate), CTP (cytidine triphosphate), GTP (guanosine triphosphate) and creatine phosphate were purchased from Sigma Chemical Company. ATP-creatine transphosphorylase was prepared from rabbit muscle by the method of Kuby et al.¹⁷⁾

Measurement of the Incorporation of ¹⁴C-Tyrosine into S-RNA.

The technique used for incorporation was based on that of Berg et al.¹⁵⁾ The standard assay condition for this measurement was as follows: The incubation mixture contained in a total volume of 1.0 ml, 100 μmoles of Tris-HCl (pH 7.8), 5 μmoles of ATP, 10 μmoles of MgCl₂, 1.2 mg of S-RNA, 0.1 μmoles of ¹⁴C-DL-tyrosine and 0.1 ml of enzyme solution containing about 400 μg of protein. The reaction was started by the addition of enzyme unless otherwise stated. The mixture was incubated at 37°C for 15 minutes. The reaction was stopped by the addition of 0.5 ml of solution containing 5 mg of carrier RNA and 5 ml of cold solution containing 0.5 M NaCl and 66 per cent ethanol. After having been left to stand for several hours at -20°C, the precipitate was centrifuged and washed three times by resuspension in the

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ethanol-salt mixture. Then the precipitate was mounted on a filter paper disk with 2 cm in diameter fixed to a glass filter. After washing by the ethanol-salt mixture and drying, it was counted in a windowless gas flow counter and the counts were corrected by self absorption curve. The specific activity of ^{14}C -tyrosine used was about 10^5 counts per minute per $0.1 \mu\text{mole}$ counted in a windowless gas flow counter. In the above procedures, the values for zero time controls were from 20 to 30 counts per minute. In all experiments, zero time controls were carried out. The results were expressed as the radioactivity (cpm) of ^{14}C -tyrosine incorporated into S-RNA and corrected for zero time controls.

RESULTS AND DISCUSSION

Requirement of the Enzyme Reaction.

The incorporation of ^{14}C -tyrosine into S-RNA was observed in the presence of ATP, MgCl_2 , S-RNA and enzyme (Table I). Omission of any one of these components resulted in a marked decrease in the rate of the reaction. These results show that the enzyme did not contain significant amounts of ATP and S-RNA. But the ratio of the optical densities at $280 \text{ m}\mu$ and $260 \text{ m}\mu$ of the enzyme preparation was very low (0.58), and this might be due to the presence of nucleotide material in the enzyme solutions. This fact was observed even in the purified amino acid activating enzyme preparations^{19,20}.

TABLE I. REQUIREMENT FOR THE INCORPORATION

Incubation mixture	Expt. No.	
	I (cpm)	II (cpm)
Complete system	356	465
— Enzyme	0	0
— S-RNA	12	0
— ATP	31	6
— MgCl_2	—	0

The incubation mixture and conditions are described under "Methods" as the standard assay condition. The reaction was started by the addition of ^{14}C -tyrosine.

General Properties of the Enzyme Reaction.

The several basic properties of the reaction

are shown as follows: The time course of the reaction showed that the maximum incorporation was achieved in 10 to 15 minutes and remained in that level (Fig. 1). The incorporation was nearly proportional to the amounts of enzyme when the amounts of enzyme were less than $400 \mu\text{g}$ under the assay condition (Fig. 2). As shown in Fig. 3, the optimum pH for the reaction was found at 7.8. This value almost coincided with that of optimum pH measured by the hydroxamate formation¹²). But the incorporation of ^{14}C -tyrosine into S-RNA seemed to be more

FIG. 1. Time Course of the Incorporation.

The standard assay condition was used except that the incubation times were changed.

FIG. 2. The Relationship Between the Incorporation and Enzyme Concentration.

Standard assay condition was used with the indicated amounts of enzyme.

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sensitive to the change of hydrogen ion concentration than in the case of hydroxamate formation, because the pH activity curve of the incorporation was sharper as compared with that of the case of hydroxamate formation. The incorporation proceeded with a

Fig. 3. Effect of pH on the Incorporation.

Standard assay condition was used with Tris buffer of different pH.

FIG. 4. Effect of S-RNA Concentration on the Incorporation.

