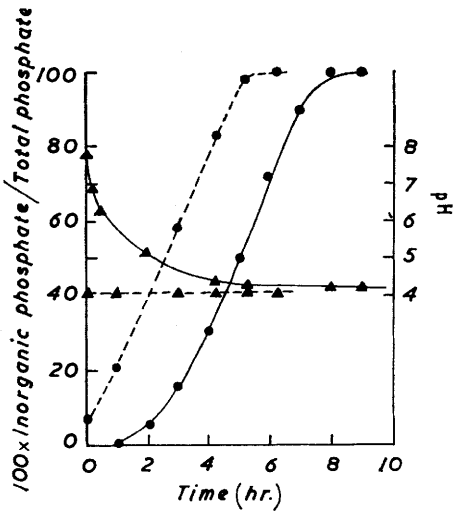
241. *The Hydrolysis of Ribonucleic Acid with Aqueous Formamide.*

By D. H. Hayes.

Ribonucleic acid may be rapidly and quantitatively degraded to ribonucleosides by hydrolysis with 50% aqueous formamide under reflux. During the hydrolysis which takes place at a slightly acid pH, considerable deamination of cytidine takes place. The ribonucleosides are isolated from the hydrolysate by ion-exchange chromatography.

The hydrolysis of ribonucleic acid to ribonucleosides and inorganic phosphate has already formed the subject of several publications.[1]

Recently the preparation of guanosine by hydrolysis of ribonucleic acid with aqueous formamide under reflux has been described.[2] This procedure seems to offer some advantages over the earlier methods and it has therefore been investigated as a means of preparing the ribonucleosides on a large scale. The course of hydrolysis was followed by periodical determination of the liberated inorganic phosphate and of the pH of the reaction



Hydrolysis of ribonucleic acid in aqueous formamide (———) *and* 8M-*ammonium formate buffer*, pH 4·1 (– – – – –).

▲ pH. ● *Liberated inorganic phosphate expressed as per cent of total phosphate.*

mixture. The results, summarized in the Figure, show that liberation of inorganic phosphate becomes rapid only after establishment of a slightly acid pH and that it is complete in 8—10 hours.

As can be seen from the yields of nucleosides obtained, hydrolysis of ribonucleic acid with aqueous formamide gives satisfactory results for adenosine, guanosine, and uridine but causes extensive deamination of cytidine. The isolated cytidine represents approximately 20% of the amount present in the intact ribonucleic acid, as determined by base analysis of this material. Similar low yields of cytidine have been obtained in all the hydrolytic procedures so far described.[1]

Since hydrolysis of ribonucleic acid with aqueous formamide takes place at a slightly acid pH, and since during the hydrolysis the formamide is largely converted into ammonium formate, it seemed probable that ribonucleic acid would be hydrolysed to ribonucleosides if heated at a slightly acid pH maintained either by use of a suitable buffer system or by

[1] Levene and Jacobs, *Ber.*, 1910, 3150; Gulland and Hobday, *J.*, 1940, 746; Bredereck, Martini, and Richter, *Ber.*, 1941, **74**, 694; Gulland and Smith, *J.*, 1947, 338; Harris and Thomas, *Nature*, 1948, **161**, 931; Harris and Thomas, *J.*, 1948, 1936; Elmore, *Nature*, 1948, **161**, 931; Elmore, *J.*, 1950, 2084; Loring and Ploeser, *J. Biol. Chem.*, 1949, **178**, 439.

[2] Dimroth *et al.*, G.P. 820,438; *Chem. Abs.*, 1945, **48**, 2091.

continuous pH adjustment. Hydrolysis of ribonucleic acid was therefore carried out in ammonium formate buffer under conditions similar to those existing in the aqueous formamide hydrolysate after pH equilibrium has been reached. As shown in the Figure the rate of liberation of inorganic phosphate observed under these conditions is the same as the equilibrium rate observed in the hydrolysis with aqueous formamide. Further work is in progress and will be reported later.

Experimental

Commerical yeast ribonucleic acid (50 g.) was dissolved in water (100 c.c.) and formamide (Eastman Kodak technical grade; 100 c.c.). Octan-1-ol (10 c.c.) was added to control the frothing which takes place during the initial stages of the hydrolysis, and the mixture was boiled under reflux for 10 hr. Aliquot parts (0·5 c.c.) of the hydrolysate were removed at intervals and suitably diluted with distilled water 10-fold for pH measurement and 500-fold for inorganic phosphate determination.[3] After complete reaction (8—10 hr.) the hydrolysate was set aside at room temperature overnight and the crude guanosine which separated was removed after addition of Hyflo filter aid (50 g.). Without addition of this large amount of filter aid filtration of the guanosine was extremely slow. After being washed with water (2 × 50 c.c.) the damp filter cake was extracted for 10 min. with boiling water (300 c.c.) and charcoal (2 g.). Rapid filtration of the boiling mixture gave a colourless filtrate from which, on cooling, guanosine crystallized (6·0 g., 21 mmoles; m. p. 228—230°). The filtrate and water washings were combined and evaporated *in vacuo* to dryness in the boiling-water bath, leaving

Table 1.

