The Liberation of Polynucleotides by the Alkaline Hydrolysis of Ribonucleic Acid from Yeast

BY KENDRIC C. SMITH AND FRANK WORTHINGTON ALLEN[1]

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Alkaline hydrolysates of yeast ribonucleic acid were separated into two fractions by two dimensional paper chromatography. Fraction 1 contained the nucleotides, while fraction 2 was composed of six substances of greater complexity than the mononucleotides.

Since the early work of Steudel and Peiser[2] ribonucleic acids have been considered to be quantitatively converted to mononucleotides by controlled alkaline hydrolysis.[3] However, in none of these studies, including the recent methods for the determination of the nucleotide content of ribonucleic acids,[4] has it been demonstrated unequivocally that mononucleotides are the sole substances produced by alkaline hydrolysis. The evidence in favor of the mononucleotides being the simplest products formed by alkaline hydrolysis seems to be fairly conclusive; however, little mention is made of complexes larger than mononucleotides being formed. Several early workers[5] claimed the isolation of such compounds, but these claims were refuted by Levene[6] on the grounds that the products described were separable mononucleotide mixtures. More recently Cohn[7] has shown the retention of small amounts of material on ion exchange columns that does not behave as do the mononucleotides, nucleosides or free bases. The finding of Becker and Allen[8] that alkaline hydrolysis liberates constituents that are in part oxidizable by the *cis*-glycol cleaving reagent lead tetraacetate also contributes information not reconcilable with our present information.

The present investigation was undertaken in an attempt to answer certain of these questions concerning the products of the alkaline hydrolysis of ribonucleic acids.

Experimental

Nucleic Acid Hydrolysis.—The sample of ribonucleic acid (purified Schwarz yeast ribonucleic acid) was the same as that used by Becker and Allen in their recent publication.[8]

The conditions of alkaline hydrolysis were as follows: 200 mg. of sodium ribonucleate was dissolved in 5 ml. of 1.0 N sodium hydroxide and allowed to stand at room temperatures (22–24°) for 24 hours. The hydrolysate was then neutralized with 5 ml. of 1.0 N acetic acid.

Paper Chromatography.—The chromatograms were developed by the ascending or descending technique on Whatman No. 1 filter paper, without prior equilibration in the solvent vapor. The developed chromatograms were dried at room temperatures under exhaust. For a permanent record of the chromatograms, a modification of the photographic technique of Markham and Smith[9] was used. The photographic paper, overlayed with the chromatogram, was placed on a convex surface and a piece of nylon netting was stretched tightly over the papers to ensure uniform contact. An eight watt, short wave, ultraviolet lamp (General Electric) served as the light source. An exposure time of 30 seconds at a distance of 7 feet was found to be sufficient.

The three solvent systems found to be of most value in this work were: solvent 1, ammonia buffered isobutyric acid as described by Magasanik, *et al.*[10]; solvent 2, isopropyl alcohol–acetic acid–water as described by Montreuil and Boulanger[11]; and solvent 3, *t*-butyl alcohol–hydrochloric acid–water as described by Smith and Markham.[4d]

Solvent 1 was useful for separating adenylic acid from cytidylic acid, leaving guanylic acid and uridylic acid occupying the same position. Solvent 2 was used to separate guanylic acid and uridylic acid. Solvent 3 was designed to separate in one dimension, adenine, guanine, cytidylic acid and uridylic acid.

Paper Ionophoresis.—The apparatus used was developed in this Laboratory by Mr. Arthur M. Crestfield.[12]

Ammonium formate buffer (0.4 M at pH 3.3) was used to prepare the nucleotides from alkaline hydrolysates of ribonucleic acid. The four nucleotides were widely separated under these conditions but there was a fifth area which appeared between the adenylic acid and guanylic acid areas (Fig. 1A). The component(s) responsible for this fifth area will be discussed in the section on fraction 2.

Sodium tetraborate buffer (0.4 M at pH 9.2) was used to separate the 3′-(or 2′)-nucleotides from their respective 5′-isomers. It is well known that borate reacts with sugars to form negatively charged sugar–borate complexes,[13] and it seemed logical to suppose that this reactivity could be used for the separation of the 3′-(or 2′)-nucleotides from their respective 5′-isomers. This indeed proved to be the case (Fig. 1B).[14]

It is interesting to note that the 3′-(or 2′)-nucleotides that have amino groups at position six are more widely separated from their respective 5′-isomers than are those with enol groups at this position.

