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The Fractionation of Dinucleoside Monophosphate and Some Trinucleoside Diphosphate Isonicotinoyl Hydrazones by Column Chromatography

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A column-chromatographic system using DEAE-cellulose and gradient elution with triethylammonium formate at pH 4.0–3.5 is described. It is capable of separating the oligonucleotide isonicotinoyl hydrazones that are produced by nuclease digestion of RNA oxidized with periodate and coupled with isonicotinic acid hydrazide. Fifteen dinucleoside monophosphate isonicotinoyl hydrazones were characterized by their elution positions on the columns, so that all but two of them could readily be identified. Twelve trinucleoside diphosphate hydrazones were also characterized by their elution positions on the column. The application of this method of fractionation to terminal-sequence studies of RNA is discussed.

The reaction of isonicotinic acid hydrazide (isoniazid) with periodate-oxidized nucleosides, nucleotides and RNA was described by Hunt (1965). In that study the isonicotinoyl hydrazones of nucleosides and oligonucleotides produced by nuclease digestion or isonicotinoyl RNA were fractionated by paper electrophoresis in order to determine the terminal nucleotide sequences of the RNA. The limitation of this method became clear, since it was difficult to separate dinucleoside monophosphate isonicotinoyl hydrazones and trinucleoside diphosphate isonicotinoyl hydrazones from nucleoside hydrazones. Midgley (1966) synthesized 15 dinucleoside monophosphate isonicotinoyl hydrazones and characterized them by paper electrophoresis, but many overlaps occurred and, although the specificity of the enzymes used to cleave RNA eliminates certain dinucleoside monophosphate isonicotinoyl hydrazones from mixtures expected from isonicotinoyl RNA, it was not easy to identify the hydrazones or to determine their relative abundance by paper electrophoresis. Consequently methods for the separation of oligonucleotide isonicotinoyl hydrazones by column chromatography have been sought, and this paper describes the chromatographic properties of 15 dinucleoside monophosphate isonicotinoyl hydrazones and many trinucleoside diphosphate isonicotinoyl hydrazones on DEAE-cellulose.

MATERIALS AND METHODS

Materials. Isonicotinic acid hydrazide was obtained from Calbiochem (Los Angeles, Calif., U.S.A.). [G-^{3}H]- and [*carboxyl*-^{14}C]-Isonicotinic acid hydrazides were obtained from Amersham–Searle (Des Plaines, Ill., U.S.A.) at specific radioactivities of 190 mCi/mmol and 9.8 mCi/mmol respectively.

Nucleosides and dinucleoside monophosphates were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Sodium metaperiodate (reagent grade) was obtained from Matheson, Coleman and Bell (East Rutherford, N.J., U.S.A.).

Buffers for chromatography were made from reagent-grade ammonium formate or redistilled triethylamine (Eastman–Kodak Co., Rochester, N.Y., U.S.A.) adjusted to the required pH with aq. 88% formic acid. In all cases the quoted molarity of buffers is that of the cation, and the pH was measured at 25°C. Serva DEAE-cellulose was obtained from Gallard and Schleisinger (Chemical Manufacturing Corp., Carle Place, N.Y., U.S.A.) and sieved dry before use. Material passing a sieve of 200 mesh and held on 325 mesh was found to give the best resolution for column chromatography.

Reaction of isoniazid with periodate-oxidized nucleosides and oligonucleotides. Periodate oxidation and reaction with isoniazid were performed as described by Hunt (1965). However, it was found necessary to remove excess of iodate and periodate to avoid formation of a degradation product of isoniazid that interfered with the chromatography of the hydrazones. This degradation product, more acidic than isoniazid, was described by McIlreavy & Midgley (1967). To avoid this problem all periodate-oxidized oligonucleotides were freed from periodate by paper electrophoresis as described by Midgley (1966), except that 0.1 M-ammonium formate, pH 3.0, was replaced by 0.1 M-triethylammonium formate, pH 3.0, to avoid breakdown of periodate-oxidized oligonucleotides. Usually portions (0.1 mg) of dinucleoside monophosphate were oxidized and eluted with 0.1–0.2 ml of water from the electrophoresis paper for reaction with 0.75 molar equivalent of [^{3}H]- or [^{14}C]-isoniazid.

