Terminal-Sequence Studies of High-Molecular-Weight Ribonucleic Acid

THE REACTION OF PERIODATE-OXIDIZED RIBONUCLEOSIDES, 5′-RIBONUCLEOTIDES AND RIBONUCLEIC ACID WITH ISONIAZID

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1. The reaction products of isoniazid with periodate-oxidized ribonucleosides and 5′-ribonucleotides have been characterized as the monohydrazones. 2. The stability, chromatographic and electrophoretic properties of the hydrazones are described. 3. ^{3}H-labelled isoniazid was shown to react with the 5′-linked terminal adenosine and cytidine groups of periodate-oxidized *Escherichia coli* transfer RNA. One mole of isoniazid reacts with 27×10^3g. of the transfer RNA. 4. One mole of ^{3}H-labelled isoniazid reacts with approx. 10^6g. of rabbit-reticulocyte ribosomal RNA. After fractionation of the RNA into its two components and treating the fractionated material with pancreatic ribonuclease and ribonuclease T_1 evidence is presented for the existence of two 5′-linked terminal sequences in the 30s fraction and only one sequence in the 17s fraction. 5. The application of this method to determining terminal sequences of high-molecular-weight RNA is discussed.

The problems involved in determining nucleotide sequences in ribonucleic acids of chain lengths greater than 100 nucleotides are mainly those of stability of the RNA and the quantity of pure material available. The first problem can be solved by methods that give nuclease-free preparations such as that of Cox & Arnstein (1963). For terminal-group analysis non-radioactive techniques (Lane, Diemer & Blashko, 1963) require amounts of about 50μg. of the terminal nucleoside, thus requiring 150mg. of RNA of mol. wt. 10^6. By using radioactive techniques, 0·2mμc of ^{14}C or 0·4mμc of ^{3}H can be detected accurately. In this case, evenly labelled RNA of mol. wt. 10^6 would be needed with a total radioactivity of between 0·6 and 1·2μc. These radioactivities are obtainable with some viral and bacterial systems (Sugiyama & Fraenkel-Conrat, 1963; Whitfeld, 1962), but it is not possible to obtain mammalian RNA of high enough specific activity to make the method of general use. Thus, to determine terminal sequences in high-molecular-weight RNA with the most economical use of the small amounts of material usually available, terminal labelling offers a universal method capable, with reagents of high specific activity, of determining sequences on milligram amounts of RNA.

RNA generally has a phosphorylated 5′-hydroxyl group at the 3′-hydroxyl-linked terminal nucleotide and free 2′,3′-hydroxyl groups at the 5′-hydroxyl-linked terminal nucleotide. Ralph, Young & Khorana (1962) condensed [^{14}C]aniline to the phosphate on the 3′-linked terminal nucleotide of yeast transfer RNA. [^{14}C]Aniline is available with a specific activity of 10mc/m-mole, or [^{3}H]aniline with a specific activity of 100mc/m-mole can be used. The method has not yet been applied to high-molecular-weight RNA.

The free 2′,3′-hydroxyl groups on the 5′-linked terminus can be oxidized with sodium metaperiodate to form a hydrated dialdehyde (Barry & Mitchell, 1953; Whitfeld, 1954; Yu & Zamecnik, 1960; Khym, 1963). Dulbecco & Smith (1960) condensed [^{35}S]-thiosemicarbazide with the dialdehyde and demonstrated terminal addition to synthetic polynucleotides, but were unable to demonstrate terminal addition to viral RNA. The short half-life of ^{35}S and the availability of material of only relatively low specific activity made it essential to find an alternative hydrazide to react with the terminal dialdehyde after periodate oxidation. ^{3}H-labelled isonicotinoyl hydrazine (isoniazid) with a specific activity up to 2c/m-mole is available, and it had already been shown by Barry & Mitchell (1953) to react with periodate-oxidized hexoses in the same way as thiosemicarbazide.

The present paper describes the reactions of isoniazid with periodate-oxidized ribonucleosides, 5′-ribonucleotides and RNA to form monohydra-

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zones with the ribonucleosides and 5′-ribonucleotides and a terminal addition product with RNA. Some of these results have been briefly reported previously (Hunt, 1963, 1964).

MATERIALS

Sodium metaperiodate (A.R.) and propan-2-ol (A.R.) were obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex; sodium acetate (A.R.), sucrose (A.R.), acetone (A.R.) and acetic acid (A.R.) were obtained from British Drug Houses Ltd., Poole, Dorset. Sephadex G-25 was obtained from Pharmacia A.B., Uppsala, Sweden. Amberlite IRA-400 was obtained from British Drug Houses Ltd. Naphthalene (scintillation grade) was obtained from Nuclear Enterprises (G.B.), Edinburgh, and 2,5-diphenyloxazole and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene from Packard Instrument Co., La Grange, Ill., U.S.A. Peroxide-free dioxan was made from British Drug Houses Ltd. laboratory-reagent dioxan by refluxing 9 vol. under nitrogen over 1 vol. of 2 N-HCl, and drying with NaOH and sodium. Adenosine and guanosine were obtained from Keighly and Keith, London, E.C.4; uridine was obtained from Roche Products Ltd., Welwyn Garden City, Herts.; cytidine, CMP, GMP and UMP were obtained from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.; AMP was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Isoniazid was obtained from E. R. Squibb and Sons Ltd., Twickenham, Middlesex, and from Macarthys Ltd., Romford, Essex. [^{3}H]Isoniazid was obtained from The Radiochemical Centre, Amersham, Bucks., and had specific activities of 603 mc/m-mole and 918 mc/m-mole when purchased.

RNA samples. Escherichia coli transfer RNA was a gift from Dr E. M. Martin.

Rabbit-reticulocyte RNA was prepared from Sandylop rabbits (preparations 1, 2 and 4) and Dutch rabbits (preparation 3) by the method of Cox & Arnstein (1963), with a modification (R. A. Cox, personal communication) in preparations 3 and 4 as follows:

Ribosome suspensions of concentration about 5 mg./ml. in sucrose (0·25 M), KCl (25 mM), $MgCl_2$ (1 mM), tris–HCl buffer, pH 7·6 (0·05 M), were mixed at 0° with 2 vol. of 6 M-guanidinium chloride (A.R.), pH 7·6; after about 10 min. 1·5 vol. of ethanol was added and the solution left until the RNA had flocculated. For preparations 1 and 2 the RNA was spun out and dissolved in water at 0° by repeatedly extracting the precipitate with water; usually on the second extraction the RNA dissolved.

