FORMATION OF METHYLENE BRIDGES IN THE REACTION OF RIBONUCLEIC ACID WITH FORMALDEHYDE

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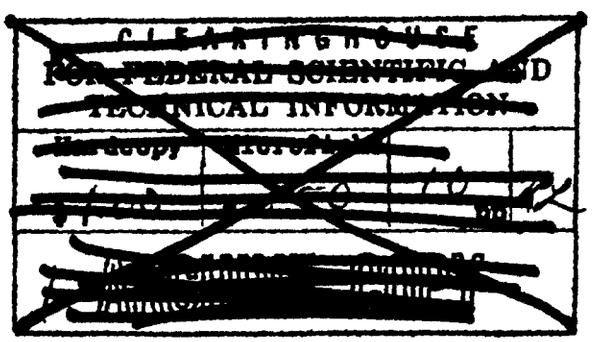

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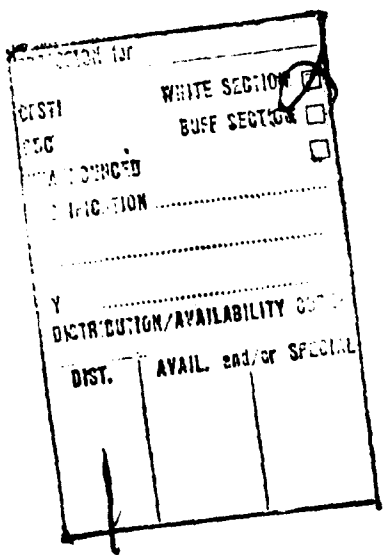

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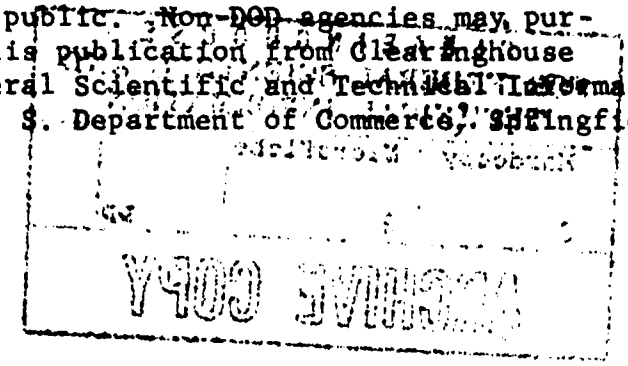

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FORMATION OF METHYLENE BRIDGES IN THE REACTION OF RIBONUCLEIC ACID WITH FORMALDEHYDE

[Following is the translation of an article by M. Ya. Feldman, Institute of Radiation and Physical-Chemical Biology, USSR Academy of Sciences, Moscow, published in the Russian-language periodical Biokhimiya (Biochemistry), 1965, Vol 30, No 1, pages 203-207. It was submitted on 29 Sep 1964. Translation performed by Sp/7 Charles T. Ostertag Jr.]

Fraenkel-Conrat [1] showed for the first time that ribonucleic acid (RNA), nucleotides, nucleosides and bases, containing an amino group, react with formaldehyde; during treatment with formaldehyde their spectra are changed. The spectral changes are reversible by dialysis or by ordinary dilution of the solutions [1-3]. We showed that the labile products of the reaction are methylol derivatives $RNHCH_2OH$ [4; 5]. The data from kinetic investigations [6; 3] conform with the formation of these structures.

In the case of adenine and adenosine, in addition to the labile methylol derivatives, following the prolonged maintenance of the solutions, secondary stable crystallizing condensation products are formed -- methylene-bis-compounds $RNH\text{-}CH_2NHR$ [5]. An analogous product is formed in the reaction of 6-aminouracil with formaldehyde [4]. A number of authors [7; 4; 8] have expressed the supposition concerning the possibility of the formation of such structures (with methylene bridges) in the reaction of RNA with formaldehyde.

In the present investigation this supposition receives experimental confirmation: From RNA, treated with formaldehyde, methylene-bis-adenosine was isolated.