Standard assay condition was used with the indicated amounts of S-RNA.

linear function, when S-RNA was used in low concentration (Fig. 4).

Requirement of ATP.

As shown in Table I, ATP was necessary for the incorporation and the extent of the incorporation depended upon the concentration of ATP (Fig. 5). But the addition of 10 μ moles of ATP showed the tendency of inhibition. The similar inhibition was observed in the hydroxamate formation¹²⁾.

FIG. 5. Effect of ATP Concentration on the Incorporation.

Standard assay condition was used with the indicated amount of ATP.

Requirement of Metal Ions.

The effect of magnesium ion on the reaction was tested (Fig. 6). 10^{-2} M of magnesium ion concentration was found to be the most effective. When the concentrations of magnesium were less than 10^{-3} M, the reaction did not proceed. At high concentration of magnesium (10^{-1} M), the stronger inhibition was observed as compared with that of the hydroxamate formation¹²⁾. The strong inhibition by the high concentration of magnesium ion may be attributed to the formation of inactive complex of S-RNA and magnesium. Other than magnesium, the stimulation of the incorporation by cobalt ion was reported on alanine activating enzyme from

FIG. 6. Effect of Mg^{++} Concentration on the Incorporation.

Standard assay condition was used with the indicated concentration of MgCl_2 .

pig liver by Webster²⁰); Everett et al.²¹) demonstrated that the incorporation of amino acid into protein by rat liver preparation was inhibited by various metals. In our experiments, manganese and calcium ions were considerably effective on the incorporation of ^{14}C -tyrosine (Table II). As shown in Table II, manganese and calcium ions had 63 and 30 per cent of activities, respectively, as compared with that of magnesium ion. In these cases, the incorporation was not found in the absence of ATP. No incorporation occurred in the presence of cadmium, zinc and nickel ions. The same effect of cobalt ion as in the case of Webster was observed also in our experiments, but in this case, zero time control was considerably high, and the incorporation was found even in the absence of ATP.

TABLE II. THE EFFECT OF Mn^{++} AND Ca^{++} ON THE INCORPORATION

Metal	Concentration (M)	Radioactivity (cpm)
Mn^{++}	0	0
	10^{-2}	193
	5×10^{-3}	174
	10^{-3}	0
Ca^{++}	0	0
	10^{-2}	93
	5×10^{-3}	85
	10^{-3}	0
Mg^{++}	10^{-2}	308

MnCl_2 or CaCl_2 was used in place of MgCl_2 . Standard assay condition was used with the indicated metals and concentrations.

The Requirement of Potassium Ion.

The requirements of potassium ion for the activation of amino acids²²⁻²⁴), and the incorporation of amino acid into protein²⁵) was demonstrated quantitatively by several authors. Under standard assay conditions, we

FIG. 7. Effect of K^+ Concentration on the Incorporation.

Standard assay condition was used with the indicated concentration of KCl .

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24) R.W. Holley, E.F. Brunngraber, F. Saad and H.H. Williams, *ibid.*, **236**, 1971 (1961).

25) M. Takanami, *Biochim. Biophys. Acta*, **39**, 318 (1960).

21) G.A. Everett and R.W. Holley, *Biochim. Biophys. Acta*, **46**, 390 (1961).

used the S-RNA as potassium salt. Therefore, as shown in Table IV, the addition of potassium ion into the reaction mixture did not show any stimulating effect as may be expected. However, with sodium salt instead of potassium salt of S-RNA under the otherwise same condition, 10^{-2} M to 10^{-1} M of potassium ion was necessary for the maximum incorporation, and the tendency of inhibition was observed at the concentration above 10^{-1} M (Fig. 7). The marked decrease of the incorporation was found in the absence of potassium ion and in this case only 10 per cent (33 cpm) of the maximum activity was found.

Effect of pCMB and Cysteine.

It was described by many authors that the activation of amino acids was inhibited by the addition of pCMB (*p*-chloromercuribenzoate). The formation of hydroxamate catalyzed by the tyrosine activating enzyme in our preparation was also inhibited by the addition of pCMB¹²⁾. Incorporation of 14 C-tyrosine into S-RNA was also strongly inhibited by pCMB in the concentration of 10^{-3} M to 10^{-4} M. Then the inhibitions were reactivated by the addition of excess amounts of cysteine. Although the formation of hydroxamate was considerably inhibited by the addition of cysteine¹²⁾, incorporation of 14 C-

tyrosine was not inhibited. These results are shown in Table III.