Column chromatography. Columns (50 cm × 0.9 cm diam.)

were poured from a slurry in starting buffer and equilibrated by passing starting buffer through them for 16–20h before use. The columns were maintained at 4°C. Flow rates were usually 30–40ml/h, maintained by a Milton Roy micropump. The effluent was split into two streams, 3–4ml/h being mixed with scintillation fluid for radioactivity counting and the remainder being passed through a Beckman DB spectrophotometer for continuous analysis of E_{260} and ^3H and ^{14}C radioactivity (Hunt, 1968).

Radioactivity counting. The radioactivity-flow monitor can be used with a variety of liquid-miscible scintillation-counting systems. The original flow cell was constructed of polyethylene and could only be used with dioxan-based scintillators. Bray's (1960) solution or Bray's solution plus 2% of a mixture of 117 parts of di-(2-ethylhexyl)-amine and 20 parts of acetic acid was suitable with solutions containing 10% of water (3ml of effluent/h plus 27ml of scintillator solution/h) (Hunt, 1968). More recently a Teflon flow cell has been constructed so that toluene-based scintillation solutions can be used. Currently a 10–15% aqueous content is used with the scintillation composition: Beckman BBS3 solubilizer (1vol.); toluene containing 0.5% of 2,5-diphenyloxazole and 0.05% of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (5vol.). Radioactivity is detected by a Packard Tri-Carb series 3314 dual-channel scintillation spectrophotometer and recorded on a Leeds and Northrop six-point multipoint recorder together with the u.v. extinction.

Digestion of RNA. Reticulocyte ribosomal RNA or yeast RNA (Sigma type XI) was digested by pancreatic ribonuclease (EC 2.7.7.16), (recrystallized from ethanol; Worthington Biochemical Corp., Freehold, N.J., U.S.A.) or by ribonuclease T_1 (EC 2.7.7.26) (Sankyo; obtained through Calbiochem) in aqueous solution at pH7.5 and room temperature for 2h at an enzyme/substrate ratio of 1:100. The pH was maintained by an autotitrator. To ensure complete digestion by pancreatic ribonuclease the digests were incubated for a further 16h at 37°C. The oligonucleotides so formed were dephosphorylated by incubation at room temperature with bacterial alkaline phosphatase (EC 3.1.3.1) (Worthington BAPC; chromatographically purified) for 2h at pH8.5. If necessary the enzymes were destroyed by incubation with Pronase (Calbiochem) for 2h at 37°C.

Preparation and determination of sequences of oligonucleotides. Oligonucleotide peaks from column chroma-