The more stable RNA preparations 3 and 4 were made by dissolving the RNA in 4 M-guanidinium chloride buffered with 0·03 M-tris–HCl, pH 7·6, at a concentration of 1 mg./ml. by warming the solution to room temperature. The RNA was precipitated by adding 0·5 vol. of ethanol, the procedure was repeated and the RNA taken up in water as above.

The $\epsilon_{1\,cm.}$ (P) values at 260 mμ of ribosomal RNA was taken as 7500 and of *E. coli* transfer RNA as 7880, 0·1% solutions of RNA in a 1 cm. cell giving extinctions of 23·2 and 24·6 respectively.

Enzymes. Pancreatic ribonuclease (EC 2.7.7.1.6) was obtained from C. F. Boehringer und Soehne G.m.b.H. Ribonuclease T_1 (EC 3.1.4.8) was prepared from Sanzyme R (Sankyo Co. Ltd., Tokyo, Japan) according to the method of Rushizky & Sober (1962) and had a specific activity of 480 enzyme units/E^{1cm}_{280} of enzyme (Rushizky & Sober, 1962).

METHODS

Periodate oxidation of nucleosides and nucleotides (*Davoll, Lythgoe & Todd*, 1946; *Khym & Cohn*, 1960). (*a*) Small scale (10 mg.). In this procedure 45 μmoles of nucleosides or nucleotides were dissolved in 2·7 ml. of water and the pH was adjusted to 5. Then 0·3 ml. of 0·2 M-sodium metaperiodate (pH 4–5) was added to these solutions and the mixtures were kept at room temperature in the dark for 1 hr. With guanosine the final volume was 6 ml. to prevent precipitation. The excess of periodate could be removed by adding butane-2,3-diol and aerating the solution, but this was not found to be necessary.

(*b*) Larger scale (100–500 mg.). In this procedure 100 mg. of the nucleosides was dissolved in 4 ml. of water (8 ml. for guanosine), 2·2 ml. of 0·2 M-sodium metaperiodate was added, the mixtures were left at room temperature in the dark for 1 hr. and then allowed to percolate through a column (6 cm. × 1 cm.) of Amberlite IRA-400 (acetate form), and the oxidized nucleosides were washed through with 20 ml. of 0·02 N-acetic acid. Guanosine was diluted with an equal volume of 0·02 N-acetic acid and then washed through with 20 ml. of 0·02 N-acetic acid. The solutions were either freeze-dried or kept for the next reaction.

Periodate oxidation of RNA (*Dulbecco & Smith*, 1960). RNA (0·5–5 mg./ml.) was treated with a 100-fold molar excess of sodium metaperiodate in 0·05–0·1 M-sodium acetate buffer, pH 5, for 1 hr. at 20° in the dark. The RNA was precipitated with 2 vol. of ethanol at 0°, redissolved in 0·1 M-sodium acetate buffer and precipitated with ethanol again.

Reaction of isoniazid with periodate-oxidized compounds. (1) Solutions containing about 100 mg. of oxidized nucleosides were freed from periodate and iodate with Amberlite IRA-400, and then mixed with 1·2-fold molar excess of isoniazid and concentrated in a vacuum desiccator to precipitate the isonicotinoyl hydrazones. The isonicotinoyl hydrazones were collected by filtration and recrystallized from minimal amounts of water until their spectra showed less than 5% of impurity by comparison with the spectrum of an equimolar mixture of the two reactants.

(2) For preparations of less than 10 mg. the oxidized nucleosides or nucleotides were treated with a 4-fold molar excess of isoniazid and stored at 4°. These solutions were used as markers in electrophoresis and chromatography.

(3) Micro-scale preparations: micromole amounts of nucleoside after reaction with a small excess of periodate were evaporated in a vacuum desiccator after the addition of an excess of butane-2,3-diol. A 4-fold molar excess of [^{3}H]isoniazid was added and the mixture incubated at 25° for 16 hr.

Reaction of [^{3}H]*isoniazid with periodate-oxidized RNA* (*Dulbecco & Smith*, 1960). The periodate-oxidized RNA was incubated in 0·1 M-sodium acetate buffer, pH 5·0, with a 100-fold molar excess of [^{3}H]isoniazid for 20 hr. at 25°. Control experiments with different periods of incubation showed this to be an optimum time for a solution of concentration 2 mg. of RNA/ml. The reaction was stopped by precipitation with 2 vol. of ethanol at 0°, and the solution washed by redissolving in 0·1 M-sodium acetate buffer and precipitation with ethanol four or five times until a control

of non-periodate-oxidized RNA showed less than 10% incorporation of the oxidized sample. Some attempts were made to wash by precipitation from guanidinium chloride or to dialyse against alternating changes of guanidinium chloride and 0·1 M-sodium acetate buffer, but the best results were obtained when material was put on a Sephadex G-25 column in 0·1 M-sodium acetate buffer, pH 5·0, after several washes by precipitation. The final material was stored at 1–5 mg./ml. in 0·1 M-sodium acetate buffer, pH 5·0, at −20°.

Enzyme digestion. All digestions were carried out by adding 2 vol. of the RNA solutions at pH 5 to 1 vol. of ribonuclease solution. Pancreatic ribonuclease had a concentration of 0·1 mg./ml. Ribonuclease T_1 had a concentration of 0·42 $E^{1\,cm.}_{280\,m\mu}$ unit/ml.

Paper electrophoresis. This was carried out on a flat-bed water-cooled machine with 0·1 M-ammonium formate buffer, pH 3·0 or 3·5, on Whatman 3MM paper. The paper was moistened in the buffer by dipping and blotting and the samples were applied in small volumes (up to 20 µl./cm.) to the moist paper. A potential gradient of up to 33 v/cm. at 44 mA/cm. width for 2½–3 hr. gave sufficient separation of the nucleoside hydrazones.

