"STUDIES ON THE DEGRADATION OF PANCREAS RIBONUCLEIC ACID"

by

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Abstract

1. Methods were developed for:
   a) the separation of free purines and pyrimidine nucleotides.
   b) the direct determination of cytidylic and uridylic acids
2. Pancreas ribonucleic acid was found to be very sensitive to aqueous hydrolysis at 25° C. as compared with yeast nucleic acid, the residue being resistant to further hydrolysis by ribonuclease.
3. Ribonuclease renders the nucleic acid soluble in glacial acetic, indicating the depolymerization of nucleic acid.
4. The depolymerized nucleic acid consists of 2 fractions:
   a) non-diffusible and
   b) diffusible through cellophane membrane, and precipitable with 3 volumes of alcohol.
5. Ribonuclease also liberates free mononucleotides, diffusible through cellophane membrane, and non-precipitable with 3 volumes of alcohol, with the exception of guanylic acid which does precipitate.
6. All four mononucleotides are found to be liberated from the nucleic acid, the amount of pyrimidine exceeding that of the purine nucleotides.

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Introduction

A. Historical

Ribonuclease (RN-ase) was discovered by Walter Jones in 1920 (26). He boiled an aqueous extract of pancreas and destroyed all enzymes except one, which decomposes ribonucleic acid (RNA) not thymus nucleic acid. Jones found that nucleic digested with this enzyme was no more precipitable with sulfuric acid. In 1923 Jones and Perkins (27) were able to separate four mononucleotides from the digestion mixture. Using Levene's procedure (41) of fractionation with alcohol they obtained guanine and adenine fractions, and concluded that the enzyme breaks inter-nucleotide linkages, although they admitted the possible presence of some substances intermediate between nucleic acid and mononucleotides. They reported that no increase in titratable acidity was produced by the enzyme action. This is now disproved, and it is contradictory to their findings, because if mononucleotides are produced on liberating internucleotide ester linkages, increase of acidity must occur.

Schmidt and Levene (55) concluded that the function of the enzyme is depolymerization, limited to the dissociation of tetranucleotides of high molecular weight into those of lower molecular weight, without the formation of mononucleotides. They considered the name "ribonucleodepolymerase" to be more appropriate for the enzyme

The first attempt to purify this heat stable enzyme

was done by Dubos and Thompson(14) by means of acetone).

Kunitz (36) succeeded in isolating crystalline ribonuclease. He separated the protein from an acid extract of pancreas by fractional precipitation with ammonium sulfate. The ribonuclease protein was found in the fraction soluble in 0.6% saturated ammonium sulfate, but insoluble in 0.8% saturated ammonium sulfate. The yield was about 3 gm. per 10 kg of pancreas. Kunitz studied extensively the chemical and physical properties of crystalline ribonuclease. The enzyme he isolated was a protein having a molecular weight of 15000$\pm$ 1000. The region of maximum stability was between pH 2.0-4.5. Heat stability was dependent upon the pH of the solution, decreasing as the pH was increased. The inactivation of the enzyme upon heating was accompanied by the gradual denaturation of the protein, as measured by its solubility in ammonium sulfate. It was also inactivated by alkali, the rate of inactivation being proportional to the rate of change of the native protein into denatured protein.

Kunitz found that ribonuclease brought about a gradual splitting of yeast nucleic acid into smaller components which diffuse through collodion membrane. This splitting was accompanied by the formation of titratable acid groups without the liberation of free phosphoric acid.

Allen and Eiler (2) studied the increase in acidity on treatment of yeast nucleic acid solutions with ribonuclease, using a glass electrode titration apparatus. They prepared a solution of nucleic acid using sodium hydroxide of known

equivalents, and adjusted the pH to 6.8, thus obtaining a buffer mixture of ribonucleic acid and sodium ribonucleate. Calculations showed that RN-ase liberated 0.95 equivalents of acid for every 4.95 equivalents of phosphoric acid per mole of tetranucleotide. The acidic group was liberated in the range of pH 6 and was therefore a secondary phosphate. These findings were confirmed later by Chantrenne (9). Allen and Eiler concluded that the action of ribonuclease could be regarded either as an opening of cyclic structure or as a depolymerization.

Since the enzyme acted upon its substrate with the liberation of titratable acidic groups, it was possible to develop a manometric method in which the acidic groups were made to evolve CO_2 from a bicarbonate buffer. This method was first used by Bain and Rush (3) for the assay of ribonuclease in biological materials, and later by Zittle (68) in his studies on the extent of hydrolysis of nucleic acid by this enzyme. Zittle suspected that the hydrolysis was stopped by the inhibitory effect of mononucleotides (68), but found that upon removal of the hydrolytic products by dialysis the hydrolysis was not increased. He compared the results of hydrolysis by ribonuclease against that caused by sodium hydroxide (70) and found that ribonucleic acid which had been hydrolyzed by treatment with normal sodium hydroxide for 0.5, 1, 2 hrs. at 25°C. was still quite reactive with the enzyme. The reactivity was measured by the amount of precipitate obtained with the uranium reagent. However, the fraction resistant to RN-ase was found to be hydrolyzed more slowly with sodium hydroxide. In order to estimate the extent of

hydrolysis, he precipitated the unchanged or resistant fraction of nucleic acid with Mac Fayden's reagent (modified by Zittle to contain (0.25% uranium acetate in 5% trichloroacetic acid). After adding an equal volume of this reagent to the hydrolysate the resulting pH was 1 (70) under these conditions about 60% of nucleic acid precipitated, while only 10% was precipitated by treatment with 7 volumes of glacial acetic acid. The fraction precipitated by acetic acid showed complete resistance to the action of ribonuclease.

These results were confirmed by Loring, Carpenter and Roll (48), who fractionated the ribonuclease resistant material into fraction A (precipitated with 7 volumes of glacial acetic acid) and fraction B, (precipitated from the acetic acid supernatant with one volume of alcohol). Both fractions were treated with ribonuclease and compared with purified nucleic acid treated with RN-ase. Fractions A and B were shown to be resistant to further treatment with enzyme. Loring concluded that ribonucleic acid contains at least 2 different types of linkages: one which is labile and one which is resistant to ribonuclease activity.

The ribonuclease resistant fraction differs in composition from the original nucleic acid in containing relatively larger amounts of purine, particularly guanine. It was suggested that ribonuclease activity was concerned to a greater extent with the liberation of pyrimidine than of purine nucleotides.

However, Loring and Carpenter (47), after treating yeast nucleic acid with ribonuclease, were able to isolate 4 mononucleotides as hydrolytic products, using Levene's method of fractionation (41, 43). They did not report any quantitative estimation of mononucleotides.

Chantrenne (9) followed the action of ribonuclease on yeast nucleic acid by measuring the opacity of the solution in Lange's electric photometer. In concentrations below 1.6 mg nucleic acid per cc., the opacity was proportional to the concentration.

A different method of studying the nature of enzyme action on nucleic acid was applied by Schmidt, Cubiles and Thannhauser (57), who introduced a combination of enzymes as tools in the analysis of the products of hydrolysis.
They separated an acid-phosphatase from hypertrophic prostate tissue. This monophospho-esterase split phosphoric acid only from monoesterofied phosphotic acid. They found that an exhaustive hydrolysis with ribonuclease rendered only 25% of the total phosphate hydrolysable with prostate phosphatase, 75% phosphate groups remaining di- or tri- esterified.
They concluded that the inorganic phosphate released by prostate phosphatase from ribonuclease treated nucleic acid originates from pyrimidine nucleotides. Hence they concluded that the action of ribonuclease involves,specifically or at least preferentially, one of the two pyrimidine nucleotides.