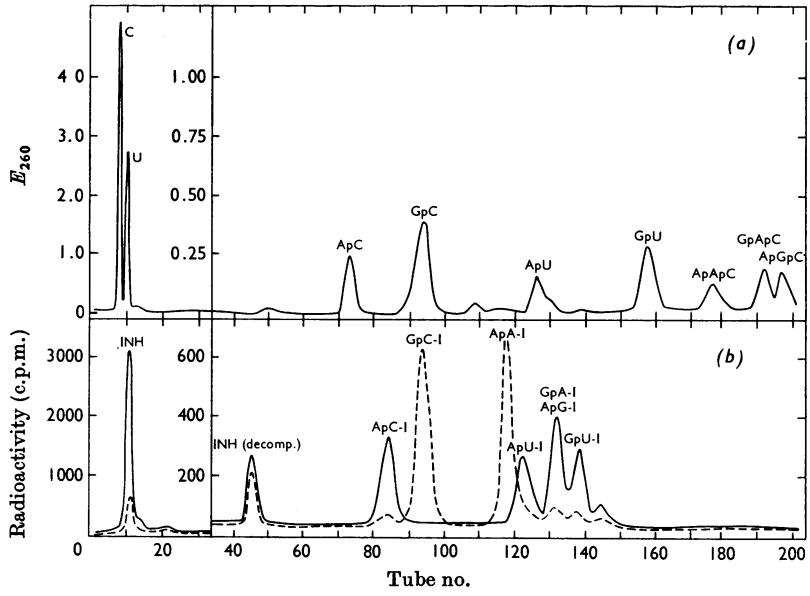

Fig. 1. Chromatography of dinucleotide monophosphate isonicotinoyl hydrazones with a pancreatic ribonuclease digest of RNA dephosphorylated with alkaline phosphatase. The extinction trace is displaced upward from the radioactivity trace to avoid confusion. (*a*) ——, E_{260} of the carrier pancreatic ribonuclease-digested RNA (4.5mg); (*b*) ——, radioactivity of ^{14}C-labelled isoniazid (INH), and ApC-, ApU-, ApG- and GpU-isonicotinoyl hydrazones; – – – –, radioactivity of ^3H-labelled isoniazid, and GpC- and ApA-isonicotinoyl hydrazones. The position of ApG- and GpA-isonicotinoyl hydrazones was determined in a separate experiment. The radioactivity-flow monitor was set to exclude cross-contamination by ^3H in the ^{14}C channel and vice versa. Bray's solution was mixed at 27ml/h with 4.5ml of the effluent from the column/h. The total flow rate on the column was 35.4ml/h, 3.5ml/tube. The counting efficiency for ^3H was about 6% and that for ^{14}C was about 40%. Conditions were: gradient at 4°C; Varigrad chambers 1–3, 5mM-triethylammonium formate, pH4.0; chamber 4, 0.5M-triethylammonium formate, pH3.5; 340ml/chamber. The extinction profile precedes the radioactivity profile by 15min. In all the figures ApC-I represents ApC-isonicotinoyl hydrazone, GpC-I GpC-isonicotinoyl hydrazone etc.

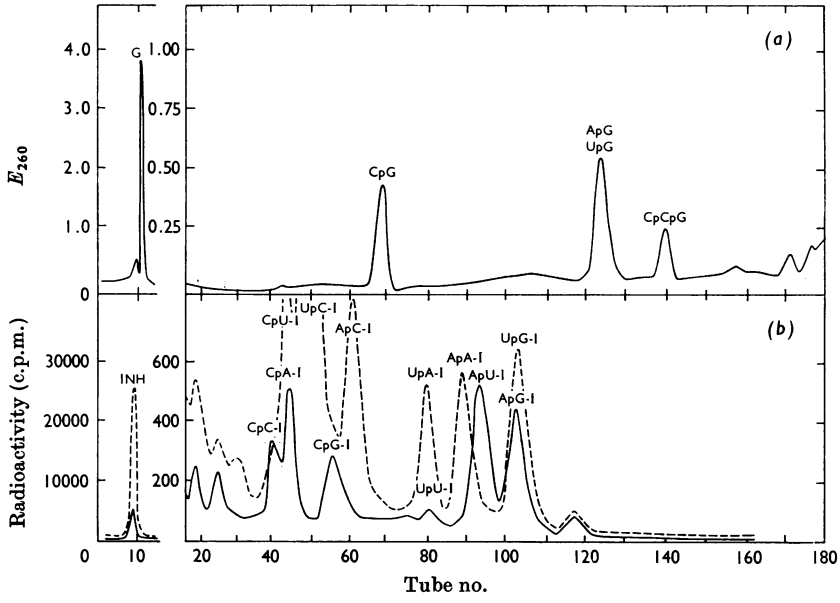

Fig. 2. Chromatography of dinucleoside monophosphate isonicotinoyl hydrazones with a ribonuclease T_1 digest of RNA dephosphorylated with alkaline phosphatase. (*a*) ——, E_{260} of the carrier ribonuclease T_1-digested RNA (4.6mg); (*b*) ——, radioactivity of ^{14}C-labelled isoniazad, and CpC-, CpA-, CpG-, UpU-, ApU- and ApG-isonicotinoyl hydrazones; ----, radioactivity of ^{3}H-labelled isoniazid, and CpU-, UpC-, ApC-, UpA-, ApA- and UpG-isonicotinoyl hydrazones. Conditions of chromatography were as for Fig. 1. The flow rate was 31.8ml/h, 3.5ml/tube.