Experimental Stage

Preparations. In all the tests we used the "highly polymeric" RNA of rabbit liver. The liver homogenate, following the precipitation of the nuclei and the incomplete destruction of the cells, was treated twice with equal volumes of water saturated phenol. The residue of phenol was removed from the aqueous phase by ether. The RNA was pre-

cipitated and re-precipitated with NaCl (end concentration -- 1.5 M). After washing the precipitate with 1.5 M NaCl and dissolving it in water, we obtained a transparent or slightly opalescent solution, from which the sodium salt of RNA was precipitated with alcohol in the presence of acetate (pH 4.8). The precipitate, washed with a mixture of alcohol with 0.1 M acetate buffer, pH 4.8 (2:1) and alcohol, was dried in a dryer at 4°. The protein content in the preparation, measured according to Loury, did not exceed 0.5%. Phosphorus of adenylic acid, isolated from the alkaline hydrolysate of RNA by chromatography on Dowex-1 in formate form [9], comprised 18.84% of the total phosphorus of the preparation, which corresponds with the data from the literature for microsome RNA of rabbit liver [10].

Formaldehyde was added to the tests in the form of a 1 M solution (in an acetate buffer, pH 4.8), prepared from paraformaldehyde. In the tests with tagged formaldehyde we used a commercial preparation of $C^{14}H_2O$ with a specific activity of 7.7 millicurie/g.

Acid phosphomonoesterase was isolated from the prostrate of a man and given to us by R. I. Tatarskaya (Laboratory of Functional Enzymology of the Institute of Radiation and Physical-Chemical Biology).

Analytical Methods. Difficulties were noted earlier in the chromatographic investigations of methylene-bis-adenosine [5; 3]; from a number of solvents tested it turned out that the most suitable for use was a mixture of 170 ml isopropanol, 41 ml of concentrated HCl, and up to 250 ml of water. We also used this solvent in our investigations. Chromatography was carried out on Vatman 4 paper in an ascending stream of the solvent. That amount of hydrolyzate was applied to the paper which would contain ~ 0.3 mg of RNA; when comparing the alkaline and phosphatase hydrolyzates they were applied in equivalent amounts. The spots on the chromatograms were determined on an ultrachemiscope or with the help of negative imprints on reflex photographic paper.

In the work with C^{14}-formaldehyde, in order to detect the position of the tag on the chromatogram, we cut out the strip of paper with the spots of the RNA hydrolyzate and cut it across into 0.5 cm sectors. For the measurement of radioactivity the strips were placed in a liquid non-polar scintillator, and the activity was determined on a "Karbotrimetr" device (firm L. I. E. Berlin, model 1959). All the data in the work was submitted for "background" deduction, that is, the activity of those sectors of the chromatogram strip which are devoid of spots (above the starting line and higher than the spots of the most mobile, compounds -- uridylic acid and uridine); apparently this background is caused by

labile bound formaldehyde and the residues of free CH_2O in the RNA hydrolyzates. In the quantitative investigations, the standard was C^{14}-methylene-bis-adenosine, obtained in the reaction of adenosine with $C^{14}H_2O$ and applied to the chromatogram in a pyridine solution.

The spectra were taken on a SF-4 device. Phosphorus was determined according to the formation of a stained molybdate complex.

Isolation and Identification of Methylene-bis-adenosine. The methylene derivative of adenosine was isolated from the formaldehyde treated RNA according to the following scheme: RNA was hydrolyzed by successive treatment with alkali and phosphatase; from the mixture of nucleosides obtained, the methylene-bis-adenosine was extracted in the form of a precipitate, not soluble in water.

Solutions were prepared in an acetate buffer, pH 4.8, with such a content of the RNA preparation that its concentration (based on phosphorus) comprised 64 mg in 10 ml (in the volume of the test); the end concentration of CH_2O in the solution -- 0.2 M, acetate -- 0.02 M. The pH of 4.8 was selected on the basis of data from a previous investigation [5], in which it was shown that this pH value is optimal during the condensation of adenine and adenosine with formaldehyde.