J. M. Gulland (21), using ribonuclease as a tool in his studies of nucleic acid structure, concluded that ribonuclease

activity varies with the sample of nucleic acid. The enzyme-resistant fraction of nucleic acid closely resembles the untreated nucleic acid, but it should not be inferred that it is an unchanged fraction of nucleic acid. There is a lack of agreement as to the changes occurring in RNA on RN-ase treatment. There are two points of view, the one supported by Jones and Perkins (27), Loring and Carpenter (47), and Zittle (68) that the products of hydrolysis are mononucleotides and that supported by Schmidt and Levene (55) and Kunitz (36), that the action of RN-ase consists in depolymerizing nucleic acid.

B. Presentation of the Problem

The increased solubility of nucleic acid in 80% acetic acid after ribonuclease treatment suggests its depolymerization. However the extent of depolymerization has not been investigated fully.

Isolation of the four mononucleotides by Loring and Carpenter (44) after treatment of RNA with ribonuclease suggests a splitting of RNA into mononucleotides but no quantitative study was made to show the extent of such splitting. The problem of whether the RNA is simply depolymerized or partially split into mononucleotides is not solved by a study of the liberation of titratable acid groups, nor by the formation of monoesterified phosphoric acid, unless the hydrolytic products are isolated after depolymerization (or splitting to mononucleotides). In both cases (liberation of a free mononucleotide

or dépolymerization of a straight chain of nucleotides) the titratable acidity would increase and phosphate radical would become monoesterified.

In view of the inadequacy of the experimental evidence explaining the nature of depolymerization, a further investigation of the problem was indicated.

In all but one of our experiments yeast nucleic acid used by previous investigators was substituted by pancreas-ribonucleic acid as the substrate for ribonuclease.

Materials and Methods

A. Ribonuclease and Nucleic Acid

Ribonuclease, prepared from 10 kg of beef pancreas by the method of Kunitz (36), was obtained in an amorphous form, as no crystals were available to seed it. This material was used in all but a few experiments. The later experiments were performed with Armour's crystalline enzyme.

The ribonucleic acid used as substrates was prepared from beef pancreas according to a method devised in this laboratory by Kerr and Seraidarian (35).

Two methods of purification of the RNA were used:

1) Levene's method (38) by precipitating with 80% acetic acid.
2) Chantrenne's method (10), by precipitating with an equal volume of a mixture of 40% acetic acid, 20% acetone and 40% water.

However, our preparations of nucleic acid when treated with this mixture resulted in a colloidal solution. To avoid this water was replaced with saline (35).

The estimation of the degree of splitting caused by ribonuclease in all experiments was made on small aliquots, i.e. by precipitating the enzyme resistant fraction with 0.25% uranium acetate in 5% trichloroacetic acid and determining the unprecipitated phosphate to total phosphate. This was compared with RNA treated in the same way in the absence of the enzyme (36, 70). In order to get full information about the action of ribonuclease, a method had to be developed for the direct determination of pyrimidine nucleotides, and for differentia-

tion between cytidylic and uridylic acids.

B. Review of Method of Preparation of Pyrimidine Compounds.

Levene's procedure for the preparation of free pyrimidine bases (38) and similarly Jones' method (28) involve drastic hydrolysis at 175°C. for 2 hours in 200 cc. of 25% sulfuric acid (per 50 mg of nucleic acid). This results in great loss of nuclear nitrogen and carbonization, hence this procedure could not be adopted for quantitative work. Attempts to modify Wheeler and Johnson's bromination color test (67) for uracil and cytosine for quantitative determination were not successful.

We found that Mac Fayden's (16) procedure of fractionation of a mixture of mononucleotides at different pH's does not separate sharply the individual constituents of nucleic acid, and so is not suited for quantitative work.

The methods applied by Plentl and Schoenheimer (54) and Barnes and Schoenheimer (4), involving hydrolysis of nucleic acid in 20% hydrochloric acid at 185°C for 3 hrs., have the same disadvantage as the hydrolysis in 25% sulfuric acid of Levene (i.e. loss of nitrogen and carbonization).

Davidson and Waymouth's method (13), designed for preparation of nucleotides, is not suitable as a method for quantitative analysis.

The biological assay for pyrimidines used by Loring (50) was not applied, because of lack of facilities. The same is true for the chromatographic method of Visher and

Chargaff (65, 66) and for the enzymatic determination of cytidylic acid by means of a special deaminase (18).

Kerr and Seraidarian's method (34), for the separation of free purines from nucleotides and nucleosides may be applied to the acid hydrolysates of nucleic acid. We noted that although the purine bases are quantitatively precipitated from acid hydrolysates of RNA by means of $AgNO_3$ in acid solutions, the unhydrolysed pyrimidine nucleotides remain in the supernatant.

Our attention was therefore directed to the finding of a reagent which would precipitate quantitatively the pyrimidine nucleotides, after hydrolysis of RNA and removal of purines by silver.

C. Precipitation of Pyrimidine Nucleotides.

Cytidylic acid, cytidine and cytosine were found to be precipitated quantitatively with silver nitrate in solutions alkaline to phenolphthalein. Mercuric nitrate also precipitates these quantitatively, while mercuric acetate only 54.5%. On attempting to precipitate uridylic acid with silver plus alkali and two volumes of ethyl alcohol, only 50% could be recovered. Finally it was found (35) that the precipitation could be made quantitatively by adding 3 volumes of isopropyl alcohol. Cytidylic acid was also precipitated quantitatively by silver in alkaline solution on adding 3 volumes of isopropyl alcohol (see Table I). For reasons to be given later in this paper, the addition of NO_3^- ions was avoided. Silver nitrate solution was therefore replaced by

silver oxide suspension, this being added to the solution containing the pyrimidine nucleotides and sulphuric acid. The precipitation was quantitative as confirmed by recovery experiments (35).

The results of these experiments taken from the unpublished work of Kerr and Seraidarian (35) are presented in Table I. The cytidylic acid was prepared by Bredereck and Richter's method (6A) with a N/P ratio of $\frac{2.9}{1}$ atoms. The uridylic acid was prepared by Kerr (35A) by deamination of cytidylic acid and had an atomic ratio of $\frac{1.95N}{1P}$.

Table I.

Comparison of $AgNO_3$, Ag_2O, ethyl alcohol and isopropyl alcohol, used as reagents for precipitation of pyrimidine nucleotides.