Effects of Various Components.

As shown in Table IV, the addition of ATP-generating system (creatine phosphate and ATP-creatine transphosphorylase) was not effective in our reaction system, and the addition of GTP which is necessary for the over all incorporation of amino acids into ribosomal protein had no effect. Furthermore, the addition of CTP, which is thought to be essential to the formation of terminal structure of S-RNA to which the amino acids are bound through an ester linkage, did not show any stimulating effect to the incorporation of 14 C-tyrosine into S-RNA. From this result, it was assumed that the S-RNA used in our experiments kept the terminal nucleotide sequence (-Cp-Cp-Adenosine) or that the enzyme which catalyzed the synthesis of terminal structure in the presence of ATP and CTP²⁶⁻³²⁾ was lacking in our preparation. In connection with this phenomenon, S-RNA prepared from the supernatant of the ground

TABLE III. EFFECT OF pCMB AND CYSTEINE ON THE INCORPORATION

Expt. No.	Incubation mixture	Radioactivity (cpm)
I	Complete system	345
	+pCMB 10^{-3} M	6
	+pCMB 10^{-4} M	3
	+pCMB 10^{-5} M	340
II	Complete system	345
	+pCMB 10^{-3} M + Cysteine 10^{-2} M	196
	+pCMB 10^{-4} M + Cysteine 10^{-2} M	296
	+pCMB 10^{-5} M + Cysteine 10^{-2} M	357
III	Complete system	439
	+Cysteine 10^{-2} M	452

The indicated amounts of pCMB and cysteine were added to the standard assay condition. In Expt. I, the reaction mixture was preincubated for 5 minutes. In Expt. II, pCMB was added first. After 5 minutes incubation, cysteine was added. Through Expt. I to III, the reactions were started by the addition of 14 C-tyrosine.

TABLE IV. EFFECT OF VARIOUS COMPONENTS ON THE INCORPORATION

Incubation mixture	Radioactivity (cpm)
Complete system	459
+KCl 10 μ moles	463
+Creatine phosphate 10 μ moles	467
+Creatine phosphate 10 μ moles +ATP-creatine transphosphorylase 100 μ g	441
+GTP 1 μ mole	467
+CTP 1 μ mole	451

The indicated amounts of test materials were added to the standard assay condition.

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- 27) J. Preiss, P. Berg, E.J. Ofengand, F.H. Bergmann and M. Dieckmann, *ibid.*, **45**, 319 (1959).
- 28) L.I. Hecht, M.L. Stephenson and P.C. Zamecnik, *ibid.*, **45**, 505 (1959).
- 29) E.S. Canellakis and E. Herbert, *ibid.*, **46**, 170 (1960).
- 30) E.S. Canellakis and E. Herbert, *Biochim. Biophys. Acta*, **45** (1960).
- 31) E.S. Canellakis and E. Herbert, *ibid.*, **47**, 78 (1961).
- 32) E.S. Canellakis and E. Herbert, *ibid.*, **47**, 85 (1961).

yeast had no capacity of the incorporation of ^{14}C -tyrosine. This fact may be caused by the method of preparing S-RNA. The method of preparation was as follows: The baker's yeast was ground with quartz sand and suspended in Chao-Schachman's medium³³⁾ under 4°C . After removal of cell fragments and large particles by centrifugation at $10,000\times g$ for 20 minutes, the final supernatant fluid was obtained by the centrifugation at $100,000\times g$

for 2 hours. From this supernatant, the so-called S-RNA was prepared by the phenol method¹⁵⁾. In the course of preparation, the structure of S-RNA might be degraded by nucleases in yeast cells.

S-RNA prepared by the method of Monier et al.¹⁴⁾ still maintained the full incorporating capacity after heating at 90°C for 10 minutes in our experiments.

Studies on the incorporation of ^{14}C -tyrosine into ribosomal protein are in progress.

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