Studies on the Amino Acids Present in Yeast RNA in Bound Form

II. Liberation of Amino Acids from RNA by Alkaline Treatment*

By SHUZO AKASHI and HANAKO ISHIHARA
(From the Department of Biochemistry, School of Medicine, Nagoya City University, Nagoya)
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In a previous paper (1) it was reported that the ribonucleic acids which were prepared from various microorganisms and purified by the combined use of the Sevag method and the continuous paper electrophoresis technique proved to contain 7 to 9 amino acids, when hydrolyzed with 6 N HCl at 100°C for 20 hours. Their amino acid contents were determined to be approximately 1 per cent on the glycine basis. Suggestion has been made that these amino acids might exist as peptides in combination with the nucleic acid. In the succeeding study (2), various purified samples of yeast RNA from different sources were hydrolyzed with HCl under much milder conditions, liberating still 6 to 7 amino acids. This made it possible to consider that these amino acids might exist linked with RNA largely as single amino acids rather than as peptides.

The present work was carried out to see whether purified yeast RNA may separate amino acids by quite a mild alkali treatment. Evidence has been offered that the RNA can release amino acids which are to be divided into at least three groups by the ease of separability, indicating the possibility that there may be several at least three kinds of linkages between amino acids and RNA, differing in susceptibility to alkali. Data were also obtained in favor of the view that the amino acids may be in association with RNA in major part as peptides and in minor part as single amino acids.

Experimental and Results

Purified RNA employed for the present study is the sample designated as sample No. 2 in the previous paper (2) i.e., a commercial yeast RNA-Na (E. Merck) which was completely removed of protein by the Sevag method, followed by dialysis against distilled water at 2°C for three days.

The purified RNA preparation was successively treated with alkali of different concentrations which were raised in a triple manner such as, 0.01 N, 0.1 N, and 1 N KOH. The treatment with any of the alkali was carried out five times repeatedly, whereby digestion time was prolonged stepwise such as, e.g., 0.5, 1.0, 2.0, 6.0 and 15 hours, the total period of time extending over about 24 hours. The details are as described below.

Estimation of Amino Acids and Inorganic Phosphate Liberated in the Dialyzates of Each Alkaline Digests—2.50 g. of the purified RNA were dissolved in 250 ml. of 0.01 N KOH and digested in a water-bath at 25°C for 30 minutes, after which the reaction mixture was placed in “Visking” dialysis tubing and dialyzed at 2~3°C against frequent change of distilled water until free of potassium ion. The combined dialyzates, in amount about 12 liters, were neutralized with perchloric acid and concentrated in vacuo to a volume of 25 ml. Insoluble potassium perchlorate separated by neutralization as well as by concentration was removed by filtration. One ml. of the concentrate was used for the determination of amino acids including peptides, according to the ninhydrin method of Moore and Stein with slight modification (3). Two ml. of the concentrate were analyzed for the content of inorganic phosphate by the method of Fiske and Subbarow. The solution remaining in the dialysis tubing...
was again made 0.01 \text{N} with respect to KOH and digested again at 25^\circ \text{C} for 1 hour and dialyzed against distilled water until free of alkali. The combined dialyzates were analyzed for amino acid and phosphate. The tubing content, after four time repetition of 0.01 \text{N} KOH treatment, the digestion time being increased stepwise as mentioned above, was made 0.1 \text{N} with respect to KOH, and the digestion and dialysis procedures were carried out five times in a similar manner. The final tubing content remaining after 0.1 \text{N} KOH treatment was raised to an alkaline concentration of 1 \text{N} KOH and then subjected to the same digestion and dialysis procedures mentioned before, and the latter procedure was followed by assay of amino acids and phosphate with the dialyzate. The data obtained are summarized in Table I.

The above data are represented in the next diagram, in which the amount of amino acids and inorganic phosphate liberated by alkali of stepwise elevated alkalinity are plotted against the time of alkaline digestion.

Proof for the Presence of Peptides together with Amino Acids in the Dialyzates of Yeast RNA—This was established by the next two experiments.