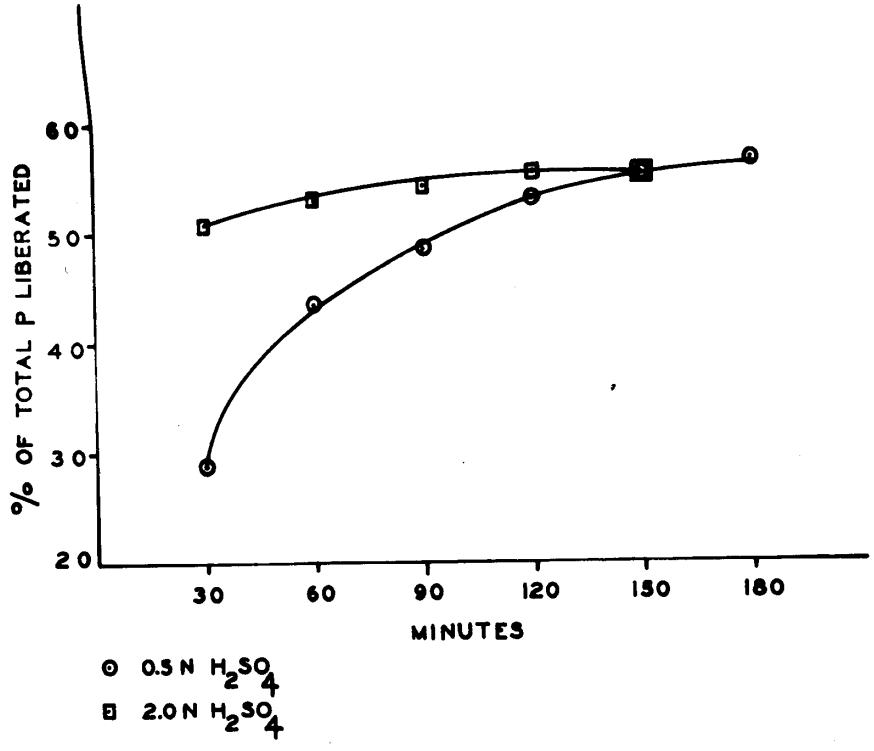
	Uridylic acid N taken mg.	Cytidylic acid N taken mg.	Total N taken mg.	Total N in alkaline silver precipitate mg.	Recovery %
Uridylic acid + Ag_2O + 2 vol. ethyl alc.	0.852		0.852	0.672	79.0
Uridylic acid + Ag_2O + 2½ vol. ethyl alc.	0.787		0.787	0.567	72.0
Uridylic acid + Ag_2O + 3½ vol. ethyl alc.	0.275		0.275	0.231	84.0
Uridylic acid + Ag_2O + 5 vol. ethyl alc.	0.734		0.734	0.515	70.0
Uridylic acid + $AgNO_3$ + 5 vol. ethyl alc.	0.734		0.734	0.515	70.0
Uridylic (Ba salt) + Ag_2O + 3 vol. isopropyl alc.	0.462		0.462	0.428	92.5
" " " " " " " " " " "	0.196		0.196	0.220	112.0
Uridylic (Ba salt) + Ag_2O + 3 vol. ethyl alc.	1.068		1.068	0.667	62.5
" " " " " " " " " " "	0.296		0.296	0.191	64.5
Cytidylic acid + Ag_2O + 3 vol. isopropyl alc.		1.26	1.26	1.26	100.0
Cytidylic acid + Ag_2O + 3 vol. ethyl alc.		0.933	0.933	0.945	101.5
" " " " " " " " " " "		1.62	1.62	1.232	98.0
Cytidylic + uridyl. + Ag_2O + 3 vol. ethyl alc.	0.777	0.458	1.235	1.098	88.8
" " " " " " " " " " "	0.413	0.630	1.043	0.858	82.0
" " " " " " " " " " "	0.534	0.777	1.311	1.005	76.7
" " " " " " " " " " "	0.296	0.630	0.926	0.793	85.5
" " " " " " " " " " "	0.630	0.196	0.826	0.755	91.0
Cytidylic + uridyl. + Ag_2O + 3 vol. isopropyl	0.413	0.630	1.043	0.987	94.4
" " " " " " " " " " "	0.288	0.630	0.918	0.925	100.5
" " " " " " " " " " "	0.630	0.196	0.826	0.810	98.0

The time required for hydrolysis of the ribonucleic acid was determined in 0.5 N and 2 N concentrations of H_2SO_4 using pancreas nucleic acid, preparation 36-17A, precipitated with an equal volume of mixture: 2 volume glacial acetic acid + 1 volume acetone + 2 volumes saline (10, 35).

It was assumed that when the liberation of inorganic phosphate from the purine nucleotides reached a constant figure, the purines would also have been completely set free. This is supported by the work of K. Lohmann (46B) and Levene and Tipson (46A).

Figure 1

Curve of Hydrolysis of Phosphoric Acid from Pancreas Ribonucleic Acid by H_2SO_4



Hydrolysis in 0.5 N H_2SO_4 was chosen for the analysis of RNA in order that the acidity might not be too great for complete precipitation of the purines by silver.

The procedure finally adopted for the ribonucleic acid analysis was as follows:

A weighed sample (60-80 mg.) of ribonucleic acid was hydrolyzed at 100° for 2½ hrs. in 0.5N H_2SO_4 in a test-tube provided with an air condensor, and the hydrolysate was diluted to 25 cc. 1 cc samples from the diluted solution were taken for total nitrogen, total phosphate, hydrolyzable phosphate and ribose determinations. The total nitrogen was determined by micro-Kjeldahl, according to Kerr and Seraidarian (33), phosphates by the methods of Fiske and Subarrow (17) and ribose by Bial's reaction as described by Kerr and Seraidarian (34).

A 15 cc sample from the 25 cc. of hydrolysate was measured for purine and pyrimidine determination.

0.02 volumes of a 1 Molar Ag_2O suspension was added with stirring to permit solution as Ag_2SO_4. The silver purines precipitate was centrifuged, the supernatant solution decanted into a 250 cc. centrifuge tube and the precipitate washed twice with distilled water. The supernatant solution and washings were combined and a few drops of Ag_2O suspension was added. The mixture was made alkaline to phenolphthalein with sodium hydroxide precipitating Ag_2O and 3 volumes of isopropyl alcohol was added to complete the precipitation of the pyrimidine nucleotides.

The precipitate was collected by centrifugation and washed twice with 3:1 isopropyl alcohol-water mixture.

The Purine and pyrimidine silver precipitates were extracted with hot 0.5 N and 1.0 N HCl (respectively) and diluted to a volume of 25 cc. with the same reagents. Total purine and pyrimidine nitrogen were determined by micro-Kjeldahl method on 1 cc samples of the extracts.

In an aliquot of purine HCl extract guanine N was determined colorimetrically (78) and adenine N was calculated by difference, (i.e., purine N - guanine N.).

In an aliquot of HCl extract containing the pyrimidine nucleotides, both cytidylic and uridylic acids were determined spectrophotometrically as described later in this paper.

The method described by Massart and Haste (61) for the determination of pyrimidine-bound ribose could not be duplicated, the recovery of pyrimidine-ribose being only 30%.

D. Picrate Precipitation Method.

With the purpose of determining cytidylic acid in the presence of uridylic acid, repeated attempts were made to precipitate cytidine with picrate. The results obtained may be of some interest, even though the attempt was unsuccessful. The procedure used for precipitating cytidine was the same as that described by Hitchings (23) for the precipitation of adenine picrate. Both cytosine and cytidine were precipitated quantitatively with picrate, cytidylic acid did not precipitate (table II).

We have not been able to find any reference in the literature to the precipitation of cytidine as picrate, although we find it

to be quantitative. The preparations used were prepared from yeast nucleic acid:

1) cytidylic acid by the method of Bredereck and Richter (6A),

2) cytidine was purchased from Hoffmann-La Roche Laboratory,

3) cytosine was purchased from Bios Laboratory.

Table II.

	Pyrimidine N taken mg.	Pyrimidine N found in picrate ppt. mg.	Recovery %
Cytosine	1.56	1.485	95.0
Cytidine	1.20	1.17	97.6
Cytidylic acid	0.94	--	--

As cytidylic acid is very resistant to hydrolysis, the only method available for splitting off the phosphate is by means of an enzyme. Prostate phosphatase splits off the phosphate radical quantitatively. This enzyme was prepared according to the method given by Schmidt and Thannhauser (57), and was further purified by precipitation with ammonium sulfate. The fraction precipitated between 0.6 and 0.8% saturation with ammonium sulphate was found to contain the phosphatase. The ammonium sulphate was then removed by dialysis. The phosphate radical of cytidylic acid was liberated quantitatively by the enzyme in 0.2M acetate buffer at pH 5.5, at 37°C. After such treatment, cytidine could not be precipitated with picrate, although, as stated above, commercial cytidine was completely precipitated.

Our interest was directed to find out whether the failure of the cytidine to precipitate was due to the presence of acetate buffer, phosphate ions or to the enzyme itself (Table III).

