# ISOLATION OF THREONYL ADENYLATE-ENZYME COMPLEX°

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The aminoacyl-RNA synthetases have been shown to carry out the following two-step reaction:

1) amino acid + ATP + enzyme $\overset{Mg}{\rightleftharpoons}$ aminoacyl-AMP-enzyme + $PP_i$

2) aminoacyl-AMP-enzyme + sRNA $\rightleftharpoons$ aminoacyl-sRNA + AMP + enzyme

Evidence for the formation of the intermediate aminoacyl-AMP-enzyme complex has been reported by several investigators (Hoagland, 1955, De Moss and Novelli, 1955, Berg, 1956). Tryptophanyl adenylate (Karasek, et al., 1958) and seryl adenylate (Webster and Davie, 1961) were isolated in trichloroacetic acid supernatant fractions after incubation of amino acid and ATP with substrate amounts of the respective enzymes. The present communication describes the isolation of enzyme-bound threonyl adenylate and the capacity of this complex to transfer the threonine directly to sRNA.

MATERIALS AND METHODS. Threonyl-RNA synthetase from rat liver was purified by the procedure of Allen et al., (1960) One hundred fold purification of the enzyme was achieved. Rat liver sRNA was prepared essentially by the procedure of Brunngraber, (1962). Threonyl adenylate was synthesized by the method of Berg (1958).

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L-Threonine-$C^{14}$ (119 μcuries/μmole) was purchased from Volk, Inc. and ATP-$C^{14}$ (10 μcuries/μmole) was from Schwartz BioResearch Inc.

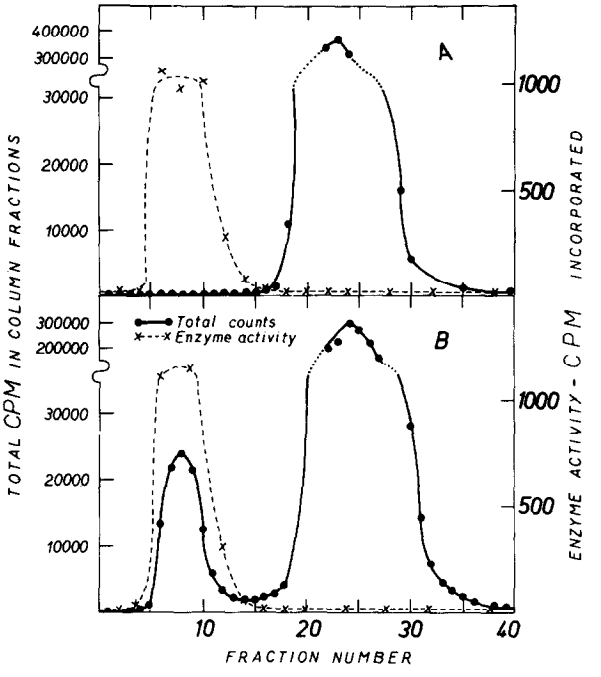


Figure 1. Separation of threonyl adenylate-enzyme complex on Sephadex G-50 columns. Ten mg of enzyme equivalent to 150 units, (a unit is defined as the amount of enzyme that can incorporate 1 mμmole of threonine into sRNA in 15 minutes under the conditions described in Table I), was incubated for 15 min. at 37° with 0.007 mM threonine-$C^{14}$ equivalent to $2.5 \times 10^6$ cpm (S.A.119 μcuries/μmole), 30 mM Tris buffer, pH 7.5, 5 mM $MgCl_2$, 1 mM GSH, 2.5 mM ATP, and 50 μg crystalline pyrophosphatase (Worthington) in a final volume of 3 ml. The mixture was poured directly on a Sephadex column 1 x 30 cm equilibrated with 0.01 M Tris, pH 7.5 and eluted with the same buffer in 0.75 ml fractions. Aliquots of 100 μl were assayed for radioactivity and 25 μl assayed for enzyme activity. A: Control incubation, without ATP. B: Experimental incubation, with ATP. The assay procedure used for enzyme determinations was as given in Table I with added ATP, 2.5 mM, and threonine-$C^{14}$ (0.01 mM, S.A. 10 μcuries/μmole).