Paper chromatography. No solvent system that separated all four nucleoside hydrazones was found. Two solvents were found to be generally useful: propan-2-ol–acetic acid–water (14:1:5, by vol.) and butan-1-ol–acetone–water (55:35:16, by vol.).

Radioactivity counting. The dioxan system of Werbin, Chaikoff & Imada (1958) was used, except that 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene was used in place of 1,4-bis-(5-phenyloxazol-2-yl)benzene. The mixture was as follows: 200 g. of naphthalene, 10 g. of 2,5-diphenyloxazole and 0·25 g. of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene to 1 l. of peroxide-free dioxan. It was shown that reagent-grade dioxan after removal of peroxides was as good as A.R.-grade material. Under these conditions 4% of water may be added without freezing at −1°. The standard assay volume was 10 ml. of scintillator plus 0·4 ml. of water containing the radioactive material in a polythene vial. The samples were counted in a Packard liquid-scintillation spectrometer (Packard Instrument Co. Inc.), a standard solution of tritiated water being used with each batch of scintillator solution. Efficiencies of 20–27% with a background of 20 counts/min. were obtained with model 314E and 30–33% with the newer model 3000. For assay of electrophoresis strips, the strip was cut into areas of 10–15 cm.² and each portion wetted with 0·4 ml. of water in a polythene vial; then 10 ml. of the scintillator was added and the vial shaken to elute the radioactivity. Under these conditions the efficiency was about 50% of that in free solution.

The presence of salt and sucrose caused a marked decrease in counting efficiency when the concentration of salt was greater than 0·1 M and that of sucrose more than 1%. Up to 0·5 mg. of RNA/ml. did not affect the counting efficiency.

Carbon, hydrogen and nitrogen analyses. These were made by Weiler & Strauss, Oxford.

Spectra. These were determined with a Unicam SP.700 recording spectrophotometer. Spectral coefficients of the nucleosides and nucleotides are those quoted in the Calbiochem catalogue (California Foundation for Biochemical Research, Los Angeles, Calif., U.S.A., 1963).

RESULTS AND DISCUSSION

Isonicotinoyl hydrazones of nucleosides and nucleotides. The isonicotinoyl hydrazones of periodate-oxidized nucleosides and nucleotides can be shown to be monohydrazones by chemical and spectrophotometric analysis. The crystalline isonicotinoyl nucleoside hydrazones contain water of crystallization that can only be removed with difficulty by prolonged heating at 80–100° *in vacuo*. The dried compounds are yellow and very hygroscopic, and show some loss of nitrogen after prolonged heating. Drying by precipitation of the hydrazones from ethanolic solution by diethyl ether met with no greater success. However, the elemental analyses shown in Table 1 agree with the proposed formulae

Table 1. *Elemental analysis of the nucleoside isonicotinoyl hydrazones*

(i) Compound crystallized from ethanol–ether; all of these compounds were hygroscopic. (ii) Compound crystallized from water. All compounds were dried at room temperature *in vacuo* except uridine (ii), which was dried at 80° *in vacuo*.

Nucleoside hydrazone		Found (%) C	H	N	C/N	Water content (moles/mole)	Calculated (%) C	H	N	C/N
Adenosine ($C_{16}H_{18}N_8O_5$): yield 80–90%						0	47·7	4·5	27·9	1·71
	(i)	46·1	5·5	26·7	1·72	1	45·7	4·8	26·7	
	(ii)	42·3	5·2	24·7	1·71	3	42·1	5·3	24·5	
Guanosine ($C_{16}H_{18}N_8O_6$): yield 45–60%						0	45·9	4·4	26·8	1·71
	(i)	44·0	5·2	25·9	1·70	1	44·1	4·6	25·7	
	(ii)	42·0	4·9	24·2	1·73	2·5	41·5	5·0	24·1	
Uridine ($C_{15}H_{18}N_5O_7$): yield 58–60%	(ii)	47·8	4·9	17·9	2·67	0	47·4	4·7	18·4	2·58
	(i)	46·0	5·5	17·4	2·67	1	45·2	5·0	17·6	
Cytidine ($C_{15}H_{18}N_6O_6$): yield 60–80%						0	47·6	4·8	22·2	2·14
	(i)	47·2	5·8	21·4	2·20	1	45·4	5·0	21·2	
	(ii)	41·9	5·1	18·4	2·27	3	41·7	5·6	19·4	

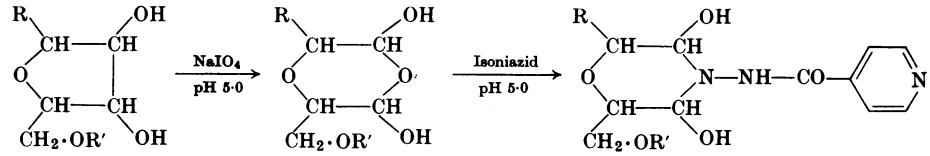
Table 2. *Spectral properties of the nucleoside isonicotinoyl hydrazones*

Spectra were measured in 0·1 N-NaOH.

Nucleoside hydrazone	$\lambda_{max.}$ (mμ)	$10^{-3} \times \epsilon_{max.}$	$\lambda_{min.}$ (mμ)	$10^{-3} \times \epsilon_{min.}$	$\frac{\epsilon_{298m\mu}}{\epsilon_{260m\mu}}$ ratio	
					Found	Calculated*
Adenosine	260	20·0	230	7·5	0·274	(i) 0·272
						(ii) 0·448
Guanosine	268	16·1	231	8·3	0·338	(i) 0·336
						(ii) 0·532
Uridine	265	12·5	244	9·8	0·476	(i) 0·452
						(ii) 0·665
Cytidine	271	12·8	249	10·0	0·531	(i) 0·522
						(ii) 0·733

* (i) Calculated for 1 isoniazid residue/mole. (ii) Calculated for 2 isoniazid residues/mole by using the following extinction coefficients:

	$10^{-3} \times \epsilon_{260m\mu}$	$10^{-3} \times \epsilon_{298m\mu}$
Adenosine	14·9	0
Guanosine	11·3	0
Uridine	7·35	0
Cytidine	7·55	0·92
Isoniazid	4·10	5·17



(I) (II) (III)

Scheme 1. Proposed reaction sequence in the formation of isonicotinoyl hydrazones of nucleosides or nucleotides. R, purine or pyrimidine base; R′, H or PO_3^{2-}.