Table III

	Pyrimidine N Taken mg	Pyrimidine N found in picrate ppt. mg.	Recovery %
Cytidylic acid and acetate buffer pH 5.5 + phosphatase	1.48	--	--
Cytidine + equivalent of PO_4 (as KH_2PO_4)	0.98	0.935	95.4
Cytidine + equivalent of PO_4 + phosphatase	0.98	0.92	94.0
Cytidylic acid + phosphatase adjusted to pH 5.5 with NaOH & HCl	1.48	1.40	94.2

As the results in Table III show, the presence of acetate prevented picrate precipitation, possibly by blocking $-NH_2$ group ($-NHCOCH_3$ formed). When the pH was adjusted to 5.5 without the addition of acetate, cytidine was precipitated by picrate. Since in the course of analysis pyrimidines nucleotides are precipitated as alkaline silver salts, it was necessary to show that the procedure does not interfere with the enzyme action or the picrate precipitation. Cytidylic acid was first precipitated with silver at alkaline pH. The precipitate was extracted with N HCl, evaporated to dryness in the Logan

evaporator (46C) dissolved in 2cc H_2O, pH adjusted to 5.5 and incubated for 1 hr. with phosphatase. Splitting of phosphate from cytidylic acid was complete, but the cytidine obtained still did not precipitate with picrate. This failure to precipitate could not be caused by deamination as on reprecipitating cytidine in the above experiment, the total nitrogen taken was recovered.

Suspecting the interference of environmental or other factors in the procedure used, the same experiment was carried on (1) in the cold, (2) in the dark, (3) in the presence of reducing agent (hydroquinone), (4) with cytidine (instead of cytidylic acid), but in no instance were the results any better than in the previous experiment. We are at a loss to explain these results. The reason is that probably cytidine causes or enhances the reduction of Ag^+ to Ag, in an alkaline medium, thus undergoing an oxidation itself; or in the presence of Ag^+, in an alkaline medium, an isomerization takes place and this isomer is no more precipitable with picrate. However, a comparison of the absorption curves in the spectrophotometer reveals no difference between silver treated and untreated cytidylic acid.

Next a trial to replace silver by mercuric nitrate was made, but it was found that the phosphatase was no more reactive under these conditions, possibly due to the presence of traces of mercury. That the ability of cytidine to precipitate with picrate does not change in contact with Ag^+ in an <u>acidic</u> medium is shown in the following experiment: A mixture of adenine and cytidine was taken and the adenine was precipitated

with $AgNO_3$ in an acid medium. The excess of Ag^+ was then removed from the supernatant solution with HCl, an aliquot was taken and treated with picrate, which gave 80% recovery of cytidine N. It is interesting to note that cytosine precipitates with picrate after Ag_2O treatment

E. Spectrophotometric Determinations.

A spectrophotometric method, to determine the amounts of cytidylic and uridylic acid, in the presence of each other, was attempted. The Beckman spectrophotometer, Model DU Quartz was used. The molar absorbtion coefficients (were calculated from the extinction measurements E of cytidylic and uridylic acids. Cytidylic acid used was purchased from Schwartz Laboratory with a N/P ratio 3.01/1 uridylic acid was prepared by Kerr (35A), with a N/P ratio of 2.01/1

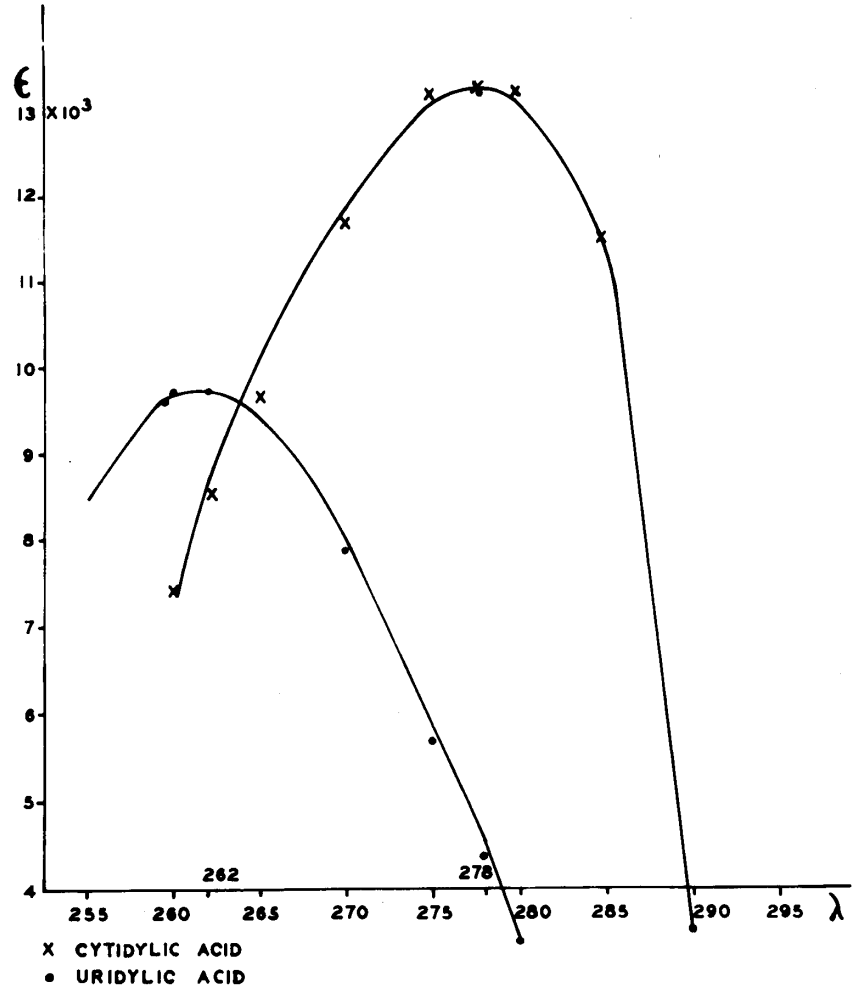
Solutions were prepared:

1) containing 8.95 - 10^{-3} moles/liter of uridylic acid, and 2) 5.8 x 10^{-3} moles/liter of cytidylic acid.

1 cc. samples were taken and diluted 50, 100, 200, 250, 400, 500 times with 0.01 N H_2SO_4. E reading were taken and the average values of ϵ plotted against λ, the curves are shown in Figure 2.

Figure 2

Molar absorbtion coefficients of cytidylic and uridylic acid.



The relationship between absorbtion and thickness of the cell and between absorbtion and concentration are as follows: $E = \log \frac{I_o}{I} = \epsilon cd$, where E is the extinction or the logarithm of the ratio of the incident to the transmitted light (measured in spectrophotometer) , d is the thickness of cell (given), c is the concentration of sample (to be calculated for the unknown) ϵ is the specific extinction, i.e. extinction for a molar concentration and unit thickness (given in figure 2). The peak of cytidylic acid curve is at λ 278 = 13.22×10^3
The peak of uridylic acid curve is at λ 262 = 9.73×10^3.
The largest difference between molar absorbtion coefficients of cytidylic and uridylic acids is at λ 278:

$$\begin{array}{r} 13.22 \times 10^3 \\ - \ 4.38 \times 10^3 \\ \hline 8.84 \times 10^3 \end{array}$$

However, when cytidylic acid is converted to uridylic by deaminization, as described below, the change in molar extinction coefficient is found to be 9.34×10^3.

The values:

ϵ 278 for cytidylic = 13.22×10^3,

ϵ 262 for uridylic = 9.73×10^3,

ϵ 278 before deaminization

ϵ 278 after deaminization = 9.34×10^3, are the average values calculated from a series of experiments (35).