RESULTS. Isolation of the threonyl-AMP-enzyme complex was achieved by gel filtration after incubation of the enzyme with ATP and L-threonine-$C^{14}$ as shown in Figure 1. A radio-

TABLE 1. TRANSFER OF THREONINE-$C^{14}$ FROM THE ENZYME-BOUND COMPLEX TO sRNA.

| | Incubation system | CPM |
|---|---|---|
| Experiment 1 | Complete | 856 |
| | -sRNA | 61 |
| Experiment 2 | Complete | 1028 |
| | -sRNA | 138 |

The incubation system included in a final volume of 0.5 ml: 50 mM Tris buffer, pH 8.0, 5 mM $MgCl_2$, 1 mM GSH, 0.3 mg rat liver sRNA and fractions from a Sephadex column containing approximately 5000 cpm and 0.2 mg protein of the threonyl-$C^{14}$ adenylate-enzyme complex. Incubation was for 15 minutes at 37° and the reaction was stopped by precipitation with cold 5% TCA containing 0.002 M threonine-$C^{12}$. Carrier sRNA was added and the precipitate was washed twice with additional TCA and once with 50:50 alcohol-ether. The final precipitate was dissolved in formic acid, plated and counted in a gas-flow windowless counter, with 39% efficiency.

active peak containing approximately 5% of the counts added moved together with the protein fraction and well ahead of the free threonine.

The following evidence indicates that this protein - bound threonine represents a threonyl-AMP-enzyme complex.

The radioactivity in the peak coincided with the threonyl RNA synthetase activity of the fractions.

The appearance of the peak depended on the presence of ATP in the incubation mixture.

An identical experiment was performed using ATP-$C^{14}$ and unlabelled threonine in the incubation mixture and a similar peak of protein-bound radioactivity appeared on gel filtration.

When peak fractions of the complex labelled with either threonine-$C^{14}$ or ATP-$C^{14}$ were precipitated with trichloroacetic acid, the supernatant fluid contained a radioactive compound that behaved identically to synthetic threonyl adenylate on column chromatography on Dowex-1-$Cl^-$ as described by

TABLE 2. EFFECT OF MAGNESIUM ON THE TRANSFER OF L-THREONINE-$C^{14}$ FROM THE ENZYME-BOUND COMPLEX TO sRNA.

| | Incubation system | CPM |
|---|---|---|
| Experiment 1 | Complete | 1218 |
| | - $MgCl_2$ | 2483 |
| Experiment 2 | Complete | 528 |
| | - $MgCl_2$ | 1090 |

The incubation mixture and procedure were the same as that given for Table 1.

Kingdon et al., (1958).

The L-threonine-$C^{14}$ present in the labelled protein peak could be transferred directly to sRNA when incubated in the absence of both ATP and free threonine (Table I). This last evidence is of considerable interest since it demonstrates that the complex isolated is enzymatically active and is capable of carrying out reaction 2. It is now possible to study the properties of the second step of the threonyl-RNA synthetase reaction independently from the first step.

Table 2 shows some preliminary experiments on the effect of magnesium on the transfer of the amino acid from the complex to sRNA. It is seen that magnesium is not required for reaction 2 and furthermore, the concentration of this ion that has been found to be optimal for the overall reaction is markedly inhibitory (50%) for step 2.

Enzyme-complex formation is dependent on the presence of magnesium and is not prevented by the presence of pancreatic ribonuclease.

In preliminary studies on the kinetics of the transfer of threonine to sRNA it was found that the reaction was

extremely rapid being complete in approximately one minute.

Complex formation has also been observed* with arginyl-RNA synthetase purified as described by Allende and Allende, 1964.

Properties of the complex with respect to stability, the nature of the interaction of the enzyme with RNA, the specificity of the aminoacyl transfer, as well as complex formation with the two chromatographic fractions of threonyl-RNA synthetase recently described (Gatica et al.,1963) are under study.

## REFERENCES

Allen, E.H., Glassmann, E., and Schweet, R.S., J.Biol. Chem., 235, 1061 (1960).
Allende, C.C. and Allende, J.E., J.Biol.Chem.,239, 1102 (1964).
Berg, P., J.Biol.Chem.,222,1025 (1956).
Berg, P., J.Biol.Chem.,233, 608 (1958).
Brunngraber, E.F., Biochem.Biophys.Res.Comm.,8,1 (1962).
De Moss, J.A., and Novelli, G.D., Biochim.Biophys.Acta, 18, 592 (1955).
Gatica, M., Allende, C.C., Matamala, M. and Allende,J.E., Annual Meeting, Soc.Biol.Stgo, Nov.1963,p.38.
Hoagland, M.B., Biochim.Biophys.Acta, 16, 288 (1955).
Karasek, M., Castelfranco, P., Krishnaswamy, P.R. and Meister, A., J.Amer.Chem.Soc.,80,2335 (1958).
Kingdon, H.S., Webster, L.T.Jr., and Davie,E.W., Proc. Nat.Acad.Sci., U.S.44, 757 (1958).
Webster, L.T.Jr., and Davie, E.W.,J.Biol.Chem.,236, 479 (1961).

*While this manuscript was being reviewed we were informed by Dr.P.Berg of the isolation of isoleucyl-AMP-enzyme complex.