(III in Scheme 1), provided that the amount of water of crystallization shown is assumed. Further evidence of the monohydrazone is given by examination of the spectral properties in 0·1 N-sodium hydroxide (Table 2), assuming that the extinction coefficients of the two combined spectra are unaltered under these conditions, when the hydrazones are likely to be broken down. (The spectra of the nucleosides and nucleotides are the same after periodate oxidation, and that of isoniazid is stable in 0·1 N-sodium hydroxide, giving some support for this assumption.) The hydrated dialdehyde structure (II in Scheme 1) for the periodate-oxidized ribose has been proposed by Barry & Mitchell (1953) for hexoses, and the structure of the isonicotinoyl hydrazones (III in Scheme 1) is similar to that proposed by Khym (1963) for the methylamine compound of adenosine 5′-monophosphate and the isonicotinoyl hydrazones of hexoses by Barry & Mitchell (1953).

The 5′-nucleotide isonicotinoyl hydrazones have not been crystallized. After reaction of the periodate-oxidized nucleotides with a 4-fold molar excess of isoniazid, the nucleotide isonicotinoyl hydrazones were isolated by paper electrophoresis at pH 3·5, and their ultraviolet-absorption spectra determined in 0·1 N-sodium hydroxide (Table 3). Quantitative recovery of the monohydrazones was obtained. The spectral ratios are similar to those of the nucleoside hydrazones and support the proposed monohydrazone structure.

Nucleoside isonicotinoyl hydrazones prepared with a 4-fold excess of isoniazid had the same spectral, electrophoretic and chromatographic properties as the crystallized compounds. The electrophoretic and chromatographic properties are given in Table 4.

Stability of the isonicotinoyl hydrazones. The nucleoside and nucleotide hydrazones were heated as the crude mixture of the periodate-oxidized compound with a 4-fold molar excess of isoniazid in buffers over the range pH 3–8 (0·04 M-disodium

Table 3. *Spectral properties of the 5′-nucleotide isonicotinoyl hydrazones purified by paper electrophoresis*

Spectra were measured in 0·1 N-NaOH.

Nucleotide hydrazone	$\lambda_{max.}$ (mμ)	$\lambda_{min.}$ (mμ)	$\frac{\epsilon_{298m\mu}}{\epsilon_{260m\mu}}$ ratio Found	Calculated*
Adenosine	259	229	0·292	(i) 0·271
				(ii) 0·447
Guanosine	256	230	0·349	(i) 0·338
				(ii) 0·533
Uridine	260	247	0·447	(i) 0·449
				(ii) 0·663
Cytidine	273	254	0·505	(i) 0·519
				(ii) 0·766

* (i) Calculated for 1 mole of isoniazid/mole. (ii) Calculated for 2 moles of isoniazid/mole by using the following extinction coefficients:

	$10^{-3} \times \epsilon_{260m\mu}$	$10^{-3} \times \epsilon_{298m\mu}$
AMP	15·0	0
GMP	11·2	0
UMP	7·40	0
CMP	7·40	0·80
Isoniazid	4·10	5·17

Table 4. *Chromatographic and electrophoretic mobilities of the nucleoside and nucleotide isonicotinoyl hydrazones*

Electrophoretic mobilities are given relative to that of cytidine hydrazone or CMP hydrazone, chromatographic mobilities relative to that of uridine hydrazone. The results in columns (*a*) and (*b*) are from separate experiments. In column (*c*) all of the nucleoside and nucleotide hydrazones were run at the same time. Where the compounds migrate on both sides of the origin, + indicates cathodic migration, − anodic migration.

Hydrazone	Relative electrophoretic mobility pH 3·0 (*a*)	pH 3·0 (*b*)	pH 3·5 (*c*)	Relative chromatographic mobility Propan-2-ol–acetic acid–water	Butan-1-ol–acetone–water
Adenosine	0·82		+0·66	0·89	0·76
Guanosine	0·40		+0·28	0·75	0·50
Uridine	0·51		+0·43	(1·0)	(1·0)
Cytidine	(1·0*)		(+1·0*)	0·89	0·61
Isoniazid	1·17		1·10	1·08	2·00
AMP		+0·64	+0·05		
GMP		−0·45	−0·21		
UMP		−0·62	−0·28		
CMP		(+1·0†)	+0·22		

* Distance travelled towards cathode after 2½–3 hr. at 26 v/cm. is 20–24 cm. at pH 3·0.
† Distance travelled towards cathode after 2½–3 hr. at 26 v/cm. is 6·4 cm. at pH 3·5.

hydrogen phosphate–0·02 M-citric acid) either for 1 hr. at 50° or 5 min. at 90°. The products were examined by paper electrophoresis at either pH 3·0 or 3·5 for the nucleoside hydrazones and nucleotide hydrazones respectively.

No detectable breakdown of the 5′-nucleotide hydrazones could be shown at 50° over the range pH 4–8. The breakdown products at pH 3·0 had the mobilities of both the free 5′-nucleotides and the free nucleosides. At 90° there is some breakdown at pH 6 and complete breakdown at pH 8. At room temperature the 5′-nucleotide hydrazones are stable for at least 48 hr. at pH 8.

The nucleoside hydrazone mixtures were heated at pH 3, 5 and 8. At 90° there is some breakdown at pH 3 and complete breakdown at pH 8, but the hydrazones are stable at pH 5.

The stability of the 5′-nucleotide hydrazones over

the range pH 4–6 is contrary to that of the methylamine compounds (Khym, 1963), indicating that the β-elimination reaction is suppressed by the acylhydrazone.

