*ISOLATION OF ADENOSINE AMINO ACID ESTERS FROM A RIBONUCLEASE DIGEST OF SOLUBLE, LIVER RIBONUCLEIC ACID**

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After it had been found that, in the process of protein synthesis in systems of animal[1] as well as bacterial[2] origin, the activated amino acid was intermediately transferred to a relatively low-molecular-weight ribonucleic acid, much interest centered on the manner in which the amino acid might be attached to this ribonucleic acid carrier. Early observations[1] on the stability of the link to heating at 100° in 10 per cent sodium chloride solution made it rather likely that the attachment was not in the form of a carboxyl phosphoanhydride, as has been shown to be the case in the initial activation step.[3] Recent observations in this laboratory[4] on the accumulation in the tryptophan-activating enzyme reaction of a 2′/3′-tryptophan ester of ATP, which will be reported on elsewhere in detail, focused attention on the possibility of an amino acid ester link to the ribonucleic acid.

The likelihood of such an ester bond was further supported by a relatively low reactivity of the aminoacyl-RNA to hydroxylamine, which, as shown in Table 1, corresponded more to that of an amino acid ester than to an anhydride. The pH of 5.5, combined with low temperature, was found to be most suitable for giving a fine gradation between the reactivities. The reactivity of the amino acid AMP ester with hydroxylamine at this low pH is considerably greater than that of the leucine ethyl ester, which, under these conditions, is very stable, although at higher pH it reacts also, as pointed out recently by Raacke.[5]

It has now been possible to clarify the linking of amino acids to RNA by degradation with ribonuclease. It appears that this enzyme liberates from RNA, charged enzymatically with radioactive amino acid, a small, basic fragment which carries practically all the radioactivity. Most experiments were done with C^{14}-leucine, but with radioactive valine analogous results were obtained. A characteristic paper electrophoresis pattern of the digestion products is shown in Figure 1. Not shown in the figure is the bulk of the ultraviolet-absorbing material which migrates in a smear towards the anode and contains little or no radioactivity. Of special interest, however, are the two cathodically migrating compounds which both contain ultraviolet-absorbing material. The faster-moving compound, B, contains practically all the radioactivity; the slower-moving compound, A, is free of radioactivity and moves in a manner roughly comparable to adenosine. Ninety-two per cent of the radioactivity of the original aminoacyl-RNA was recovered in compound B.

When tested on the paper with periodate according to H. T. Gordon *et al.*,[6] compound B was periodate-negative, while A was positive. The eluate of B showed an absorption maximum at 258 mμ in acid and at 260 mμ in alkali, indicating adenosine. The eluate from A, according to the ultraviolet spectrum, contained adenosine and probably smaller amounts of other nucleosides. The absence of

periodate reaction in conjunction with the presence of amino acids in B indicated strongly that either the 2′- or the 3′-hydroxyl of the ribose was blocked.

For direct comparison with compound B, 2′/3′-leucyl adenosine was prepared by incubation of 2′/3′-leucyl AMP [7] with prostate phosphatase.[8] Compound B and the synthetic compound showed the same electrophoretic mobility.

In another experiment compound B was eluted and kept for 1 hour in 0.2 *M* ammonia to liberate the amino acid and was subsequently hydrolyzed for 1 hour at 100° with N HCl, to liberate adenine. This hydrolyzate was subjected to electrophoresis under the conditions of Figure 1. It showed a single ultraviolet-absorbing compound, electrophoretically identical with adenine and showing, on elution, the adenine absorption maxima of 263 mμ in acid and 269 mμ in alkali. The radioactivity now was separated from the ultraviolet-absorbing material and

