between 5 and 10 kcal. higher than E_1 for denaturation.

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[CONTRIBUTION FROM THE DEPARTMENTS OF CHEMISTRY AND BIOLOGICAL CHEMISTRY OF WASHINGTON UNIVERSITY]

The Mechanism of the Alkaline Hydrolysis of Ribonucleic Acids

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Yeast ribonucleic acid was subjected to hydroxide ion catalyzed hydrolysis in H_2O^{18}. The phosphate groups of the mononucleotides formed in the hydrolysis were found to contain one atom of O^{18} per atom of phosphorus. A mechanism for the hydrolysis of ribonucleic acid which is in accord with these data is discussed.

The discovery of two isomeric forms of each mononucleotide,[2] as well as cyclic nucleoside-2′,3′-phosphoric acids,[3] in alkaline hydrolysates of yeast ribonucleic acid stimulated interest in the mechanism of alkaline hydrolysis of this acid. To explain the formation of isomers and cyclic phosphates, Brown and Todd[4] invoked Fonó's interpretation of the alkali lability of ribonucleic acids[5] and elaborated a hydrolysis mechanism which involved the intermediate formation of a cyclic *triester*. In an effort to obtain more detailed information concerning the reaction mechanism, the alkaline hydrolysis of ribonucleic acid has been carried out in H_2O^{18}. The results of this work, taken in conjunction with the known properties of phosphate esters, are in agreement with a modification of the mechanism originally suggested by Brown and Todd.[4]

Experimental

Materials.—A sample of yeast ribonucleic acid (Schwarz Laboratories, Inc., Mount Vernon, N. Y.) was purified by precipitating twice with sodium chloride–ethanol.[6] This purified product (RNA), which was shown by dialysis to contain no free mononucleotides, was used in the hydrolysis experiments.

Dry Dowex A-1 chloride (Dow Chemical Company, Midland, Mich.), 200–400 mesh, was extracted with 95% ethanol in a Soxhlet extractor. The extraction was continued until the optical density at 260 mμ of two successive samples of ethanol from the extraction thimble was the same.[7] After drying the extracted resin to constant weight, it was converted to the formate form by means of a solution 1 *N* in both formic acid and sodium formate.

The charcoal used for adsorbing the nucleotides from the ion exchange column eluates was prepared as follows: About 50 g. of Darco G-60 (Darco Corporation, New York, N. Y.) was added to 1 l. of 6 *N* hydrochloric acid. After boiling this mixture for 45 minutes, the supernatant liquid was removed by decantation. This operation was repeated twice. The charcoal next was boiled for 45 minute periods with 1-l. portions of distilled water until the supernatant gave a negative chloride test. Finally, the charcoal was removed by filtration through a sintered glass funnel and air-dried at 120° for 24 hours. Samples of Darco G-60 treated in this way were found to contain 0.0% phosphorus.

The intestinal phosphatase employed in these experiments was a commercial preparation (Armour Laboratories, Chicago, Ill.) containing 15 units/mg.[8]

The O^{18}-enriched water used in this work contained 1.97 atom per cent. excess O^{18}.

Hydrolysis of RNA.—A 350-mg. sample of the sodium salt of RNA was dissolved in 20 ml. of 0.5 *M* sodium hydroxide in H_2O^{18}. This solution was kept at 37° for 17 hours. The resulting hydrolysate was concentrated to a small volume *in vacuo* at room temperature.

Chromatographic Separation and Isolation of the Nucleotides.—The concentrated hydrolysate was taken up in water and chromatographed on a 3.8 sq. cm. × 15 cm. long Dowex A-1 formate column.[2c] For the elution, the following solutions were used successively to displace the stated nucleotides from the column: cytidylic acids, 0.01 *N* formic acid; adenylic acids, 0.070 *N* formic acid plus 0.0070 *N* sodium formate; uridylic acids, 0.0017 *N* formic acid plus 0.030 *N* sodium formate; guanylic acid-*a*, 0.0042 *N* formic acid plus 0.103 *N* sodium formate; and, finally, guanylic acid-*b*, 0.0042 *N* formic acid plus 0.171 *N* sodium formate. All of the nucleotides except the uridylic acids-*a* and -*b* were eluted as distinct peaks. The uridylic acids were eluted as a single peak with two distinct maxima. The fractions containing this peak were divided at the midpoint between the maxima to yield two fractions, one containing predominantly *a*-isomer the other predominantly *b*-isomer.

The individual nucleotides in the various fractions of the ion exchange column eluate were concentrated as follows: A portion of acid-treated Darco G-60 was added to a given fraction[9] and the mixture stirred for a few minutes. The charcoal[5] containing the adsorbed nucleotides then was removed by filtration, was washed free of formic acid and formate with two portions of distilled water and finally was dried in a vacuum desiccator containing activated alumina.[7]

The nucleotides were recovered from the charcoal samples by exhaustive elution with a 5% aqueous solution of pyridine. The combined pyridine eluates from a given charcoal sample were extracted three times with equal volumes of chloroform to remove the pyridine and then were distilled almost to dryness under reduced pressure. These concentrates, containing individual nucleotides, subsequently were hydrolyzed in order to convert organically-bound phosphate to inorganic phosphate.

Hydrolysis of the Mononucleotides.—A concentrate containing a single nucleotide was dissolved in 10 ml. of a solution containing 0.1 *M* veronal buffer, *p*H 8.9, and 0.008 *M* $MgCl_2$. The resulting mixture was incubated for 2 hours at 37° with 5 mg. of intestinal phosphatase. At the end of the incubation, the protein was precipitated with trichloroacetic acid and the inorganic phosphate was isolated and analyzed for O^{18} as described previously.[10]

(1) Part of this work was done during the tenure by one of us (M.C.) of an Established Investigatorship of the American Heart Association.