In order to calculate the total number of mols of pyridines, cytidylic acid had to be converted to uridylic and readings taken at λ 262, using for calculation the molar absorbtion coefficient 9.7×10^3. As a routine readings were taken on both sides of this peak, i.e., at λ 259 and λ 265. A peak at λ 262 was taken as an index of complete conversion of cytidylic acid to uridylic. On the other hand, the number of mols of cytidylic acid could be calculated from the change in the absorbtion at λ 278 on converting the cytidylic to uridylic acid, using the factor of 9.34×10^3 per mole. The amount of uridylic acid was assumed to be the difference between the total number of mols and the number of mols of cytidylic acid.

Calculations:

$$\frac{\text{E 262 after deamination}}{9.73 \times 10^3} \times \frac{\text{volume}}{1000} = \text{total number of moles in the sample taken}$$

$$\frac{\text{E 278 before deamination} - \text{E 278 after deam.}}{9.3 \times 10^3} \times \frac{\text{volume}}{1000} = \text{number of moles of cytidylic acid.}$$

The best method for determining cytidylic acid would seem to be by deaminization, using a deaminase specific for cytidylic acid (31, 32). Greenstein (18) has described such an enzyme, found in the kidney of a certain stram of mouse. As this was not available, a different method had to be developed. The deamination was done by Van Slyke's method (63) by using equal volumes of 50% sodium nitrite and glacial acetic acid.

A 1 cc sample of pyrimidine HCl extract was taken, evaporated to dryness, next 1 cc H_2O added then 0.25 cc of 50% $NaNO_2$ and 0.25 cc. of glacial acetic acid. After standing at room

temperature for 1½ hours cytidylic acid was found to be deaminized. The excess of nitrite was destroyed by the cautious addition of 1cc 20 % NH_2OH (35), this was done in the cold to avoid axime formation. The sample was diluted to 100 cc and absorbtion determined at 262 and 268. On another aliquot of pyrimidine original HCl extract i.e. not deaminized, the absorbtion was determined at 278.

The samples taken usually contained about 0.2 mg of N and gave E readings of about 0.400 at 278 before deamination. A blank containing 1 cc of N HCl in the place of pyrimidine extract and then treated in the same way as the unknown sample was run in each case and the absorbtion of the unknown sample was measured against a reference cell containing the blank. The recovery of total pyrimidine nitrogen in a series of experiments averaged 90%. It was suspected that the loss (about 10%) was due to deamination or to some other changes taking place in the course of analysis. Fearing that traces of nitrous acid might be formed from nitrate in the presence of a silver precipitate and light, and thus some cytidylic acid might be deaminized, it was decided to use Ag_2O in place of $AgNO_3$, although the results given in the experiment below revealed no difference when performed in the dark. It was also important, to avoid the presence of NO_3^- ions, considering the fact that NO_3^- absorbs ultraviolet light.

The following experiments were performed, with the idea of increasing the percentage of recovery:

1. 1 cc cytidylic acid solution (3.7×10^{-3} moles/liter) was precipitated with Ag_2O + 3 volumes of isopropyl alcohol; the precipitate was extracted with hot HCl and diluted to 100 cc with H_2O. The absorbtion values were read in Beckman's spectrophotometer.
2. Ditto in the dark
3. Ditto in the cold

1a. Same as 1 but precipitated with $AgNO_3$

2a. " " 2 " " " "

3a. " " 3 " " " "

4. 1 cc. cytidylic acid solution (3.7×10^{-3} moles/liter) diluted to 100 cc with 0.01 N HCl. Results are shown on Table IV.

Table IV.

	E 1.	E 2.	E 3.	E 1a.	E 2a.	E 3a	E 4
280	.455	.465	.450	.440	.465	.460	.480
278	.460	.470	.455	.445	.470	.467	.485
275	.450	.460	.447	.440	.461	.457	.475
270	.400	.415	.400	.390	.415	.410	.430
265	.320	.335	.323	.311	.335	.330	.350
262	.270	.285	.274	.260	.285	.278	.295
260	.230	.250	.240	.227	.250	.242	.260

The calculations from table IV, namely $\frac{E\ 278}{13.22 \times 10^3} \times \frac{\text{volume}}{1000}$, showed errors of no more than 10% in each case (1, 2, 3, 1a, 2a, 3a) as compared with unprecipitated cytidylic acid (see Exper. 4 of the same table.).

The recovery of cytidylic acid in the experiment 4 as compared with the amount taken is 99.5%. It may be of significance that the values obtained when the experiment was performed in the dark are definitely higher than in the presence of light, although it seemed to make no difference whether $AgNO_3$ or Ag_2O was used.

The absorbtions of cytidylic acid in acid and alkaline solutions differed to a great extent, but on acidification the values were again the same as in acid solutions (Table V). Therefore, the alkaline medium did not change the molecule permanently.

Table V

Comparison of E values of the same amounts of cytidylic acid in 0.01 N H_2SO_4, 0.01 N NaOH and acidified after 3 hrs. of alkaline treatment.

	1 cc cytidylic acid → 100 cc in 0.01 N H_2SO_4 E	1 cc cytid. → 100 cc in 0.01N NaOH E	1 cc cytid. + 1 cc 1N NaOH, left for 3 hrs., then 2 cc. 1 N H_2SO_4 → 100 cc with H_2O E
280	.480	.230	.480
278	.485	.262	.485
275	.475	.300	.480
270	.430	.325	.430
265	.350	.311	.350
262	.295	.295	.295
260	.260	.280	.265

Experimental

A. Analysis of the Ribonuclease Resistant Fraction

Before a detailed method of analysis was worked out, some preliminary experiments with ribonuclease were performed. The procedure applied was as follows:
A 2% pancreas ribonucleic acid solution was divided into two parts, to one of which ribonuclease was added, while the other was kept as control. Both solutions were incubated in 0.1 M acetate buffer pH 5.3 at 25° C.

The extent of splitting of ribonucleic acid, based on precipitation with McFaddens reagent, discussed under "Materials and Methods"-A, was determined. After 24 hrs. of incubation 3 volumes of alcohol were added to both solutions, the precipitates were washed and dried in vacuum over P_2O_5 at 78°C. Analyses were made of weighed samples of the ribonuclease-resistant fraction, the control incubated without enzyme, and the original nucleic acid which had not been incubated.

Table VI

The preparation (36-4) used for this experiment was a pancreas RNA, purified per Levene(38) by precipitation with 80% acetic acid. This precipitate was then dissolved in water and dealyzed in a cellophane bag, then reprecipitated by 3 volumes of alcohol and treated with the enzyme, as described above. (excep.: incubat. for 5 hrs.

	Controls		Ribonuclease treated
	not incubated	incubated	
	millimoles per 100 mg NA		
% of total P not precipitated by uran. reagent	0%	3.08%	11.3%
Total N (TN)	1.085%	1.13	1.1
Total P (TP)	.267	.266	.230
Hydrolysable P.	.146	.157	.160
Ribose	.146	.150	.147
Purine N	.161	.149	.149
Guanine	.104	.0992	.108
Adenine	.051	.0498	.041
Purine N/Total N	74.2	66.0	67.4
% Hydrol.P.	54.1	59.0	67.4
Guanine/Adenine	1.82/1	2.02/1	2.70/1

Table VII

Pancreas ribonucleic acid, preparation 36-15 used for this experiment was precipitated with an equal volume of a mixture of 2 volumes acetic acid + 1 volume of acetone ~~+ 2 volume of acetone~~ + 2 volumes saline, per Chantrenne (10, 35)

	Controls		Ribonuclease treated
	not incubated	incubated	
	Millimoles per 100 mg NA		
% of total P not precipitated by uran. reagent	0	20.4	55.0
T. N.	1.15	1.17	1.19
T. P.	.29	.273	.263
Hydr. P.	.16	.175	.178
Ribose	.159	.189	.194
Purine N	.145	.167	.173
Guanine	.101	.0845	.136
Adenine	.044	.0825	.037
Purine N/T.N.	81.5%	71%	72.5%
% Hydr. P.	55.4%	64.4%	67.5%
Guanine/Adenine	2.29/1	1.03/1	3.66/1

Table VIII

Pancreas ribonucleic acid, preparation 36-17A was precipitated as in 36-15.