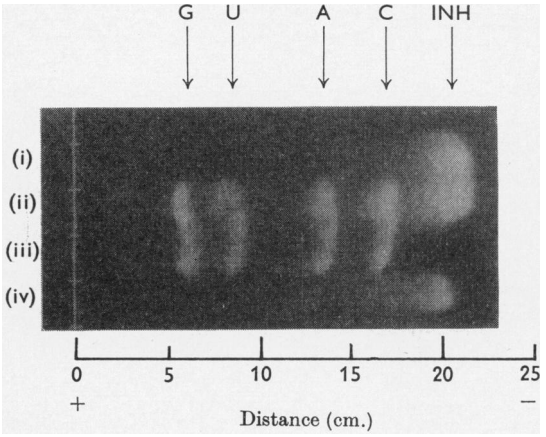


Fig. 1. Separation of the nucleoside isonicotinoyl hydrazones by paper electrophoresis in 0·1 M-ammonium formate buffer, pH 3·0, at 26 v/cm. for 3 hr. (i) Isoniazid (INH); (ii) isoniazid + isonicotinoyl hydrazones of guanosine (G), uridine (U), adenosine (A) and cytidine (C); (iii) isonicotinoyl hydrazones of guanosine, uridine, adenosine and cytidine; (iv) isoniazid.

Separation of the nucleoside isonicotinoyl hydrazones. Since the products of ribonuclease digestion of periodate-oxidized RNA that has been terminally labelled with isoniazid will be isonicotinoyl nucleoside hydrazones and isonicotinoyl oligonucleotide hydrazones, it is necessary to separate the nucleoside hydrazones from each other and from the oligonucleotide hydrazones. This can be accomplished partially by paper electrophoresis at pH 3·0 in 0·1 M-ammonium formate. Under these conditions the nucleoside hydrazones migrate to the cathode (Fig. 1) and the unlabelled oligonucleotides towards the anode. It is not possible to predict the exact mobility of oligonucleotide hydrazones except to say that, since they have one less phosphate group and an additional basic group on the isoniazid, some dinucleotides will have a net positive charge and will migrate slowly towards the cathode. Thus it is not possible by using electrophoresis to be sure that guanosine hydrazone and uridine hydrazone would be separated from the dinucleotide hydrazones of adenosine and cytidine (i.e. ApApINH, ApCpINH etc.), but it is unlikely that the adenosine hydrazone and cytidine hydrazone could be thus contaminated.

Reaction of [^{3}H]*isoniazid with periodate-oxidized RNA.* Table 5 shows that oxidation of ribosomal RNA with a 100-fold molar excess of metaperiodate (assuming mol. wt. 10^6 for the RNA) is complete after 20 min. at 25°, but that interaction with a

Table 5. *Uptake of isoniazid into RNA after various periods of treatment with periodate and* [^{3}H]*isoniazid at* 25°

The specific activity of [^{3}H]isoniazid was 591 mc/m-mole. The efficiency of counting was 27·0%.

Reaction time with isoniazid (hr.)	Specific activity (counts/min./$E_{260m\mu}$ unit) Time of reaction with periodate.....20 min.	40 min.	60 min.	(mean counts/min.$E_{260m\mu}$ unit corrected for blank*)	Isoniazid uptake (μmole/g. of RNA)
2	8490	7300	5980	3370	0·22
4	12600	12060	12590	8530	0·56
20	17120	17820	16560	13280	0·87

* The blank was not treated with periodate but incubated with isoniazid for 20 hr.; it had 3890 counts/min./$E_{260m\mu}$ unit.

Table 6. *Reaction of* [^{3}H]*isoniazid with periodate-oxidized* E. coli *transfer RNA*

Isoniazid was diluted to a specific activity of 32·2 μc/m-mole. Incubation with isoniazid was for 21 hr. at 25°. The efficiency of counting was 22·1%.

	Specific radioactivity (counts/min./mg. of RNA)	Isoniazid uptake (μmoles/g. of RNA)
Periodate-oxidized	673000	53·3
Non-oxidized	83900	6·6
After chromatography	461500	36·6

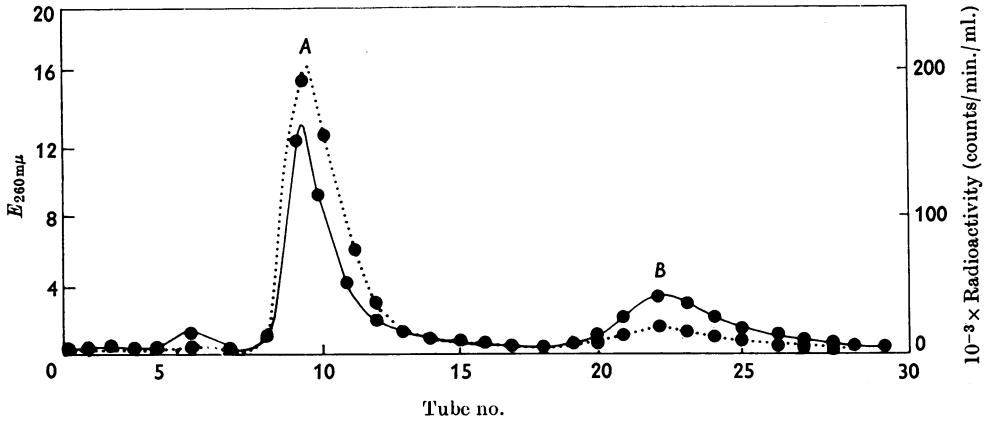


Fig. 2. Chromatography of 2·4mg. of [^{3}H]isoniazid-labelled *E. coli* transfer RNA (*A*) with 0·8mg. of carrier isoniazid (*B*) on a column of Sephadex G-25 in 0·1M-sodium acetate buffer, pH5·0 (2ml. per tube). ●—●, $E_{260m\mu}$; ●····●, radioactivity.