The practical application of the separation by the use of borate buffers will be discussed in the section on fraction 1.

Separation of Alkaline Hydrolysates into Two Fractions.—In the course of two dimensional paper chromatographic studies on the quantitativeness of the alkaline hydrolysis of ribonucleic acid it was found that the hydrolysates could be separated into two fractions.

A chromatogram which held a centimeter spot of hydrolysate containing 400 micrograms of nucleic acid was developed for 12 hours with solvent 1 and for 12 hours in the second dimension with solvent 2. The four nucleotides were resolved; however, there were seven additional spots which

(1) Supported in part by Grant-in-aid #RG 2496, U. S. Public Health Service.

(2) H. Steudel and E. Peiser, *Z. physiol. Chem.*, **120**, 292 (1922).

(3) W. Jones and M. E. Perkins, *J. Biol. Chem.*, **55**, 567 (1923); W. Jones and M. E. Perkins, *ibid.*, **62**, 357 (1925); G. Schmidt and P. A. Levene, *ibid.*, **126**, 423 (1938); C. A. Zittle, *ibid.*, **163**, 119 (1946); G. Schmidt and S. J. Thannhauser, *ibid.*, **161**, 83 (1945); E. Volkin and C. E. Carter, THIS JOURNAL, **73**, 1516 (1951).

(4) (a) W. E. Cohn, *ibid.*, **72**, 1471 (1950); (b) W. E. Cohn, *ibid.*, **72**, 2811 (1950); (c) E. Chargaff, B. Magasanik, E. Vischer, C. Green, R. Doniger and D. Elson, *J. Biol. Chem.*, **186**, 51 (1950); (d) J. D. Smith and R. Markham, *Biochem. J.*, **46**, 509 (1950).

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(6) P. A. Levene, *J. Biol. Chem.*, **33**, 229 (1918); **40**, 415 (1919).

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(9) R. Markham and J. D. Smith, *Biochem. J.*, **45**, 294 (1949).

(10) B. Magasanik, E. Vischer, R. Doniger, D. Elson and E. Chargaff, *J. Biol. Chem.*, **186**, 37 (1950).

(11) J. Montreuil and P. Boulanger, *Compt. rend.*, **231**, 247 (1950).

(12) Unpublished data.

(13) J. Boeseken, *Advances in Carbohydrate Chem.*, **4**, 189 (1949).

(14) We wish to express our gratitude to Dr. Waldo E. Cohn for generous samples of the 5′-phosphates of guanosine, adenosine, cytidine and uridine.
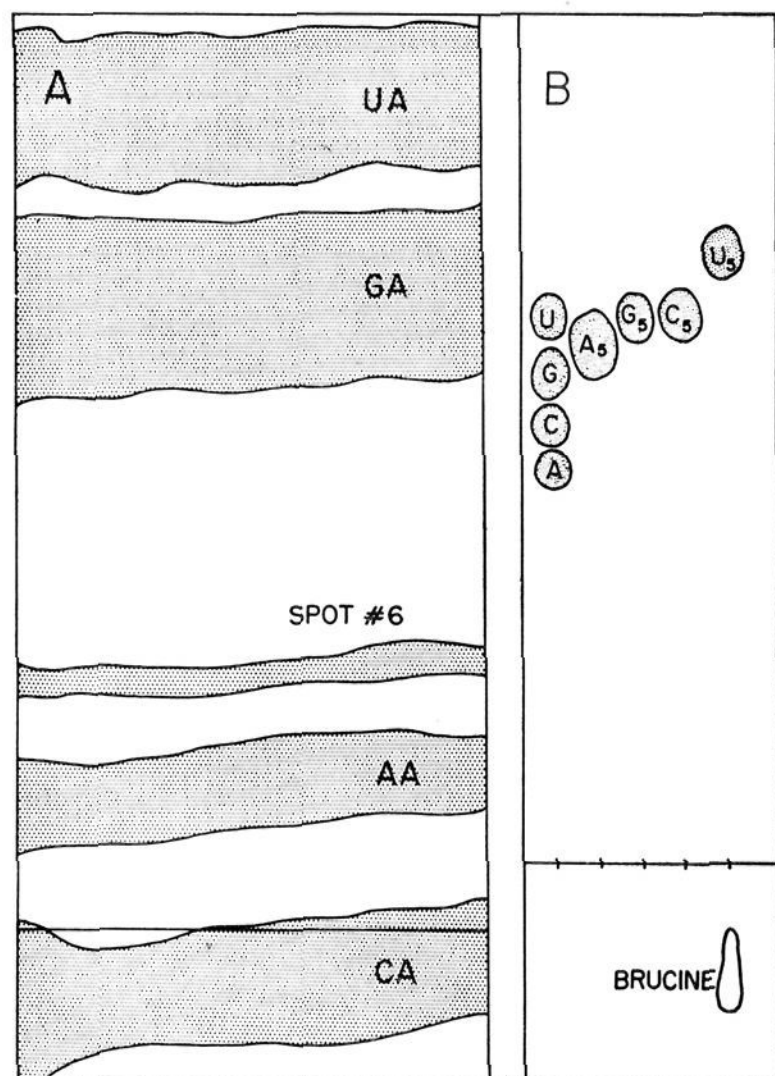
Fig. 1.—A, separation of 3.3 mg. of an alkaline hydrolysate of yeast ribonucleic acid by paper ionophoresis in 0.4 M ammonium formate buffer (pH 3.3) at 80 milliamp. and 950 volts for 5 hours on Whatman No. 1 filter paper. CA, AA, GA and UA refer to cytidylic, adenylic, guanylic and uridylic acids. Spot 6 refers to substance 6 in fraction 2. B, separation of the 5'-nucleotides from their respective 3'-isomers by paper ionophoresis in 0.4 M sodium tetraborate buffer (pH 9.2) at 30 millamp. and 850 volts for 2 hours on Whatman No. 1 filter paper. A, C, G and U refer to the respective 3'-nucleotides, while A_5, G_5, C_5 and U_5 refer to the respective 5'-nucleotides. U_5 was used as the brucine salt.