	Controls		Ribonuclease treated
	not incubated	incubated	
	Millimoles per 100 mg NA		
% of total P not precipitated by uran. reagent	0	26.3	40.
T.N.	1.14	1.13	1.165
T.P.	.294	.267	.254
Hydr. P	.165	.165	.182
Ribose	.162	.145	.160
Purine	.150	.150	.169
Guanine	.1075	.109	.129
Adenine	.0425	.041	.04
Purine N/ T.N.	66%	67%	72.5%
% Hydr. P	50.5%	62%	72%
Guanine/Adenine	2.54/1	2.63/1	3.21/1
Cytid./urid.		1.77/1	.62/1

Table IX

Pancreas ribonucleic acid preparation 36-17B constitutes the supernatant of 36-17A, precipitated with alcohol.

	Controls		Ribonuclease treated
	not incubated	incubated	
	Millimoles per 100 mg NA		
% of total P not precipitated by uran. reagent	0	36.0	42.3
T.N.	1.1	1.11	1.18
T.P.	.275	.256	.254
Hydr. P	.152	.163	.174
Ribose	.145	.144	.149
Purine	.138	.152	.165
Guanine	.037	.114	.121
Adenine	.041	.038	.044
Purine N/ T.N.	62.5%	68.7%	72%
% Hydr. P.	55.2%	63.5%	68.4%
Guanine/Adenine	2.34/1	3.0/1	2.75/1

Discussion of Tables VI-IX

In the four experiments presented in Tables VI, VII, VIII, IX, the percentage of hydrolysable P to total P and of purine N to total nitrogen was found to be increased after ribonuclease treatment, the same was true for the ratio of guanine to adenine. The increase in the percentage of hydrolysable P is more than the increase in the percentage of purines. This is expected when pyrimidines are lost. One pyrimidine nucleotide lost from purine-pyrimidine dinucleotide represent a 50% loss of phosphate but only 28.5-37.5% loss of nitrogen. At this point the following two factors should be considered:

1) the percentage of splitting of different samples treated with ribonuclease in the same way, is not uniform.

2) ribonucleic acid solution splits on incubation without the

addition of enzyme.

In two cases (preparations: 36-15 unquoted and 36-24 to be described later in this paper) the splitting of ribonucleic control and ribonuclease treated sample were the same. The following possible explanations should be considered:

1) The RNA used (pancreas) differs from yeast RNA, being resistant to ribonuclease treatment. The resistance was, however, not uniform in the different preparations:
2) Ribonuclease may have been present in these preparations as a contamination due to the method of preparation of the nucleic acid from pancreas.

The following experiment shows that the second alternative cannot be accepted, unless the ribonuclease present were an enzyme different from that described by Kunitz.

Exper.: To a solution containing 84.7 mg of ribonucleic acid, 5 cc 0.2 M acetate buffer pH 5.3 and 0.2 cc 1% $CuSO_4$ solution were added and the mixture was diluted to 10 cc. To 1 cc of this solution 1 cc of uranium reagent was added and the mixture was centrifuged. The supernatant solution contained no phosphate.

A 9 cc portion of the solution was incubated at 25° C. for 24 hrs. After 24 hrs. 1 cc of this was taken and treated with 1 cc. of uranium reagent. The supernatant contained 31.2% of the total phosphate.

Cu^{++} is known to inhibit ribonuclease activity (69), therefore if Kunitz' ribonuclease were present in our preparations, its action would be inhibited and no splitting would occur.

An attempt was made to precipitate the RN-ase resistant fraction and the fraction resistant to hydrolysis im the control, with 80%

glacial acetic acid, but no precipitate was obtained, indicating that the resistant fraction was not the unchanged N.A.

B. Fractionation of Ribonuclease Treated Ribonucleic Acid With Determination of Pyrimidine Nucleotides.

The analysis of the ribonuclease resistant fraction gives only indirect evidence as to the nature of material which is split off. It does not answer the question whether ribonuclease action consists in the splitting of mononucleotides from RNA or only in depolymerization.

Since fractionation of the ribonuclease treated samples would give a more direct answer to this question, the following procedure was used in the last series of experiments:

The ribonuclease treated material and the control were put in cellophane bags and dialyzed for 24 hrs. The contents of the cellophane bags were then concentrated to a small volume in Logan evaporator and analyzed. The dialysate was evaporated in vacuum then treated with 3 volumes of ethyl alcohol, precipitating the low polymer fraction. The supernatant fluid, (believed to contain mononucleotides) was evaporated to a volume convenient for analyses.

In order to determine whether or not the alcohol supernatant consists of mononucleotides, the action of the mono-phosphoesterase of prostatic tissue was studied on this fraction, as follows: Both the ribonuclease treated sample of RNA and a control specimen were incubated and dialyzed. The dialysate was fractionated with 3 volumes of alcohol and the total phosphate content in the alcohol supernatant was determined. Next prostate

phosphatase was added to the bag containing RNA plus RN-ase, and the mixture was incubated for another 2 hrs.

Phosphate taken	Phosphate split off by phosphatase	% P split off
.5 mg	.335 mg	67.0
.5 mg	.325 mg	65.0
.5 mg	.225 mg	45.0
.5 mg	.235 mg	47.0
AVERAGE 5	.28	56.0

56% of the total phosphate was split off from the alcohol soluble fraction, meaning that a mixture of mono- and poly-nucleotides was present (or less probably dinucleotides). No phosphate is split off by the phosphatase from the alcohol insoluble fraction.

Table X

	Original		Control			
	per cent	millimoler per 100 mg NA	non-diffusible	Diffusible Alc. insoluble	Diffusible Alc. soluble	Total
			Millimoles			
T.N.	15.6	1.11	1.65	.348	.77	2.768
T.P.	8.35	.275	.335	.0845	.222	.642
Hydr. P	5.05	.163	.254	.0495	.083	.387
% Hydr. P	59.2%		75.7%	58.5%	37.4%	60%
Ribose	19.8	.132	.26	.0495	.081	.39
Purine	10.01	.143	.248	.052	.073	.373
Guanine N	7.50	.101	.182	.046	.040	.268
Adenine N	2.51	.0359	.067	.006	.033	.106
Guanine Adenine	2.99/1		2.71/1	7.2/1	1.73/1	2.5/1
Pyrimidine N	4.94		.1084	.0234	.1474	.273
Cytidylic N			.064	.011	.085	.160
Uridylic			.039	.012	.062	.113
Cytid. Urid.			1.65/1	.941/1	1.36/1	1.42/1
Purine N T.N.	64.8%		75.5%	75.5%	47%	60.5%
Purine Pyrimidine		1.015/1	2.42/1	2.22/1	.495/1	1.37/1

Pancreas ribonucleic acid, preparation 36-20, precipitated with an equal volume of a mixture of 2 volumes acetone + 1 volume glacial acetic acid + 2 volumes saline. (For nucleic acid original analysis see Table X). Splitting of ribonuclease treated sample after 24 hrs. incubation was 35.6%
Splitting of ribonuclease control sample after 24 hrs. incubation was 32.5%.
After 24 hrs. of dialysis 51% of T.P. was dialyzed out in ribonucl. treated sample.
After 24 hrs. of dialysis 42% of T.P. was dialyzed out in control.
The results of analysis are shown on Table X, and XI.

Table XI

Analysis of ribonucleic acid 36-20, ribonuclease treated.