Results are given as millimoles of base, nucleoside, or phosphorus per 50 g. of ribonucleic acid or hydrolysate therefrom.

		RNA Hydrolysate		
		Pyridine-insoluble	Pyridine-soluble fraction	
	Intact RNA	fraction	Paper †	Ion-exchange †
Adenine		—	4	—
Adenosine	33 *	—	22	21
Guanine		—	0·7	—
Guanosine	34 *	—	3·3	4·4
Cytidine	23 *	—	9·2	6·8
Uridine	28 *	—	39	34
Phosphorus	145	110	—	—

* Results derived from base analysis of intact RNA.
† Paper, results obtained by paper-chromatography of hydrolysis products.
Ion-exchange, results calc. from the amounts of nucleosides found by ion-exchange analysis of hydrolysis products (cf. Table 2).

Table 2.

Eluant	Fractions	Compound	Range of fractions	Content of peak (micromoles)	$\varepsilon_{280}/\varepsilon_{260}$
0·01M-$K_2B_4O_7$	1—11	—	—	—	—
0·09M-$H{\cdot}CO_2NH_4$–0·01M-$K_2B_4O_7$	12—73	Cytidine	13—18	13·6	0·90—0·94
		Adenine			
		Guanine	20—22 *	—	—
		Adenosine	21—35	42·6	0·13—0·15
		Uridine	38—62	68	0·32—0·35
0·09M-$H{\cdot}CO_2NH_4$ (pH 8·3)	74—103	Guanosine	80—87	8·8	0·63—0·66
		Inosine	88—98	0·4	
0·09M-$H{\cdot}CO_2NH_4$ (pH 4·3)	104—145	Xanthosine	105—107	1·0	—
		Cytidylic acids	108—115	Traces	—
		Uridylic acids	127—133		

* Estimated by paper chromatography of fractions 20—25.

a voluminous semisolid residue consisting chiefly of ammonium formate. This residue was extracted under reflux for successive periods of 10 min. each with pyridine (200, 100, 100 c.c.). After each extraction the mixture was cooled to room temperature and filtered. The combined pyridine extracts were evaporated *in vacuo* on the water-bath, leaving a glassy solid (25 g.).

[3] Lowry and Lopez, *J. Biol. Chem.*, 1946, **162**, 421.

This product and the pyridine-insoluble residue were each dissolved in distilled water, and the solutions, after dilution to 100 c.c., were examined for nucleoside and inorganic phosphate content.[3] Nucleoside determinations were carried out by two-dimensional paper chromatography of the hydrolysis products [Whatman No. 1 paper; developing solvents, first direction aqueous ammonia, pH 10[4]; second direction, 5% aqueous disodium hydrogen phosphate–isopentyl alcohol[5] (3 : 2)]. Spots on the developed chromatograms corresponding to adenine, guanine, adenosine, and guanosine were eluted with 0·01N-hydrochloric acid (5·0 c.c.), and the eluates were assayed spectrophotometrically. Resolution of cytidine and uridine was usually poor. These compounds were therefore eluted together from the chromatogram with 0·01N-hydrochloric acid (5·0 c.c.) and determined by differential spectrophotometry.[6] Samples of the ribonucleic acid used for hydrolysis were analysed for purine and pyrimidine base content by Marshak and Vogel's method[7] and for phosphorus content by Fiske and SubbaRow's procedure.[8] The results of the analyses are combined in Table 1.

Chromatographic Separation of the Nucleosides.—(a) *Separation on an analytical scale.* An aliquot part (0·20 c.c.; nucleoside content approximately 150 micromoles) of the solution of pyridine-soluble material obtained by hydrolysis of 50 g. of ribonucleic acid was mixed with 0·4M-potassium borate (0·5 c.c.). The pH was adjusted to 10 by addition of ammonia, and the solution was passed on to a column (15 × 1·0 cm.) of Dowex 2 × 8 ion-exchange resin (200—400 mesh; formate form) which had been previously washed with 0·01M-potassium borate (200 c.c.). The buffers used for elution of the column, and the results, are summarized in Table 2. The course of elution was followed by determining the optical densities of individual fractions at 260 and 280 mμ.