1) Preliminary Test for Peptides—The final dialyzed, namely, No. 5 dialyze of each of the three fractions as designated I, II, and III in Table I was employed for this purpose. A given amount (1 ml.) of the dialyzates (25 ml.) was hydrolyzed with 3 \text{N} HCl at 100^\circ \text{C} for 20 hours and upon removal of the HCl and ammonia by distillation in vacuo, the amino acids present were determined by the ninhydrin method. The amounts of amino acids in the dialyze before and after acid hydrolysis were estimated, the data of which are recorded in Table II, indicating a considerable, i.e., 2.5 to 6.2 fold increase in ninhydrin value after acid hydrolysis, therefore the presence of not a few peptides.

2) Experiment by the Combined Use of Paper Chromatography and Paper Electrophoresis—All of the remaining portion of No. 5 dialyze of Fraction I (0.01 \text{N} KOH digest) was desalted by passing through resin columns of Dowex-I (OH) and Dowex-50(H). The final eluate was concentrated to an appropriate volume and applied to paper electrophoresis, yielding the result shown in Fig. 2-A.

| TABLE I |

**Estimation of Amino Acids (Including Peptides) in Term of Glycine and Inorganic Phosphate Separated in the Dialyzates of Alkaline Digests of Yeast RNA (2.5 g.)**

<table>
<thead>
<tr>
<th>KOH concentration</th>
<th>Fraction I</th>
<th>Fraction II</th>
<th>Fraction III</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of dialyze</td>
<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>Time of digestion (hour)</td>
<td>0.5 1.0 2.0 3.0 6.0 18.0</td>
<td>0.5 1.0 2.0 3.0 6.0 15.0</td>
<td>0.5 1.0 2.0 3.0 6.0 15.0</td>
</tr>
<tr>
<td>Time from the start (hour)</td>
<td>0.5 1.5 3.5 9.5 14.5 27.5</td>
<td>0.5 1.5 3.5 9.5 14.5 24.5</td>
<td>0.5 1.5 3.5 9.5 14.5 24.5</td>
</tr>
</tbody>
</table>

| Amino acid liberated* (mg.) | 431 548 356 330 258 | 287 523 454 237 232 | 736 1,299 1,178 701 707 |
| (mg./100g. RNA) | 17.3 22.0 14.3 13.2 10.3 | 11.5 21.0 18.2 9.5 9.3 | 29.5 52.1 47.2 28.0 28.3 |
| Total (mg.) | 1,923 | 1,733 | 4,621 |
| (mg./100g. RNA) | 77.1 | 69.5 | 185 |

| Inorganic P (mg.) | 6.38 1076 388 306 369 | 430 375 373 405 373 | 149 279 165 131 121 |
| (g./100g. RNA-P) | 0.32 0.54 0.19 0.16 0.19 | 0.22 0.19 0.19 0.20 0.19 | 0.073 0.014 0.083 0.066 0.061 |
| Total inorg. P liberated (mg.) | 2,777 | 1,956 | 845 |
| (g./100g. RNA-P) | 1.40 | 0.99 | 0.43 |

* Estimation of amino acids was carried out on the glycine basis, in which correction was made for the ninhydrin value due to the perchloric acid which was used for neutralization of alkali, since significant amounts of ninhydrin positive substance especially ammonia was present in the reagent.
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pattern, which was corresponding to neutral amino acid and was assigned with an arrow mark, was cut off from the paper and extracted with water. The extract was developed by two-dimensional paper chromatography to find an unknown spot in addition to those spots for glycine, serine, valine, etc. as given in Fig. 2-B.

This unidentified spot suspected of peptide was cut off from the paper and extracted with water. The extract was hydrolyzed with 3 N HCl at 100°C for 20 hours. The hydrolyzate was rechromatographed to identify the spot for serine, glycine, glutamic acid and histidine. The relevant chromatogram is illustrated in Fig. 2-C.

The same experiment was also made with each of No. 5 dialyzates of Fraction II (0.1 N KOH digest) and Fraction III (1 N KOH digest), obtaining results similar to those found with Fraction I.