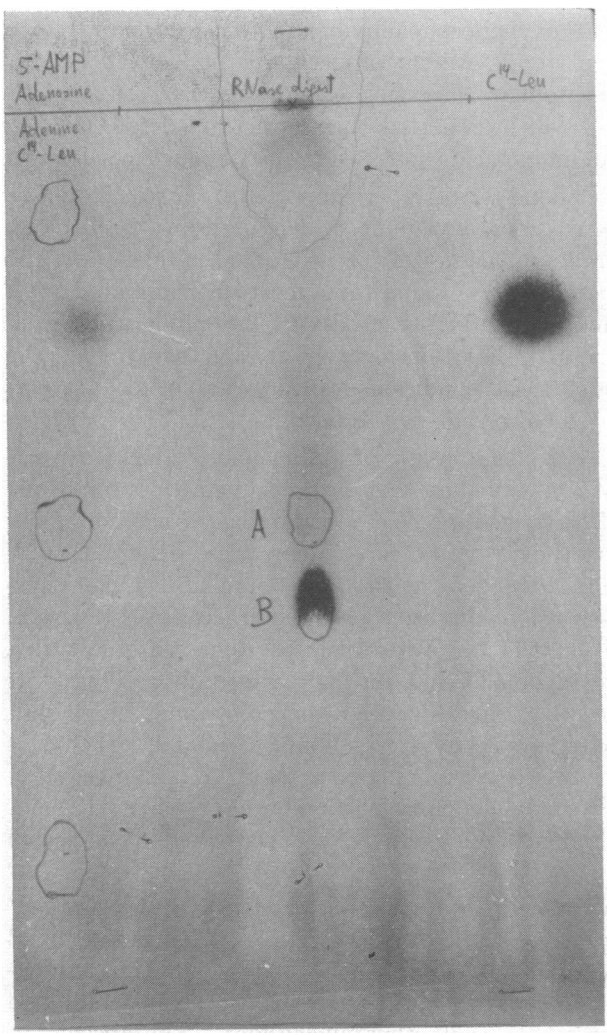

FIG. 1.—Electrophoresis pattern of RNase digest of C^{14}-leucine–labeled soluble RNA. The 100,000 × *g*. supernatant of a 20 per cent homogenate (Waring Blendor, 1 min.) of 4 rat livers was used for phenol treatment (see Schuster, Schramm, and Zillig, *Z. Naturforsch.*, **11b**, 339, 1956; and Kirby, *Biochem. J.*, **64**, 405, 1956). The extract, when dialyzed overnight and lyophilized, yielded 120 mg. of RNA fraction. The whole fraction, dissolved in 4 ml., was used for incorporation of amino acid, mainly C^{14}-leucine. The mixture contained 500 μM Tris buffer, pH 7.2, 50 μ*M* $MgCl_2$, 100 μ*M* ATP, and 2.5 μmoles C^{14}-D,L-leucine, 5.6 μC/μmole, 1 ml. of pigeon pancreas supernate (1 mg. protein) (Weiss, Acs, and Lipmann, these PROCEEDINGS, **44**, 189, 1958) and was incubated with the rat liver RNA in a total volume of 10 ml. for 10 minutes at 37°. The charged RNA was isolated as acid alcohol precipitate, which, after washing with 0.2 *M* perchloric acid and water and redissolving in 0.1 *M* ammonium acetate solution, was dialyzed overnight and lyophilized, and the residue was used for the digestion with RNase. The residue was dissolved in 0.25 ml. water and incubated, after adding 100 μg. of RNase (Worthington) in 0.05 ml., for 10 minutes at 25°, the pH of the solution being about 6.

Paper electrophoresis was carried out according to Markham and Smith (*Biochem. J.*, **52**, 552, 1952), using a 0.05 *M* ammonium acetate buffer of pH 3.2. 11 *V*/cm were applied for 16 hours. The electropherogram was placed on X-ray-sensitive film for 2 days.

moved less rapidly and corresponded to a leucine marker. A rough estimation in the respective eluates of adenine by ultraviolet absorption and ninhydrin-positive material yielded nearly equivalent amounts, the amino acid being somewhat higher.

The following experiment further confirms the equivalence of ninhydrin-positive material and adenosine. To liberate the amino acid in this case, the compound was hydrolyzed briefly with 0.1 *N* sodium hydroxide, lyophilized, and kept in the desiccator over P_2O_5 to remove ammonia contained in the buffer. The residue was dissolved, and the adenosine content determined to be 42 mμmoles by ultraviolet absorption at 258 mμ. In the same solution, amino acid was determined according to Moore and Stein,[9] and 53 mμmoles of amino acid were found, corresponding to a ratio of adenine to amino acid of 1 to 1.25. The excess of ninhydrin-positive over ultraviolet-absorbing material was probably due to the methods of determination against a paper blank and to a slight deamination of adenosine, which could not be excluded under the conditions used here. From the radioactivity of this material, however, only 4 mμmoles of C^{14}-leucine were accounted for in this solution, corresponding to 7.5 per cent of the total amino acids as determined by ninhydrin. It appears, therefore, that the soluble amino-acid charged, ribonucleic acid contained a large amount of other amino acids in addition to leucine-C^{14}. It should be mentioned that, under the electrophoretic conditions used here, most amino acids were found to migrate in a cluster.

For the further identification of adenosine, the eluate, after hydrolysis with 0.2 *M* ammonia, was incubated with adenosine deaminase, following the procedure of Kornberg and Pricer[10] and using a crude, intestinal phosphatase (Pentex) as a source of deaminase. The eluate showed formation of inosine, as determined by a characteristic change in ultraviolet absorption at 240 and 260 mμ. This further confirms the compound to be adenosine and excludes adenylic acid, which did not react under these conditions.

Experiments are being carried out with the isolated soluble ribonucleic acid directly, as obtained from fresh rat liver for ribonuclease digestion, and aminoacyl adenosine is isolated, in complete analogy in the experiments with RNA previously charged with radioactive amino acids. Hoagland[11] independently observed that freshly isolated RNA is fully charged with amino acids.