were resolved in the first dimension but did not move out in the second dimension. One of these spots did not move in either solvent (Fig. 2A).

The chromatographic results offered a method for the preparation of sufficient quantities of this immobile phase for preliminary characterization. This was accomplished by applying the hydrolysate along the paper as a streak (400 micrograms per centimeter) and developing with solvent 2 until a clean separation of the mobile and immobile phases was obtained (72 hours).

The two phases were eluted from the paper with water and the fractions collected by lyophilization. The nucleotide, or mobile phase, will be termed fraction 1, while the immobile phase that was eluted from the paper with water will be termed fraction 2.

There remained on the paper a substance in the area of fraction 2 that was not eluted from the paper with water but could be eluted with dilute ammonia. This substance gave a positive test for desoxyribose[15] and was the substance that did not move in solvents 1[4c] or 2. Thus, it proved to be desoxyribonucleic acid, a well known contaminant of ribonucleic acids.

Relative Concentration of the Two Fractions.—Following this chromatographic procedure quantitatively by measuring the ultraviolet absorption at 260 mμ and by phosphate analysis, using a modification of the method of Griswold,

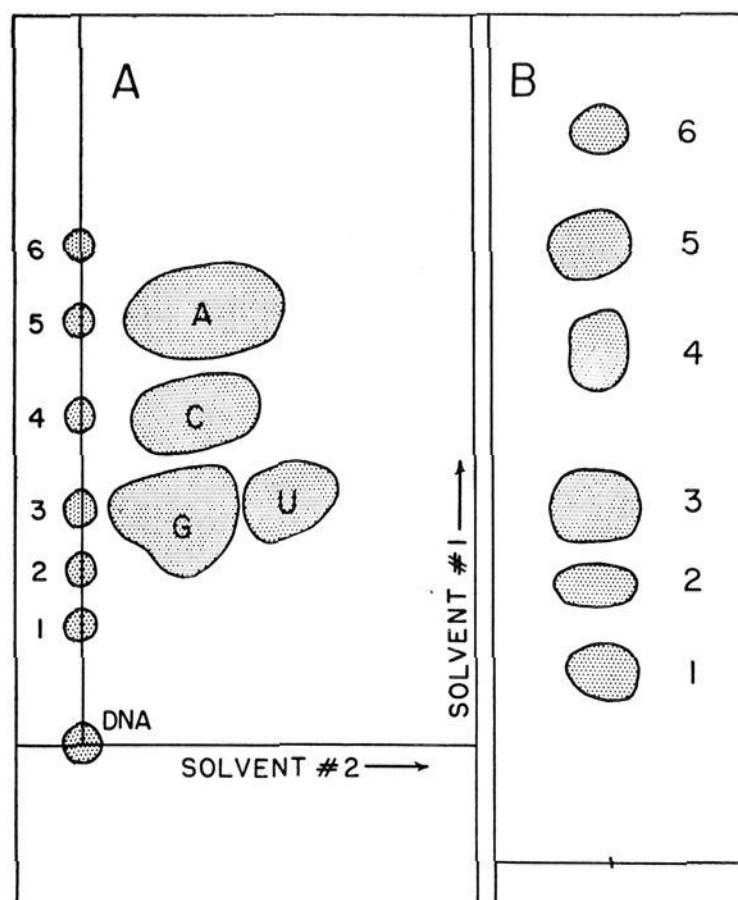(15) P. K. Stumpf, *J. Biol. Chem.*, **169**, 367 (1947).