The mixture was maintained for 20 days at room temperature and after the addition of acetate (pH 4.8) to a concentration of 0.1 M the ribonucleic acid was precipitated with a double volume of ethyl alcohol (80-85% of the RNA passed into the precipitate). For hydrolysis to nucleotides, 5 ml of 0.5 M KOH was added to the RNA, and the test was maintained for 20 hours at 36°.

In order to separate the excess of K^+, 57% $HClO_4$ up to pH 7-8 was added to the solution. The supernatant liquid was mixed with an equal volume of water and with the help of 0.5 M HCl the pH of the solution was brought to 5. After the addition of phosphatase preparation (1 mg for 10 mg of RNA nucleotides) the mixture was incubated in a water bath at 37°. During the process of incubation, 0.05 ml samples were taken in which the inorganic phosphate was determined. As a rule the increase of inorganic phosphate ceased after 30 minutes. In the termination of incubation the temperature of the bath was raised up to 100° and the mixture was maintained at this temperature for 5 minutes. The precipitate of denatured proteins was discarded. The completeness of phosphatase hydrolysis was controlled chromatographically. In the event of incomplete hydrolysis the incubation with ferment was repeated.

The mixture of nucleosides was maintained for a period of several days or evaporated after neutralization (KOH) in a rotor evaporator

under vacuum. In both cases the resulting precipitates were washed with water many times (1.5 -- 2 ml each of five - eight times) with chromatographic control. Left in the precipitate was methylene-bis-adenosine (see identification below) without admixtures of other nucleosides. In the process of isolation it was not possible to avoid significant losses of methylene-bis-derivative. The yield comprised 0.2 - 0.3 mg.

For chromatographic identification an aqueous suspension of the resulting precipitate was applied on paper; methylene-bis-adenosine (standard) was also applied in the form of an aqueous suspension (the method of applying a substance in the form of an aqueous suspension or in a pyridine solution does not reflect on its mobility [3]. As is seen from figure 1, based on mobility on the chromatogram the investigated substance corresponds to methylene-bis-adenosine.

On chromatograms, obtained directly after completion of phosphatase hydrolysis (before the precipitation of the substance), spots were also detected which corresponded in mobility to methylene-bis-adenosine -- standard (R_f 0.25). On the ascending chromatogram (a drawing of it is presented in figure 2,b) this spot is located lower than the spots of the other nucleosides and is clearly separate from the neighboring spot of guanosine (R_f 0.39); it is absent on control chromatograms of RNA hydrolyzates not treated with formaldehyde.

The UV spectrum of the isolated substance corresponded with the spectrum of methylene-bis-adenosine (table 1).

For the identification of the bases found in the composition of the isolated product, it was hydrolyzed in 1 M HCl at 100°. Hydrolysis was performed in a sealed tube and the solution contained 0.1 mg of substance in a volume of 0.1 ml. Chromatographically one base was detected in the hydrolyzate -- adenine (figure 1).

In the tests with C^{14}-formaldehyde (figure 2,b and table 2) the spot of the substance being identified on the chromatogram of the phosphatase hydrolyzate contained a tag in quantities considerably exceeding its content on other sectors of the chromatogram.

Thus, the substance detected by us in RNA, treated with formaldehyde and hydrolyzed to nucleosides, is a product of the condensation of adenosine with formaldehyde; based on chromatographic mobility and UV spectrum it is identified as methylene-bis-adenosine.

Identification of Methylene-bis-adenylic Acid. In the tests of the alkaline hydrolyzate of RNA, treated with formaldehyde under the accepted test conditions (pH 4.8, prolonged maintenance at room

temperature), a chromatographically unchanged "supplementary" spot was detected which was absent in the case of non-formaldehyde treated RNA. Just as in hydrolysis to nucleosides (see above), on an ascending chromatogram the "supplementary" spot is located lower than the other spots. However in contrast to the nucleosides, the "supplementary" spot of the alkaline hydrolyzate was not completely separated from the neighboring spot of guanylic acid (figure 2). The spots of the substances are distinguished from each other due to the characteristic blue shimmer of guanylic acid. With the help of other systems of solvents we were not able to achieve a more complete division of nucleotides of RNA treated with formaldehyde.