(b) *Separation on a large scale.* A column (35 × 5·8 cm.) of Dowex 2 × 8 ion-exchange resin (200—400 mesh; formate form) was washed with 0·01M-potassium borate (1 l.). The remainder of the solution of pyridine-soluble material obtained from the hydrolysate of 50 g. of ribonucleic acid (nucleoside content approximately 75 millimoles of mixed nucleosides) was mixed with 0·4M-potassium borate (250 c.c.), and the pH was adjusted to 10 with ammonia. This solution was passed on to the column and elution carried out with 0·01M-potassium borate (2 l.) followed by ammonium formate–potassium borate buffer (0·09M with respect to formate, 0·01M with respect to borate; pH 8·3; 38 l.). Fractions of 500 c.c. were collected at a flow rate of 300 c.c. per hr. and examined for ultraviolet absorption at 260 and 280 mμ. Cytidine was eluted in fractions 7—13, adenosine in fractions 19—35, and uridine in fractions 36—72.

Cytidine. Fractions 7—13 were combined, acidified to pH 2 with hydrochloric acid, and treated with two portions of charcoal (10 g., 5 g.) with 10 minutes' stirring after each addition. The ultraviolet absorption of the solution at 260 mμ was thereby reduced to less than 1% of its original value. The charcoal was removed, washed with water (200 c.c.), and eluted with 50% aqueous-ethanolic 0·3M-ammonia (2 × 1·5 l.) with 6 hours' shaking at room temperature for each extraction. Evaporation of the combined charcoal eluates to dryness *in vacuo* on the water-bath left a solid which was dissolved in dry methanol (20 c.c.). The solution was filtered and the filtrate added to dry methanolic 6·8M-hydrogen chloride (7·0 c.c.). Cytidine hydrochloride crystallized and was collected (1·2 g.). Concentration of the mother-liquors caused deposition of further cytidine hydrochloride (0·15 g., total yield 1·35 g., 5 millimoles), m. p. 200—210° (decomp.). Recrystallized from methanol, cytidine hydrochloride had m. p. 209—212° (decomp.).

Adenosine. Fractions 19—35 were combined, evaporated *in vacuo* on the water-bath to 350 c.c., filtered, and set aside in the refrigerator. Adenosine monohydrate crystallized and was collected (5·0 g., 17·5 millimoles; m. p. 202—206°). Recrystallization from water followed by drying (P_2O_5) at 80°/1 mm. for 12 hr. gave adenosine, m. p. 227—228°.

Uridine. Fractions 36—72 were combined, acidified to pH 2 with hydrochloric acid, and treated with charcoal (3 × 10 g.) as described for the cytidine eluate. The charcoal was collected, washed with water (400 c.c.), and eluted with 50% aqueous-ethanolic 0·3M-ammonia (3 × 2 l.) with 6 hours' shaking for each extraction. Evaporation of the combined

[4] Levenbook, quoted by Wyatt, "The Nucleic Acids," Academic Press, New York, 1955, Vol. I, pp. 252—253.
[5] Carter, *J. Amer. Chem. Soc.*, 1950, **72**, 1466.
[6] Loring, Fairley, Bortner, and Seagram, *J. Biol. Chem.*, 1952, **197**, 809.
[7] Marshak and Vogel, *J. Biol. Chem.*, 1951, **189**, 597.
[8] Fiske and SubbaRow, *J. Biol. Chem.*, 1925, **66**, 375.

eluates left uridine which crystallized from 95% ethanol as needles (6·1 g., 25 millimoles), m. p. 163—165°), and after recrystallization from 95% ethanol had m. p. 165—166·5°.

Hydrolysis of Ribonucleic Acid in Ammonium Formate Buffer. Ribonucleic acid (1·0 g.) was heated in ammonium formate buffer (8M-formate; pH 4·0; 4·0 c.c.) at 140° (bath-temperature). The acid dissolved in 5—10 min. and the resulting solution had pH 4·1. Periodic determinations of the inorganic phosphate content of the hydrolysate and of its pH were made as previously described, measurement of time beginning when the ribonucleic acid had completely dissolved. The results obtained are summarized in the Figure.

The work was carried out partly at the Institut de Recherches sur le Cancer, Villejuif, Seine, France, during tenure by the author of an appointment as Attaché de Recherches of the Centre National de la Recherche Scientifique; and partly at The University of Michigan. The author thanks Mlle. E. LeBreton, Sous-Directeur of the Institut de Recherches sur le Cancer, and Professor G. R. Greenberg of the Department of Biological Chemistry of the University of Michigan for their interest. This study was supported in part by a research grant to G. R. Greenberg from the National Institute of Arthritis and Metabolic Diseases, United States Public Health Service.

THE DEPARTMENT OF BIOLOGICAL CHEMISTRY, THE UNIVERSITY OF MICHIGAN,
ANN ARBOR, MICHIGAN, U.S.A. [*Received, July 1st,* 1959.]