Fig. 2.—A, Two dimensional chromatogram of 400 micrograms of an alkaline hydrolysate of ribonucleic acid, run first in solvent 1 for 12 hours and then in solvent 2 for 12 hours (ascending technique). Whatman No. 1 filter paper was used. A, G, C and U refer to the respective nucleotides. DNA refers to desoxyribonucleic acid. Numbers 1–6 refer to the various substances in fraction 2. B, A sample of fraction 2 run in solvent 1 for 23 hours (ascending technique) using Whatman No. 1 filter paper. Numbers refer to the various substances in this fraction based on their chromatographic behavior.

Humoller and McIntyre,[16] the relative concentrations of the two fractions and the desoxyribonucleic acid content were determined (Table I).

TABLE I

RELATIVE CONCENTRATIONS OF THE TWO FRACTIONS OBTAINED IN THE CHROMATOGRAPHY OF ALKALINE HYDROLYSATES OF RIBONUCLEIC ACID

Fraction	% by ultraviolet absorption at 260 mμ Optical density[a]	% of total hydrolysate	% by phosphate analysis Micromoles phosphorus[a]	% of total hydrolysate
Total hydrolysate	291.00	...	51.13	....
Fraction 1	267.00	91.75	48.87	95.58
Fraction 2	8.75	3.01	1.65	3.23
Desoxyribonucleic acid	3.00	1.03	0.71	1.39
	Recovery: 95.79		Recovery: 100.20	

[a] Each datum is the average of three separate experiments with corresponding blanks, and is corrected for 1.0 ml. of alkaline hydrolysate.

Studies on Fraction 1.—The amount of fraction 2 present (3%) cannot account for the total lead tetraacetate titer (12%) found by Becker and Allen.[8] The majority of the compounds labile to oxidation by lead tetraacetate must therefore be components of fraction 1. Lead tetraacetate titrations[17] were performed on the two fractions, but because of the high paper blanks only qualitative results were

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(17) G. Fawaz and K. Seraidarian, THIS JOURNAL, **69**, 966 (1947).

obtained. The data did, however, indicate that the titrating material was located primarily in fraction 1. The absence of free nucleosides was confirmed both by paper chromatography[10] and by paper ionophoresis. The absence of nucleoside-5'-phosphates was demonstrated using the paper ionophoretic technique previously described. From this one must conclude that there are compounds present in ribonucleic acid which are as yet uncharacterized.

Studies on Fraction 2.—The chromatographic results suggest that this fraction is composed of substances of greater complexity than mononucleotides. It gives a negative test for desoxyribose[15] and is therefore apparently derived from ribonucleic acid. When resubmitted to chromatography using solvent 1, this fraction separates into five major spots and one minor spot with reference to relative concentration (Fig. 2B). The six spots were eluted with water and the ultraviolet absorption curves run for each, using corresponding paper blanks (Fig. 3).

Samples of each of the six spots were hydrolyzed, chromatographed and analyzed according to the method of Smith and Markham.[4d] The molar ratios of the six spots are given in Table II. The curves calculated from these data when compared with the experimental curves were in close agreement.

TABLE II

NUCLEOTIDE COMPOSITION OF THE COMPOUNDS IN FRACTION 2

Nucleotide acid	1	2	3	4	5	6
Guanylic	3	7	10	1	..	..
Adenylic	..	..	2	4	19	1
Cytidylic	..	1	1	3	1	..
Uridylic	1	2	1	..	..	..