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Results and Discussion

The O^{18} concentration in the inorganic orthophosphate obtained from each nucleotide is given in Table I. The sample of cytidylic acid-*a* was lost. The values found vary between 80 and 90% of the theoretical value for one atom of oxygen introduced into the phosphate. The reason for this deviation is not understood; the exchange observed by Stein and Koshland[11] between inorganic phosphate and water, with alkaline phosphatase as catalyst, is too small to explain this deviation.

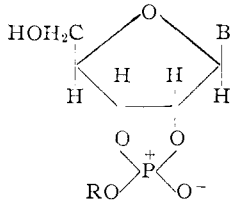
TABLE I

OXYGEN 18 CONCENTRATION IN NUCLEOTIDES OBTAINED BY ALKALINE HYDROLYSIS OF RIBONUCLEIC ACID IN H_2O^{18}[a]

Nucleotide	Atom per cent. excess O^{18}	No. of atoms O^{18} introduced
Adenylic acid-*a*	0.436	0.89
Adenylic acid-*b*	.409	.83
Guanylic acid-*a*	.406	.83
Guanylic acid-*b*	.389	.79
Cytidylic acid-*b*	.393	.80
Uridylic acid-*a*	.438	.89
Uridylic acid-*b*	.446	.91

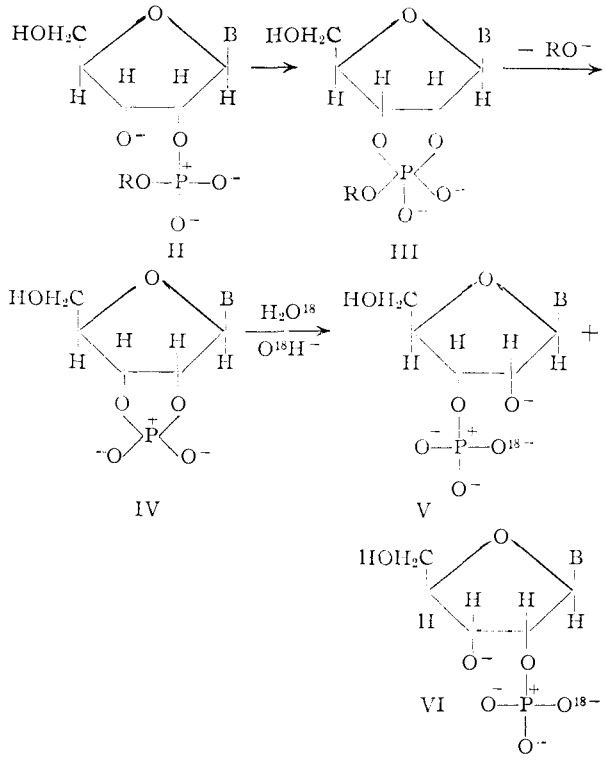
[a] The H_2O^{18} contained 1.97 atom per cent. excess O^{18}.

Brown and Todd[4] proposed that the first step in the hydrolysis of ribonucleic acids is the formation of a cyclic triester of orthophosphoric acid (I)



where R represents a nucleoside and B represents a purine or pyrimidine base. This step involves, in essence, the conversion of a dialkylphosphoric acid to a trialkyl phosphate in alkaline solution. Since no examples of the esterification of an orthophosphate anion by an alcohol are known, this conversion is very unlikely. Further, alkaline hydrolysis of I in H_2O^{18} will give IV, with one of the negative oxygen atoms replaced by O^{18}, since it is known that hydroxide ion catalyzed hydrolysis of phosphate esters takes place with cleavage of the O–P, rather than the C–O, bond.[12] Subsequent hydrolytic cleavage (in H_2O^{18}) of this cyclic phosphate, already containing one O^{18} attached to phosphorus, will then yield the isomeric mononucleotides V and VI, each with two of the negatively charged oxygen atoms on the phosphorus replaced by O^{18}. This conclusion is not in agreement with our finding that only one O^{18} atom per phosphorus atom is introduced into the phosphate group of the nucleotides.

A mechanism[7,13] which is consistent with the data, in that it predicts the appearance of only one O^{18} atom per phosphorus atom in the final mononucleotides (V and VI), is



It should be noted that II is written with the hydroxyl group on C_3'(or C_2') as an alkoxide ion, since in 0.5 *N* hydroxide this species would be present in appreciable concentration.[14] Nucleophilic attack on the positively charged phosphorus atom (II → IV) certainly would take place at a more rapid rate with alkoxide ion than with the corresponding alcohol. In going from II to IV, whether or not a pentacovalent phosphorus compound such as III is an intermediate,[15] is immaterial. The formation of an intermediate triester (I), originally suggested by Brown and Todd[4] and more recently designated by these authors[13] as a "classical" version of the mechanism represented above, is excluded, however, by the data given in this paper.

The mechanism presented here is also in agreement with the observation that the methoxide ion catalyzed methanolysis of yeast ribonucleic acid results in the formation of monomethyl esters of the constituent nucleotides.[16] Dimethyl esters would have been expected on the basis of Brown and Todd's original mechanism[4] for the base-catalyzed degradation of ribonucleic acids. Further, since the mechanism assumes O–P, rather than C–O, bond cleavage it also explains why xylo- or arabinonucleotides are not obtained in the isomerization of ribonucleotides or in the alkaline hydrolysis of ribonucleic acids.

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