	Ribonuclease treated			
	Non-Diffusible	Diffusible Alc.insoluble	Diffusible Alc.soluble	Total
	M i l l i m o l e s			
T.N.	2.63	.72	1.36	4.71
T.P.	.555	.156	.348	1.059
Hydr. P	.398	.0995	.111	.609
% Hydr. P	71.6%	61.6%	33.8%	57.5%
Ribose	.403	.118	.127	.648
Purine	.382	.099	.115	.596
Guanine	.372	.075	.068	.515
Adenine	.010	.024	.048	.082
Guanine / Adenine	3.7/1	3.08/1	1.42/1	6.3/1
Pyrimidine	.144	.0517	.243	.439
Cytidylic	.072	.028	.156	.256
Uridylic	.072	.024	.087	.183
Cytidylic / Uridylic	1/1	1.19/1	1.9/1	1.4/1
Purine N/T.N.	72.5%	68.8%	42.5%	63.5%
Purine/pyrim.	2.65/1	1.92/1	.472/1	1.34/1

Table XII

	Original		Control			
	per cent	milimoler per 100 mg NA	non-diffusible	Diffusible Alc. insoluble	Diffusible Alc. soluble	Total
			Milimoles			
T.N.	16.15	1.18	2.5	.9	.865	4.265
T.P.	8.53	.275	.54	.21	.271	1.021
Hydr. P	5.12	.165	.368	.144	.052	.464
Ribose	25.80	.172	.416	.153	.0785	.648
Purine	11.	.157	.402	.134	.0317	.568
Guanine	8.3	.118	.312	.11	.006	.428
Adenine	2.7	.0386	.090	.024	.026	.14
Pyrimidine	4.96	.122	.169	.070	.219	.458
Cytidylic	3.02	.072	.103	.0499	.110	.264
Uridylic	1.39	.0496	.066	.0201	.109	.194
Purine N/T.N.	68%		74%	74.5%	18.3%	66.5%
% Hydr. P	60%		68%	68.5%	19.2%	45.2%
Guanine / Adenine		3.06/1	3.47/1	4.6/1	.234/1	3.06/1
Cytid. / Urid.		1.42/1	1.56/1	2.43/1	1.01/1	1.36/1
Purine / Pyrimid.		1.29/1	2.38/1	1.91/1	.145/1	1.24/1

Pancreas ribonucleic acid, preparation 36-24, precipitated as in 36-20 (the analysis of original see table XII). Before incubation RNA was dialyzed in cold 5% of T.P. dialyzed out. Splitting of ribonuclease treated sample after 36 hrs. of incubation was 29.0% Splitting of ribonuclease control sample after 36 hrs. of incubation was 29.0%. After 24 hrs. of dialysis after incubation 49.5% of T.P. was dialyzed out in ribonuclease treat. sample.
After 24 hrs. of dialysis 60.0% of T.P. was dialyzed out in ribonuclease + phosphatase sample.
After 24 hrs. of dialysis 49.0% of T.P. was dialyzed out in ribonuclease control.

The results are shown in Tables XII and XIII

Table XIII

	Ribonuclease treated				Ribonuclease and Phosphatase			
	non-diffusible	Diffusible Alc. insoluble	Diffusible Alc. soluble	Total	non-diffusible	diffusible alc.insoluble	Diffusible Alc. soluble	Total
	Millimoles				Millimoles			
T.N.	2.68	.606	1.35	4.636	2.88	.55	1.54	3.97
T.P.	.56	.136	.37	1.066	.546	.1345	.461	1.142
Hydr. P	.394	.09	.052	.536	.45	.116	.322	.888
Ribose	.480	.111	.146	.737	.432	.119	.172	.723
Purine N	.402	.093	.092	.587	.38	.085	.123	.588
Guanine	.312	.073	.012	.397	.317	.068	.015	.400
Adenine	.090	.020	.079	.189	.063	.017	.108	.188
Pyrimidine	.156	.042	.263	.461	.168	.0334	.241	.442
Cytidylic	.094	.027	.138	.259	.105	.021	.139	.265
Uridylic	.062	.015	.125	.202	.063	.012	.102	.177
Purine N/T.N	75.3%	76.5%	33.9%	63.5%	66%	77.2%	40%	74%
% Hydr. P	70.5%	66.5%	14%	50.3%	82.4%	86.1%	70%	77.5%
Guanine/Adenine	3.47/1	3.65/1	.157/1	2.09/1	5.03/1	4.06/1	.137/1	2.12/1
Cytidyl./Urid.	1.52/1	1.89/1	1.1/1	1.29/1	1.67/1	1.7/1	1.36/1	1.5/1
Purine/Pyrim.	2.58/1	2.22/1	.348/1	1.27/1	2.26/1	2.55/1	.51/1	1.33/1

Results:

Tables X, XI, XII, XIII show that about 50% of the RNA dialyzed out of the sac in both control and RN-ase treated samples and about twice as much was found in the alcohol supernatant than in the alcohol precipitate. The ratios Purine N/T.N., Purine/pyrimidines and hydrolysable P/T.P. show that the alcohol supernatant contained twice as much pyrimidines as purines, while the results obtained in the alcohol precipitate was the opposite. On reviewing the results of these experiments one must bear in mind the fact that guanylic acid is less soluble than adenylic acid in 75% alcohol. Therefore, the relative increase of guanine in the alcohol precipitate (which we believe consists mainly of a low polymer fraction of RNA), may be due to the low solubility of guanylic acid rather than to a loss of adenine nucleotide from the nucleic acid. Adenylic, uridylic and cytidylic acids are soluble in 3 volumes of alcohol. The ratio of cytidylic acid to uridylic acid did not show any outstanding changes, but guanine to adenine ratio was decreased in the alcohol supernatant.

In order to compare the ribonuclease action on pancreas ribonucleic acid and yeast nucleic acid, yeast nucleic acid was treated with the enzyme in the same way as ribonucleic acid in the above experiments.

(Table XIV)

Table XIV

	Original		Control			
	per cent	Milimoles per 100 mg NA	non diffusible	Diffusible Alc. insoluble	Diffusible Alc.soluble	TOTAL
			Milimoles			
T.N.	16.20	1.16	1.29	.58	.77	2.64
T.P.	8.62	.278	.466	.138	.208	.814
Hydr. P	5.52	.178	.198	.093	.0508	.342
Ribose	27.85	.185	.248	.109	.0915	.449
Purine	11.7	.167	.194	.093	.0292	.316
Guanine	6.24	.0892	.113	.058	.003	.174
Adenine	5.46	.078	.081	.035	.0262	.142
Pyrimidine	4.42	.115	.077	.273	.1634	.513
Cytid.	1.92	.0457	.026	.213	.063	.302
Uridyl.	1.93	.069	.049	.060	.1	.209
Purine N/T.N	72%		75.5%	80%	18.9%	60%
% Hydr. P.	64%		42.5%	67.4%	24.4%	42%
Guanine/Adenine		1.14/1	1.39/1	1.68/1	.115/1	1.22/1
Cytidyl./Urid.		.67/1	.539/1	3.56/1	.0635/1	1.44/1
Purine/Pyrimid.		1.45/1	2.52/1	.341/1	.179/1	.616/1

Yeast nucleic acid preparation 33-11 was purified by Levene's method and dialyzed(for analysis see Table XIV), in cold for 24 hrs. 10% of T.P. dialyzed out, before incubation.
Splitting of ribonuclease treated sample after 24 hrs. incubation was 18.5%.
Splitting of ribonuclease control sample after 24 hrs. incubation was traces on
After 24 hrs. dialysis 49.0% was dialyzed out in the ribonuclease treated sample.
After 24 hrs. dialysis 0.0% was dialyzed out in the control.

the dialysate fraction was absent in the yeast nucleic acid control sample on incubation at 25° C, indicating that yeast nucleic acid does not decompose on incubation without the enzyme and (or) yeast nucleic acid is much more sensitive to ribonuclease treatment than pancreas ribonucleic acid.