In Figure 2 the action of ribonuclease on amino acid–charged RNA is depicted. This formulation takes into account the findings of Hecht and Zamecnik[12] that the amino acid attachment requires an end-group configuration of two cytidylic acids followed by a terminal adenylic acid. The liberation by ribonuclease of a 2′/3′-aminoacyl-adenosine indicated strongly that the amino acids in aminoacyl-RNA are bound to a ribose hydroxyl of the terminal adenosine, which is in accord with and amplifies the earlier finding.[12] Ribonuclease obviously is not prevented by amino acid esterification from its attack on a pyrimidine-purine phosphate bridge. It should be mentioned that amino acid–containing nucleotide fractions have been described by Koningsberger *et al.*[13] These fractions may be related to the soluble RNA. However, they do not show the same characteristics, and their identity has to be further established.

It appears, then, that the initial activated amino acid is transferred from the phosphoanhydride to the 2′- or 3′-hydroxyl of the terminal adenosine of RNA and

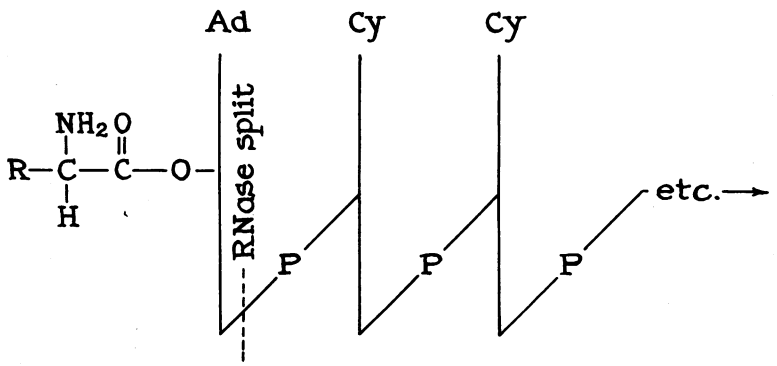

FIG. 2.—Formulation of the linking of amino acid to the terminal adenosine of the soluble RNA.

enters, in this form, the further steps in protein synthesis. The fact that this amino acid–charged soluble ribonucleic acid will react with pyrophosphate and adenylic acid to reform ATP, as has been shown previously by Schweet,[14] Berg,[15] and in our laboratory,[16] indicates that the amino acid ribose ester link must be a rather energy-rich bond, on the energy level of the phosphoanhydride link in ATP.

TABLE 1

REACTIVITY OF AMINO ACID DERIVATIVES WITH 1 *M* HYDROXYLAMINE AT pH 5.5 AND 0°

Compound*	Per Cent Decomposition after 1 Hour
Leucine ethyl ester	0
2′/3′-Leucine ester of AMP	28
C^{14}-leucyl-RNA	41
Leucyl-AMP anhydride	100

* The leucine ester of AMP was prepared according to Wieland *et al.*, *Advances in Enzymol.*, **19**, 235, 1957 and the leucyl-AMP anhydride by a modification of the procedure of Berg (*Federation Proc.*, **16**, 152, 1957). With the synthetic compounds, hydroxamate formation was measured (Lipmann and Tuttle, *J. Biol. Chem.*, **159**, 21, 1945). With C^{14}-leucyl-RNA, the liberation of radioactivity was followed.

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The following abbreviations have been used: RNA, ribonucleic acid; AMP, adenosine monophosphate; ATP, adenosine triphosphate; Tris, tris(hydroxymethyl)amino methane; RNase, ribonuclease.

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*THE GENETIC STRUCTURE OF THE INCOMPATIBILITY FACTORS IN SCHIZOPHYLLIUM COMMUNE**

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The genetic nature of the incompatibility factors in the higher fungi has been the subject of considerable interest for many years. Multiple-allelic incompatibility systems of two types, bifactorial [1, 2] and unifactorial,[3] were described in 1920–1924 and have since been found to be common in all groups of the Basidiomycetes except the rusts and possibly the smuts. The number of alternate factors at the incompatibility loci varies from ten or so in the Gasteromycetes [4, 5] to a hundred or more in the Hymenomycetes. [6, 7] The occurrence among the progeny of a single fruit of occasional non-parental factors, interfertile with both parental factors, was originally interpreted as mutation at the incompatibility loci.[8]

Schizophyllum commune typifies the bifactorial incompatibility system. Alternate incompatibility factors of two series, A and B, assort independently at meiosis to yield progeny of four distinct, self-sterile, *mating types*.

$$A^1A^2B^1B^2 \rightarrow A^1B^1,\ A^1B^2,\ A^2B^1,\ A^2B^2.$$

Sexual interaction between mycelia belonging to different mating types to yield dikaryotic mycelia is restricted to those conbinations that are heterozygous for both A and B factors. This pattern of interaction is termed *tetrapolar sexuality* or *tetrapolarity*.

The single-locus nature of the incompatibility factors and the mutative origin of non-parental factors were generally accepted until 1950, when Papazian [9, 10] found evidence, via tetrad analysis, that the A factor of *S. commune* comprised two or more loci, between which crossing over produced non-parental A factors. The six presumed recombinant classes reported by Papazian[9] were interpreted by Raper [11] as reflecting an A factor of at least four loci.