The unknown spot appearing between adenylic acid and guanylic acid on a formate (pH 3.3) ionophoretic separation of total alkaline hydrolysates, when eluted and resubmitted to chromatography in solvent 1 moved with the same R_f as Spot 6 of fraction 2.

This small amount of fraction 2 present in the total alkaline hydrolysates of ribonucleic acid should not detract from its significance and importance in the theory and scope of nucleic acid chemistry. The results presented here not only cast doubt upon the quantitativeness of alkaline hy-
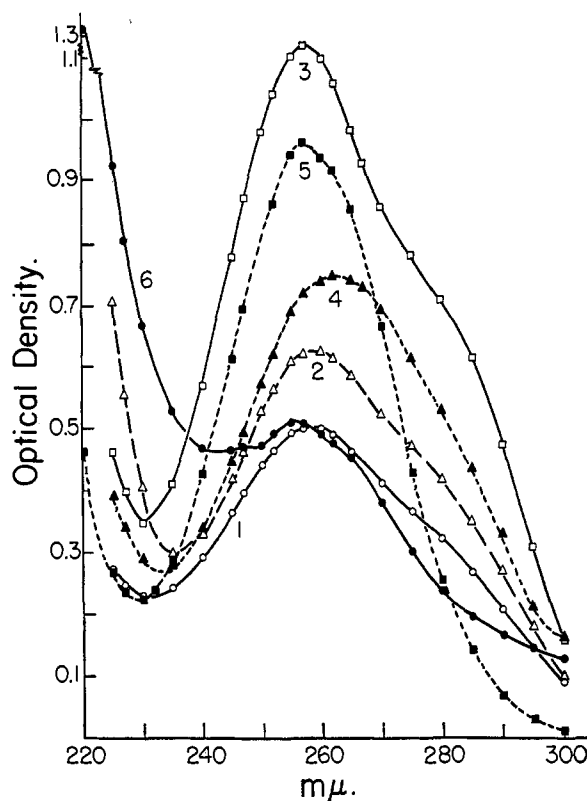

Fig. 3.—Ultraviolet absorption curves of the polynucleotides. Numbers refer to the various substances in fraction 2.

drolysis to form mononucleotides, but also indicate the future trend in the investigation of the chemical hydrolysis products of ribonucleic acids, that of studying the non-mononucleotide fraction of the hydrolysate which contains linkages stable to alkali at room temperatures.

BERKELEY, CALIFORNIA

Alkaloids of *Veratrum eschscholtzii* Gray. I. The Glycosides*

BY M. W. KLOHS, M. D. DRAPER, F. KELLER, W. MALESH AND F. J. PETRACEK

Isorubijervosine ($C_{33}H_{53}O_7N$), a new glucosidic alkaloid, as well as the known glucosides, pseudojervine and veratrosine, has been isolated from *Veratrum eschscholtzii* Gray. On acid hydrolysis, isorubijervosine yielded the aglycone isorubijervine, and D-glucose. Isorubijervosine was shown to be 3(β]-D-glucosyl-Δ^5-solanidene-18-ol.

Isorubijervosine, a new glucosidic alkaloid, has been isolated from the hitherto uninvestigated species, *Veratrum eschscholtzii* Gray, as well as the known glucosides, pseudojervine and veratrosine.

Pseudojervine was originally isolated by Wright and Luff,[1] from *Veratrum viride* Ait. and *Veratrum album* L. The empirical formula ($C_{29}H_{43}O_7N$) advanced by these workers was later revised to $C_{33}H_{49}O_8N$ by Poethke[2]; however, the glycosidic character of this alkaloid was not revealed until the work of Jacobs and Craig[3] showed it to be the gluco-

side of the alkamine jervine. The latter investigators also succeeded in isolating veratrosine[3] (C_{33}-$H_{49}O_7N$), and showed it to be the glucoside of the alkamine, veratramine.

After investigating the hypotensively active amorphous bases[4] of *Veratrum eschscholtzii* Gray, our attention was directed to the more hydrophilic fractions herein designated as II and IV.[5] On dissolving fraction I in boiling ethanol, pseudojervine rapidly separated as an insoluble white powder. The mother liquors were evaporated to dryness and

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(2) W. Poethke, *Arch. Pharm.*, **276**, 170 (1938).

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(5) Fraction IV when treated in the same manner as Fraction II yielded additional quantities of isorubijervosine and pseudojervine.