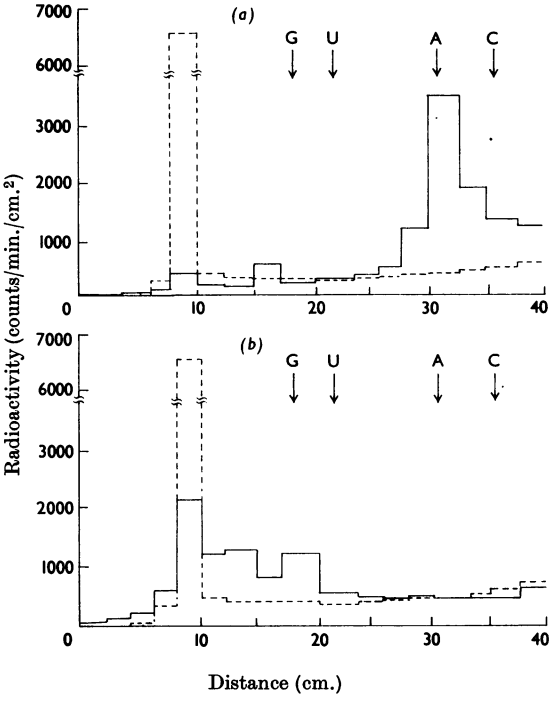


Fig. 3. Separation of the ribonuclease-digestion products of [^{3}H]isoniazid-labelled *E. coli* transfer RNA by paper electrophoresis at pH3·0. (*a*) After digestion with pancreatic ribonuclease; (*b*) after digestion with ribonuclease T_1. ----, Control incubated without enzyme;—, enzymic digest. The arrows marked G, U, A and C refer to the positions of the marker isonicotinoyl hydrazones of guanosine, uridine, adenosine and cytidine respectively (Fig. 1).

100-fold molar excess of [^{3}H]isoniazid is only complete after about 20hr. at 25°.

E. coli transfer RNA appeared to have fully reacted after a similar time under similar conditions, judging by the uptake of isoniazid after chromatography on Sephadex G-25 (Table 6 and Fig. 2). The uptake of 36·6moles of isoniazid/10^6g. of RNA is equivalent to mol.wt. $27{\cdot}0 \times 10^3$, which compares favourably with the reported values of 23×10^3–28×10^3 (see Brown, 1963).

E. coli transfer RNA has the sequences pCpCpA and pCpC at the 5′-linked terminus (Hecht, Stephenson & Zamecnik, 1959). Digestion of the isoniazid-labelled RNA with pancreatic ribonuclease, which is specific for pyrimidine nucleotides, should yield the adenosine hydrazone and cytidine hydrazone, whereas ribonuclease T_1, which is specific for guanylic acid residues (Sato-Asano, 1959), should yield only oligonucleotide hydrazones. Isoniazid-labelled *E. coli* transfer RNA was digested with either pancreatic ribonuclease or ribonuclease T_1 for 2hr. at 25° at pH5·0. The reaction products were separated by paper electrophoresis at pH3·0 and the radioactivity was assayed by cutting the paper into strips and counting in a liquid-scintillation counter (Fig. 3). In two separate experiments it was shown that more than 80% of the bound label released by pancreatic ribonuclease migrates with the adenosine hydrazone and cytidine hydrazone, approx. 75% of this being with the adenosine hydrazone (Fig. 3*a*). By contrast no radioactivity is found in these regions after electrophoresis of the ribonuclease-T_1 digests (Fig. 3*b*), although some label has the same electrophoretic mobility as the guanosine hydrazone.

Table 7. *Incorporation of* [^{3}H]*isoniazid into different preparations of RNA after 20 hr. at 25°*

A control of non-oxidized RNA was treated in exactly the same way as the oxidized sample.

RNA preparation	Isoniazid uptake corrected for control (μmoles/g. of RNA)	Isoniazid uptake by non-oxidized control (% of that by oxidized sample)
1	0·87	29
	0·88	29
	0·89	16
2*	0·75	13
3†	1·20	11
4†	1·24	13

* Incubated for 17 hr. with isoniazid.

† These samples were incubated with a new batch of [^{3}H]isoniazid.

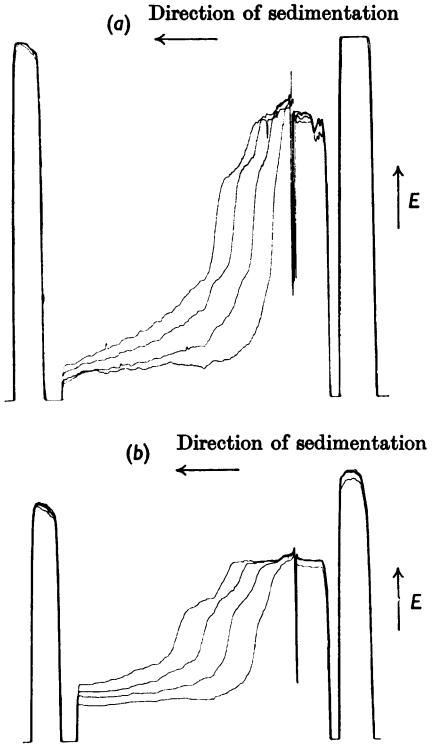


Fig. 4. Densitometer tracings of the sedimentation patterns of ribosomal RNA in a Beckman Spinco ultracentrifuge, equipped with u.v. optics, in 0·1 M-phosphate buffer, pH 7·0, at 42040 rev./min. Tracings were taken at 8 min. intervals. (*a*) Untreated RNA, at 5°; (*b*) RNA after reaction with periodate and isoniazid (preparation 4, in Table 7), at 20°.

[^{3}H]*Isoniazid-labelled rabbit-reticulocyte RNA.* Earlier preparations of isoniazid-labelled ribosomal RNA (preparations 1 and 2 in Table 7, when fractionated by sucrose-density-gradient centrifugation or analytical ultracentrifugation, showed some degradation of the RNA. The most recent preparations of RNA prepared by precipitation three times from 4 M-guanidinium chloride at pH 7 (preparations 3 and 4 in Table 7) showed minimal degradation and a higher level of incorporation (Table 7 and Fig. 4).