As is seen from figure 2,A, almost all the radioactivity of the products of alkaline hydrolysis of RNA which has been treated with $C^{14}H_2O$ is concentrated in the "supplementary" spot. The activity, arriving at the spot of GMF [*], as this follows from the position and form of the peak, belongs apparently not to GMF, but to the "supplementary" substance; a new peak is lacking, the symmetrical form of the curve is not altered.

[* GMF -- guanosine monophosphate, and in figure 2, A -- AMF -- adenosine monophosphate; CMF -- cytidine monophosphate; UMF -- uridine monophosphate.]

From figure 2,b it is seen that after phosphatase hydrolysis the activity transfers to the already identified (see above) spot of methylene-bis-adenosine (the non-symmetrical form of the peak is probably explained by the small admixture of the initial phosphorylized product). Thus, the initial phosphorylized compound, corresponding to this derivative (methylene-bis-adenosine) and which above was designated as "supplementary," is methylene-bis-(adenosine-3'- phosphate).

This conclusion is supported in the second test (table 2), set up in a somewhat different variation: The Concentration of $C^{14}H_2O$ is 0.04 M; the activity was determined in the cut out spots (and not in the strips of an evenly cut chromatogram) on an end-window counter (T-25-BFL). And in this case the radioactivity of the "supplementary" spot of the alkaline hydrolyzate following dephosphorylation of the nucleotides passes over to the activity of methylene-bis-adenosine.

The position of the phosphate groups (3') in the methylene-bis-adenylic acid results from the known mechanism of alkaline hydrolysis of RNA. The amount of adenylic acid in the composition of the methylene-bis-derivative (measures by C^{14}) in the test with 0.2 M CH_2O (figure 2) comprised 21.8% of the total amount of adenylic acid in the preparation of RNA, in the test with 0.04 M CH_2O (table 2) -- 8.8%.

In the control tests with RNA, not treated with formaldehyde, and RNA, precipitated and hydrolyzed immediately after the addition of formaldehyde (0.2 M), the "supplementary" spot was absent on the chromatograms of the alkaline hydrolyzate.

Summary

From RNA, processed with formaldehyde, a product of the condensation of adenosine with formaldehyde was isolated. It was identified by chromatographic mobility and UV spectrum as methylene-bis-adenosine.

In the alkaline hydrolyzate of RNA, treated with $C^{14}H_2O$, C^{14}-methylene-bis-adenylic acid was detected chromatographically.

Thus, experimental confirmation was obtained of the earlier expressed supposition concerning the formation of methylene bridges in the reaction of RNA with formaldehyde.

We express our thanks to R. I. Tatarskaya for giving us the preparation of phosphatase and to M. R. Shubinaya for assistance in the work.

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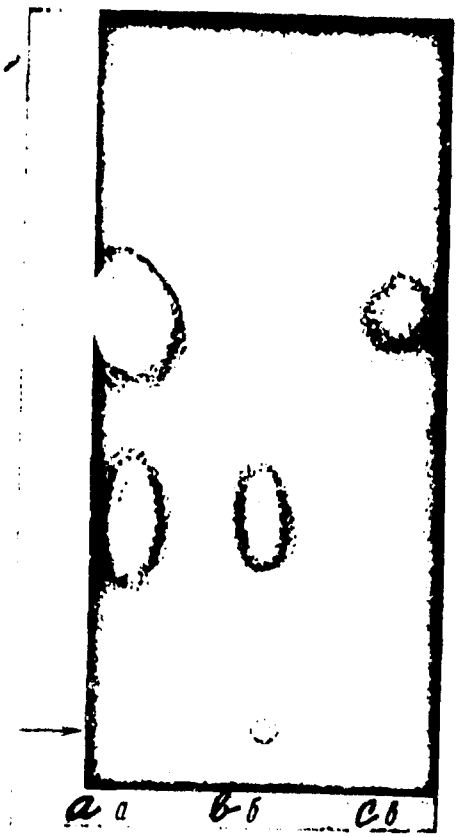

Figure 1. Identification of the insoluble product, isolated from the alkaline-phosphatase hydrolyzate of RNA, treated with formaldehyde. a - standards: Lower spot -- methylene-bis-adenosine, upper -- adenine; b - substance being identified; c - product of its acid hydrolysis. The arrow indicates the starting line.