Formation of Histidine Hydroxamate—One g. of purified RNA was dissolved in 4 ml. of water and 10 mg. of hydroxylamine hydro-

Fig. 1. Liberation of amino acids and inorganic phosphate from the purified yeast RNA by alkaline treatment, the alkalinity of which being elevated stepwise. The hatched areas denote amino acids liberated and the dark ones inorganic phosphate released. The area which is situated above No. 5 dialyze of each fraction and enclosed by a dotted line indicates the increments of ninhydrin values produced by total acid hydrolysis of that portion of dialyzates.

|TABLE II|Increase in Ninhydrin Values in Term of Glycine of Dialyzates by Acid Hydrolysis|
|Dialyze*|  |  |  |
|  | No. 5 (Fraction I) | No. 5 (Fraction II) | No. 5 (Fraction III) |
|Ninhydrin value before acid hydrolysis | µg. | µg. | µg. |
|after acid hydrolysis | 10.3 | 9.28 | 28.3 |
|  | 63.8 | 9.28 | 70.7 |
|  | (10.3×1.2) | (9.28×5.4) | (28.3×2.5) |

* Analysis was made using 1 ml. portion of each 25 ml. dialyzates.
chloride were added. The reaction mixture, upon adjustment of pH at about 5.5, was allowed to stand overnight at 2–3°C with occasional shaking. The solution was next dialyzed against distilled water and the dialy- zate was evaporated in vacuo to dryness. The residue was dissolved in a little water and applied to paper electrophoresis. The pattern developed with ferric chloride solution revealed a single spot of reddish-brown color in accord with the check spot of histidine hydroxamate, as shown in Fig. 3.

**DISCUSSION**

It has been experimentally evidenced that the purified yeast RNA is able to separate very small amounts of amino acid as well as inorganic phosphate in three steps corresponding to varying alkalinity, when treated with dilute alkali such as, 0.01 N, 0.1 N, and 1 N KOH at 20°C. The separated amount of amino acid in each of the three fractions I, II, and III was in the order of III > I > II, whereas that of inorganic phosphate was I > II > III.

The fact that amino acids appear forming three sharp peaks in the alkaline digest of RNA seems to suggest that at least three different alkali susceptible links may exist between amino acid and nucleic acid. With regards the inorganic phosphate, which was liable to be liberated mainly in Fraction I and II, is considered to be of alkali labile nature.

According to the work by Potter and Dounce (5), it was pointed out that firmly bound amino acids are present in the alkali stable fractions of the Schmidt-Thannhau ser digest of RNA from calf pancreas, rabbit, and yeast. The amino acids were
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postulated to be linked with phosphate group through their amino group as phosphoamide. However, they failed to observe amino acids in the mononucleotide, or alkali labile fraction. This alkali labile fraction was meant by those fractions which can be split off as mononucleotide, when RNA is treated with 1N NaOH at 37°C for 15 hours. In the present study, however, an alkali labile fraction does mean those fractions which are released by treatment with alkali having concentration equal to or smaller than 1 N KOH, e.g., 0.01N and 0.1N KOH at 20°C for about 24 hours.

Concerning the other type of the bond involved between amino acids and the nucleotide, carboxy-phosphoryl or carboxy phosphoanhydride type was postulated as initial activation step of amino acid prior to protein synthesis by Hoagland (6). Meanwhile Lipmann et al. (7) made an experiment which demonstrated, using C14-labelled leucine, the formation of amino acid ribose ester bond in the soluble RNA of the rat liver, an important finding from the point of protein synthesis.

The other type of combination of amino acid with nucleic acid or nucleotide will be via pyrophosphate as found in uridine-diphosphate compounds. The latter compounds, however, usually readily release inorganic phosphate as well as component sugar by mild acid hydrolysis, but not by mild alkali treatment, since the glycosidic linkage between phosphoric acid and sugar is rather resistant to alkali. In this connection the work of this laboratory (8) should be noticed, according to which from a unique strain of Alcaligenes foecalis was isolated a peculiar kind of nucleic acid, conveniently named as “alcaligenic acid”, and the substance contained about 8.5 per cent of sugar-peptide complex in bound form together with labile phosphate, both of which were split off when heated for 7 minutes at 100°C in 1 N HCl. The present data which showed that mild alkali treatment of yeast RNA is able to liberate simultaneously both inorganic phosphate and amino acids may possibly be accounted for by assuming that the amino acids and pyrophosphate are bound together either by an aminoacyl or an amido-phosphoryl group. Since no information is as yet available concerning such kind of compound, these types of combination seem to be possible, but less probable. Anyway, the nature and origin of alkali labile phosphate in yeast RNA still remain to be elucidated.