The distribution of radioactivity in a sucrose gradient of RNA preparation 4 is shown in Fig. 5. The fractions marked I–V (Fig. 5*b*) were isolated by precipitation with 2 vol. of ethanol after the addition of carrier RNA and salt to 0·1 M. The RNA from fractions II and III corresponds to the two major components of ribosomal RNA with sedimentation coefficients 30s and 17s respectively. These fractions were digested with pancreatic ribonuclease and ribonuclease T_1. Fig. 6 shows the analysis of the radioactivity after electrophoresis of these digests compared with the unfractionated RNA. It is clear that the distribution of labelled isoniazid is different from that of digests of *E. coli* transfer RNA (Fig. 3) and is different in each of the ribosomal-RNA components. With allowance for the fact that there has been some breakdown of the 30s RNA, it is clear that there are two different terminal sequences in the 30s RNA and probably only one in the 17s RNA, as can be demonstrated by closer analysis of the digests of fractions II and III. In the pancreatic-ribonuclease digest of fraction II (30s RNA) (Fig. 6*c*) about 40% of the bound radioactivity has the mobility of the uridine hydrazone, and a similar amount has the same mobility in the ribonuclease-T_1 digests (Fig. 6*e*). This indicates that there are two terminal sequences, one of the type pyrimidine-pU and the other GpU. Since only two main peaks can be identified in each of the two digests these are most probably the only two terminal sequences in the 30s fraction. In fraction III (16s RNA) only one major peak is evident in both digests (Figs. 6*d* and 6*f*). That obtained from pancreatic ribonuclease (Fig. 6*d*) has the mobility of the adenosine hydrazone as about 60% of the bound radioactivity. This indicates the presence of the sequence pyrimidine-pA, and it is likely that this is the only terminal sequence in this fraction

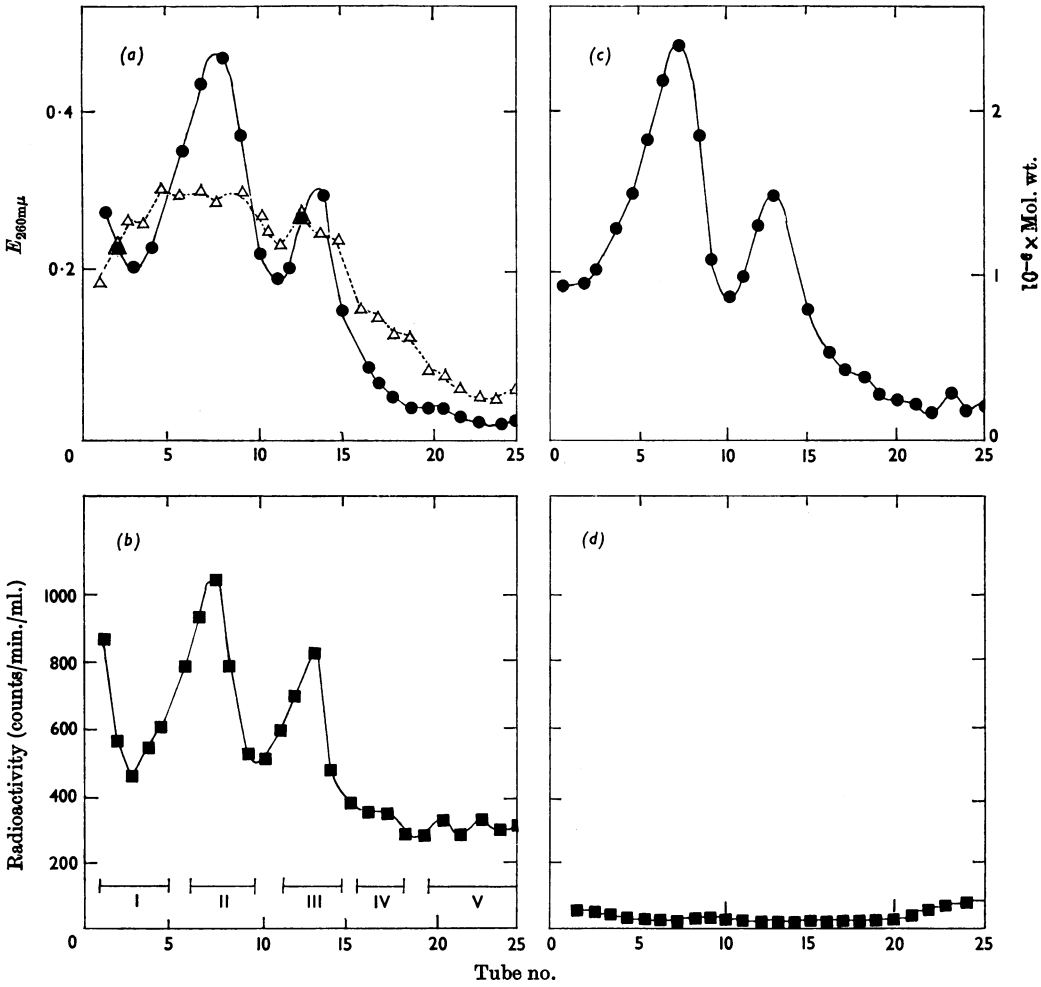


Fig. 5. Distribution of [^{3}H]isoniazid on labelled ribosomal RNA in the sucrose gradient (5–20% of sucrose in 0·1 M-sodium acetate buffer, pH 5·0) for 14 hr. at 25000 rev./min. at 0° in a Spinco SW 25 swinging-bucket rotor (1·6 mg. of RNA per tube). The efficiency of counting was 30·6%. (*a*) Distribution of RNA and (*b*) distribution of ^{3}H in RNA after periodate oxidation and interaction with [^{3}H]isoniazid; the fractions marked I–V indicate how the tubes were combined to isolate the various components. (*c*) Distribution of RNA and (*d*) distribution of ^{3}H in RNA after interaction with[^{3}H]isoniazid without periodate oxidation. ●, $E_{260m\mu}$; ■, radioactivity; △, mol.wt. estimated from the amount of isoniazid bound to the RNA. The samples were diluted 13-fold for analysis; this factor is omitted from both the extinction values and the radioactivity measurements.

since the ribonuclease-T_1 digest has about 60% of its radioactivity as a homogeneous oligonucleotide fraction. The sequences of the oligonucleotide hydrazone fractions have not yet been determined.

The presence of differing terminal sequences in the two components of ribosomal RNA is in agreement with the results of other workers who used less-specific techniques. Differences in base composition of the two components of ascites-cell ribosomal RNA have been demonstrated (Montagnier & Bellamy, 1964), but the only evidence for differing sequences in the two components of ribosomal RNA is given by Aronson (1963), who compared the relative frequency of various oligonucleotide fragments in ribonuclease digests of the RNA from the two components of *E. coli* ribosomes.