Discussion

Pancreas ribonucleic acid was found to be easily decomposed in aqueous solutions on incubation at 25°C at pH 5.3 (acetate buffer).

Treatment with ribonuclease did not reveal any additional changes, except in the adenylic acid content (Tables X, XI, XII, XIII), the loss of which was greater in the ribonuclease treated sample (see Table XV).

The hydrolysis of RNA in aqueous solution cannot be explained by the presence of Kunitz' enzyme, since addition of copper ions did not inhibit the decomposition.

It seems that we are dealing with either (a) a mixture of a labile and a resistant fraction of pancreas ribonucleic acid or else (b) the original RNA has labile groups and a resistant residue. The labile groups are easily split off on incubation in aqueous solution, and the fraction which remains seems to be resistant to ribonuclease.

However, yeast ribonucleic acid, purified by precipitation with 80% acetic acid and dialyzed to get rid of low polymer fraction, was resistant to incubation in aqueous solution, i.e. no splitting took place, and was sensitive to ribonuclease. The hydrolysis products were the same as in the pancreas RNA. The changes produced in pancreas RNA on incubation and those caused by RN-ase on yeast RNA consist of the depolymerization of the nucleic acid into smaller fractions which dialyze out

Table XV

Distribution of Nucleotides after Dialysis of Ribonuclease-treated and Control Specimens of Pancreas RNA, with Molar Ratio of Nucleotides in Each Fraction.

	Data from Table No.	Percentage Distribution						Molar Ratios			
		Adenine	Guanine	Cytosine	Uracil	Total P	Hydrol P TP	Adenine	Guanine	Cytosine	Uracil
Control-Diff.Alc.sol.	10	31.1	15.	53.1	54.8		37.4	1	1.2	2.6	1.9
" " insol.	10	5.7	17.2	6.9	10.6		58.5	1	7.7	1.8	2.0
Diffusible Total	10	36.8	32.2	60.0	65.4	42.		1	2.2	2.5	1.9
Non-diffusible	10	63	67.8	40.0	34.6		75.7	1	2.7	1	0.6
RN-ase Diff.Alc.sol.	11	58.5	13.2	60.8	47.5		33.8	1.0	1.4	3.3	1.8
" " insol.	11	29.3	14.6	11.0	13.1		61.6	1.0	3.1	1.2	1.0
" total	11	87.8	27.8	71.8	60.6	51		1	2.0	2.6	1.5
Non-diffusible	11	12.2	72.2	28.2	39.4		71.6	0.14	5.2	1.0	1.0
Control Diff.Alc.sol.	12	18.6	1.4	41.8	56.0		19.2	1	0.23	4.2	4.2
" " insol.	12	17.2	25.6	19.0	10.3		68.5	1	4.6	2.1	0.8
Diff. Total	12	35.8	27.0	60.8	66.3	49		1	2.3	3.2	2.6
Non-diffusible	12	64.2	73.0	39.2	33.7		68.1	1	3.5	1.1	0.7
Sum								1	3.1	1.9	1.3
RN-ase Diff.Alc.Sol.	13	41.8	3.0	53.2	61.9		14.	1	0.15	1.8	1.6
" " Insol.	13	10.6	18.4	10.4	7.4		66.5	1	3.7	1.4	0.8
" Total	13	52.4	21.4	63.6	69.3	49.5		1	0.9	1.7	1.4
Non-diffusible	13	47.6	78.6	36.4	30.7		70.5	1	3.5	1.0	0.7
								1	2.1	1.4	1.1
RNase + Prostate-Base	13										
Diffusible-Alc.Sol.		57.4	3.8	52.4	57.6		70.	1	0.14	1.3	0.9
" " Insol.		9.1	17.0	7.9	6.8		86.1	1	4.0	1.2	0.7
Total Diffusible		66.5	20.8	60.3	64.4	60.0		1	0.7	1.3	0.9
Non Diffusible		33.5	79.2	39.7	35.6		82.4	1	5.0	1.7	1.0
Yeast RNA + RN-ase	14										
Diffusible-Alc.Sol.		18.3	1.7	20.8	47.7		24.4	1	0.11	2.4	3.8
" Alc.Insol.		24.6	33.3	70.5	28.7		67.4	1	1.7	6.1	1.7
Total Diffusible		42.9	35.0	91.3	76.4	49.0		1	1	4.5	2.6
Non-Diffusible		57.1	65.0	8.7	23.6		42.5	1	1.4	0.32	0.60
								3.1	4.3	1.0	1.9

through cellophane membrane (about 50% of the nucleic acid taken) the dialysate containing 1/3 of the original purine and 2/3 of the pyrimidine nucleotides. 26.3% - 51% of the diffusible fraction was precipitable with 3 volumes of ethyl alcohol, but no phosphate could be split off by prostatic phosphatase, indicating that this fraction must be a relatively high polymer, but small enough to diffuse through cellophane membrane.

The alcohol soluble fraction when treated with the phosphatase, liberated 56% of the phosphoric acid groups. If this fraction were a mixture of tetra- and mononucleotides we could conclude that about 50% of it consists of mononucleotides. This confirms the work of Loring and Carpenter (47) who were able to isolate the mononucleotides, but did not report ~~report~~ any quantitative data.

During the decomposition of the RNA pyrimidine nucleotides are split off to a greater extent than purine nucleotides, increasing in the alcohol soluble fraction. This is revealed by the ratios of $\frac{\text{Purine N}}{\text{Total N}}$, $\frac{\text{Hydrolys. P}}{\text{Total P}}$ and $\frac{\text{Purine}}{\text{Pyrimidine}}$, each of which is at the minimum value in the alcohol supernatant. The ratio of $\frac{\text{cytidylic acid}}{\text{uridylic acid}}$ shows that ribonuclease does not specifically split off only one of the pyrimidine nucleotides. The decrease in $\frac{\text{guanine}}{\text{adenine}}$ ratio in the alcohol supernatant might be due to the low solubility of guanylic acid in alcohol, but not to the increased liberation of adenine nucleotide.

The non-diffusible fraction differs from the original nucleic acid in being a smaller polymer, no longer precipitable by 80% acetic acid.

The action of ribonuclease was found to involve both the depolymerization of nucleic acid into smaller fractions non-diffusible and diffusible through cellophane membrane and in the liberation of free mononucleotides, preferentially but not specifically pyrimidine nucleotides.

The liberation of mononucleotides suggests that either both purine and pyrimidine nucleotides (the latter to a greater extent) form the side chains in the nucleic acid molecule, or else that they are split off from the depolymerization products, without being necessarily the side chains in the original molecule.

Summary and Conclusions

1. Methods were developed for:
   a) the separation of free purines and pyrimidine nucleotides.
   b) the direct determination of cytidylic and uridylic acids.
2. Pancreas ribonucleic acid was found to be very sensitive to aqueous hydrolysis at 25°C as compared with yeast nucleic acid, the residue being resistant to further hydrolysis by ribonuclease.
3. Ribonuclease renders the nucleic acid soluble in glacial acetic, indicating the depolymerization of nucleic acid.
4. The depolymerized nucleic acid consists of 2 fractions:
   a) non-diffusible and
   b) diffusible through cellophane membrane, and precipitable with 3 volumes of alcohol.
5. Ribonuclease also liberates free mononucleotides, diffusible through cellophane membrane, and non-precipitable with 3 volumes of alcohol, with the exception of guanylic acid which does precipitate.
6. All four mononucleotides are found to be liberated from the nucleic acid, the amount of pyrimidine exceeding that of the purine nucleotides.

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