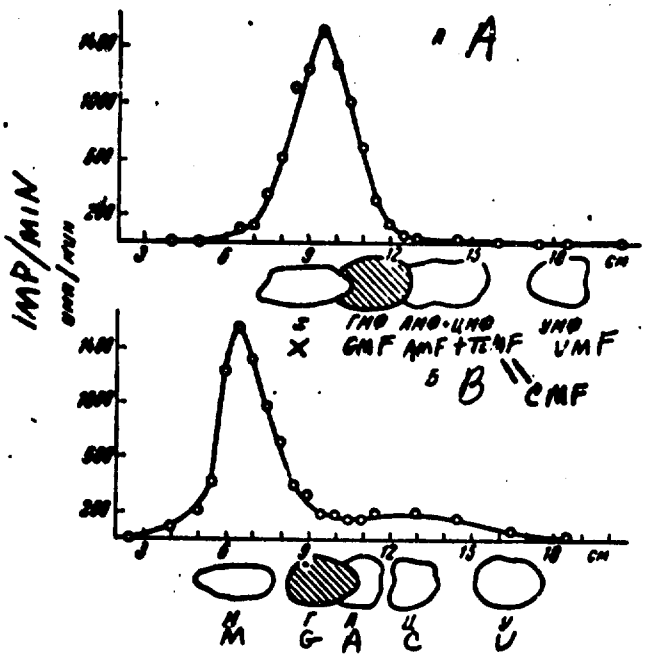

Figure 2. Radioactivity of the hydrolysis products of RNA treated with $C^{14}H_2O$

The mixture, containing 4.1 mg RNA, 0.2 M $C^{14}H_2O$ and 0.01 M acetate buffer, pH 4.8, in a volume of 0.55 ml, maintained for 5 months at room temperature. A -- alkaline hydrolyzate of RNA, B -- alkaline-phosphatase hydrolyzate. Under the curves for radiation -- their appropriate arrangement on the chromatogram, x -- "supplementary" spot of alkaline hydrolyzate, Methylene--bis-adenosine; G, A, C, U -- other nucleocides. The activity of the cut strips was measured on a scintillation counter. "Background" (see text) -- 294 imp/min. Axis of abscissae -- distance from starting line. Luminescent spots are cross hatched.

Table 1

Characteristics of the spectrum of the substance being identified in 0.25 M KOH

Index	Investigated substance	Methylene-bis-adenosine [3]	Index	Investigated substance	Methylene-bis-adenosine [3]
λ_{max}, mmk	270*	270**	E_{250}/E_{260}	0.71	0.68
λ_{min}, mmk	232--233	231	E_{280}/E_{260}	0.93	0.94

* Optical density of the solution at 270 mmk in the test comprised 0.984.
** ε_{max} 30 800

Table 2

Radioactivity of the hydrolysis products of RNA treated with $C^{14}H_2O$ (in imp/min)

Reaction mixture contained 0.04 M $C^{14}H_2O$; other conditions the same as figure 2. Radioactivity of the spots cut from the chromatograms was measured on an end-window counter., "Background" -- 42 imp/min.

Derivative	Alkaline hydrolyzate (nucleotides)	Alkaline phosphatase hydrolyzate (nucleosides)
Uracil	13	14
Cytosine	28	7
Adenine		8
Guanine	62*	14**
Methylene-bis-adenine	157***	153

* On the lower half of the GMF spot, combining with the "supplementary" spot, it is 57 imp/min.
** The sector of the chromatogram between the spots of methylene-bis-adenosine and guanosine weakly absorbed UV rays and possessed an activity equal to 50 imp/min.
*** Activity of the identified "supplementary" spot of alkaline hydrolyzate.