It is clear from these results that a great deal of sequence information can be obtained from the terminal-labelling technique. Already it is possible to ascribe certain terminal sequences to the frac-

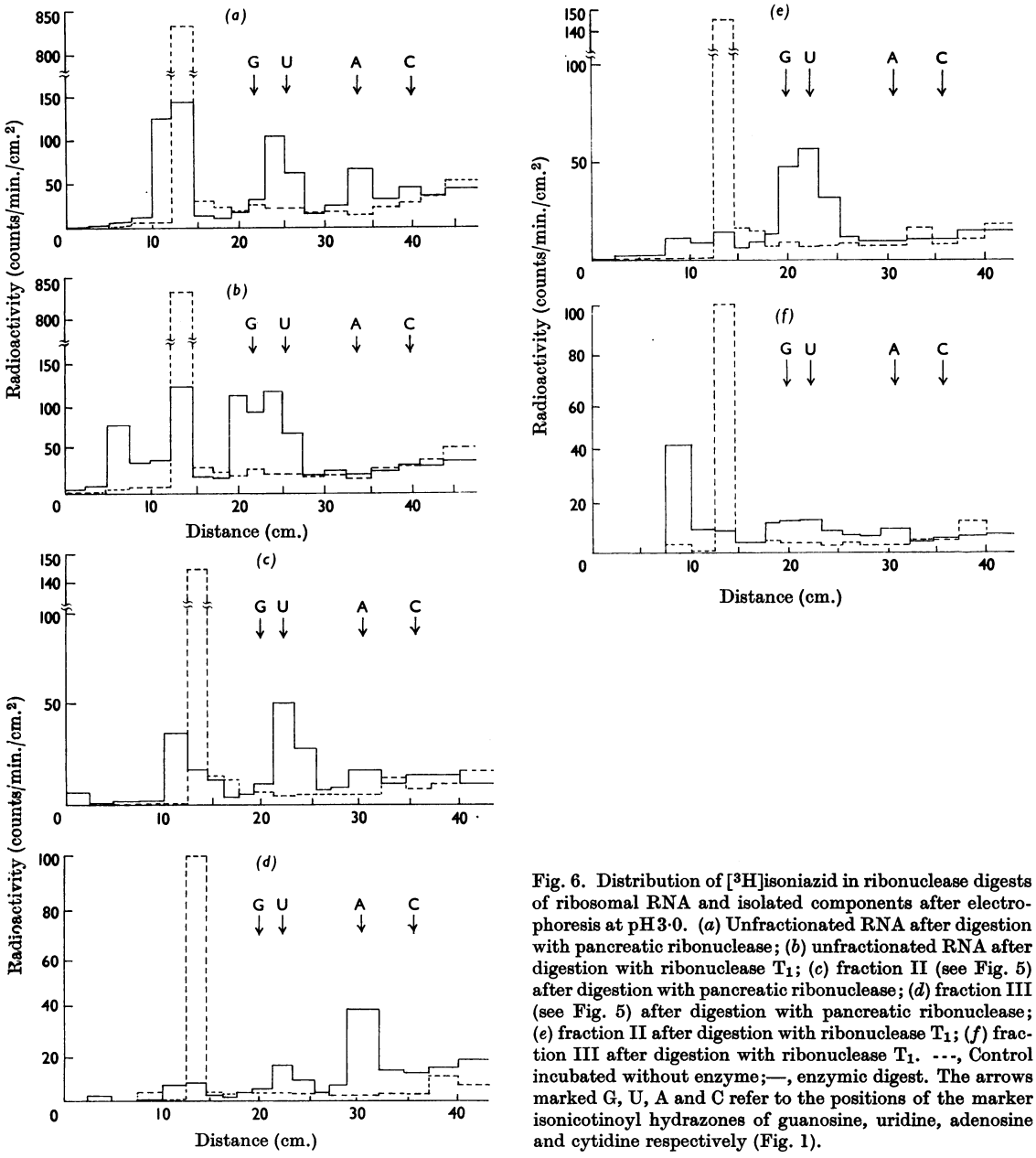


Fig. 6. Distribution of [³H]isoniazid in ribonuclease digests of ribosomal RNA and isolated components after electrophoresis at pH 3·0. (*a*) Unfractionated RNA after digestion with pancreatic ribonuclease; (*b*) unfractionated RNA after digestion with ribonuclease T_1; (*c*) fraction II (see Fig. 5) after digestion with pancreatic ribonuclease; (*d*) fraction III (see Fig. 5) after digestion with pancreatic ribonuclease; (*e*) fraction II after digestion with ribonuclease T_1; (*f*) fraction III after digestion with ribonuclease T_1. ---, Control incubated without enzyme;—, enzymic digest. The arrows marked G, U, A and C refer to the positions of the marker isonicotinoyl hydrazones of guanosine, uridine, adenosine and cytidine respectively (Fig. 1).

tions of RNA, and further knowledge of the size of the oligonucleotide hydrazone fractions that will be obtained on ion-exchange columns in buffers containing urea (Tomlinson & Tener, 1963) should make the task of elucidating these sequences much easier.

By combining the technique of stepwise degradation after periodate oxidation (Ogur & Small, 1960; Yu & Zamecnik, 1960; Whitfeld, 1954; Brown, Fried & Todd, 1955) with the terminal-labelling technique it should be possible to penetrate further

into the molecule than by using either technique alone. This is best illustrated by the following hypothetical example.

An RNA molecule has the following sequence at its 5′-linked terminus:

–pCpApGpUpA

On reaction with [^{3}H]isoniazid (INH*) after periodate oxidation it would have the following structure:

–pCpApGpUpAINH*

After enzymic digestion the following compounds would be isolated: (i) ribonuclease T_1 would give UpAINH*; (ii) pancreatic ribonuclease would give AINH*.

If the original sequence were subjected to stepwise degradation by one step we would have the following terminal sequence:

–pCpApGpU

Reaction with [^{3}H]isoniazid after periodate oxidation would give:

–pCpApGpUINH*

and after ribonuclease digestion the following fragments would be isolated: (i) ribonuclease T_1 would give UINH*; (ii) pancreatic ribonuclease would give ApGpUINH*.

Hence it would be possible to determine the sequence pyrimidine-ApGpUpA with checks on the sequence after only one stepwise degradation.

The use of new specific enzymes would facilitate the work still further, but at present the enzyme available, together with stepwise degradation, should enable sequences up to heptanucleotides to be established without having to identify sequences bigger than di- and tri-nucleotides.

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