In view of those mentioned above, it appears likely that at least four types of linking may be involved between amino acid and RNA, namely, 1) N-phosphoryl type, 2) amino acid-phosphoanhydride type, 3) amino acid ribose ester type, and 4) diphosphate or pyrophosphate type. Moreover, the possibility cannot be excluded that amino acids are linked with the base components of RNA.

These various types seem to differ from each other in lability to alkali. Among them the most labile ones will be the aminoacyl-phosphoanhydride type and the diphosphate type because of their acid anhydride nature. The less labile one will be the N-phosphoryl type, since according to the study of Chantrenne (9) as cited by Potter and Dounce (5) the phosphoamide bonds of the type

$$\text{R-O-P-NH-R}$$

are more stable than

$$\text{OH}$$

the simple phosphoamide bonds that occur in phosphocreatine and phosphoarginine. The least labile one will be the ribose ester type. One of the reasons for this is, as reported by Lipmann (7), that between 2/3-leucine ester of AMP and leucy-AMP anhydride there is found considerable difference of reactivity of these amino acid derivatives with 1M hydroxylamine at pH 5.5 and 0°C, the former having values much lower than the latter.

Based on this premise the data of the present study can be accounted for as follows. Fraction III seems to include those amino acids which are not so readily liberated by dilute alkali, therefore being of the ribose ester type. Fraction I, on the contrary, may contain the most labile amino acids, accordingly, those belonging to the aminoacyl-phosphoanhydride and the diphosphate type. Fraction II, being the intermediate of Fraction
I and II, may represent the less labile amino acid involved in the N-phosphoryl type. Whether or not this conjecture is real, is a problem to be solved experimentally by future study.

However, it can be said undoubtedly that small amounts of amino acids bound to RNA are set free at least in three groups by various mild alkali treatments, owing to the different susceptibility to alkali of the linkages involved. A support for the presence of labile amino acid has been provided by the formation of histidine hydroxamate. However, none of the other amino acid hydroxamate has yet been obtained, for which further detailed studies are required. The reason for the hydroxamate formation limited to histidine may be partly due to the fact that histidine is apparently the predominant amino acid bound to the RNA in concern. That the free amino group is present in the various amino acids linked with RNA has been demonstrated by the unpublished data obtained in this laboratory, according to which dinitrophenylated RNA preparations were treated in a similar manner with different mild alkali, yielding several kinds of DNP-amino acids in the dialyzates.

With regard to the occurrence of peptide in bound form, the amino acid found in the RNA being studied seems to be associated in major part as peptide and in minor part as amino acid, as shown in Table II, which represents a considerable increase in ninhydrin value of No. 5 dialyzate of each fraction as a result of acid hydrolysis of the dialyze. The study is now in progress, to elicit the nature of links between amino acid and nucleotide with the fraction which appears as nucleotides in Fraction III.

SUMMARY

1. Yeast RNA, completely purified by the Sevag method and prolonged dialysis, separated in the dialyzates very small amounts of mixture of amino acid and peptide together with a trace of inorganic phosphate, forming three sharp peaks of Fraction I, II and III, when treated consecutively at 20°C with dilute alkali of varying concentrations such as, 0.01 N, 0.1 N, and 1 N KOH in a triple manner and each time dialyzed. Treatment with the same alkali concentration was carried out repeatedly five times, the digestion time of which being prolonged stepwise such as, 0.5, 1.0, 2.0, 6.0, and 15 hours.

2. The nature of links between amino acid and RNA involved in three fractions and also the origin of inorganic phosphate were conjectured.

3. Fraction I, the dialyzate of 0.01 N digest, comprising the most alkali labile amino acids, presumably deals with the aminoacyl-phosphoanhydride type and the diphosphate type. Fraction II, the dialyzate of 0.1 N KOH digest, being less alkali labile, may represent the N-phosphoryl type. Fraction III, the dialyzate of 1 N KOH digest, being relatively alkali stable, may include the amino acid ribose ester type.

4. A greater part of the bound amino acids seems to be in peptide form.

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