tography were concentrated by freeze-drying. Each peak was further purified by paper electrophoresis in 0.1M-ammonium acetate, pH3.5, on a water-cooled flat-bed instrument (Savant Instruments, Hicksville, N.Y., U.S.A). Each individual band was eluted with water and digested with snake venom phosphodiesterase (EC 3.1.4.1) (Worthington VPH89) for base analysis. The phosphodiesterase digests were fractionated by paper electrophoresis in 0.1M-ammonium formate, pH3.5, and the nucleosides and nucleoside 5′-phosphates formed were eluted with 0.1M-HCl and their extinctions read at 260 and 280nm. Sequences of di- and tri-nucleotides could be determined by assuming that the specificities of pancreatic ribonuclease and ribonuclease T_1 are such that they produce 3′-terminal pyrimidine 5′-phosphates and 5′-guanylic acid respectively, and that nucleosides produced by venom phosphodiesterase digestion were at the 5′-termini.

RESULTS AND DISCUSSION

Fractionation of dinucleotide monophosphate isonicotinoyl hydrazones. First attempts to separate the dinucleoside monophosphate isonicotinoyl hydrazones by chromatography on DEAE-cellulose were made by using a concave-to-linear gradient of ammonium formate, pH3.5. Although this gradient resulted in excellent separation of the pancreatic ribonuclease digestion products of RNA (Hunt, 1968), several of the early-eluted dinucleoside monophosphate isonicotinoyl hydrazones were crowded together and their separation could not be detected. This problem was resolved by raising the pH of the starting buffer to 4.0. The standard elution gradient adopted was: chambers 1–3 of a multichamber gradient former (Varigrad; Buchler Instruments, Fort Lee, N.J., U.S.A.), 5mM-triethylammonium formate, pH4.0 (1020ml); chamber 4, 0.5M-triethylammonium formate, pH3.5 (340ml). This resulted in retention of the weakly acidic dinucleoside monophosphate isonicotinoyl hydrazones sufficiently to effect a reasonable separation (Figs. 1 and 2). All the dinucleoside monophosphate isonicotinoyl hydrazones were available except GpG, and they were divided into two groups representing the dinucleoside monophosphate hydrazones expected to be produced from isoniazid-labelled RNA by pancreatic ribonuclease (ApC, GpC, ApA, GpA, ApG, ApU and GpU) and by ribonuclease T_1 (CpC, UpC, ApC, CpU, UpU, ApU, CpA, UpA, ApA, CpG, UpG and ApG). The separation of the pancreatic ribonuclease dinucleoside monophosphate isonicotinoyl hydrazones relative to a pancreatic ribonuclease digest of RNA is shown in Fig. 1. Clear separations were obtained for all except GpA-isonicotinoyl hydrazone

and ApG-isonicotinoyl hydrazone. However, these can be differentiated by use of ribonuclease T_1, which only destroys GpA-isonicotinoyl hydrazone. The position of each hydrazone was further checked by simultaneous chromatography of two or three derivatives labelled with either [^{14}C]- or [^{3}H]-isoniazid. The 12 dinucleoside monophosphate isonicotinoyl hydrazones to be separated from the ribonuclease T_1 digest presented a more complex picture (Fig. 2). In this separation the assignment of the exact position of CpC-isonicotinoyl hydrazone is still not absolute, since its position was subject to some variation. Another factor that complicated the location of the early-eluted dinucleoside monophosphate isonicotinoyl hydrazones in synthetic mixtures was the presence of some isoniazid decomposition product in each preparation, eluted between tubes 40 and 50 (Fig. 1). This increased in amount as more dinucleoside monophosphate isonicotinoyl hydrazones were added to the mixture. It is also increased in amount with storage. Consequently it was essential to chromatograph the ribonuclease T_1 dinucleoside monophosphate isonicotinoyl hydrazones from CpC, CpU, CpA and CpG individually and with one or two others to establish their exact elution position. On some occasions CpC-isonicotinoyl hydrazone produced another peak eluting between tubes 20 and 30 (see Fig. 2), and of all the dinucleoside monophosphate hydrazones it was the most difficult to locate exactly. In double-labelling experiments CpU-isonicotinoyl hydrazone ran slightly ahead of CpA-isonicotinoyl hydrazone but only by about 4–5ml. UpU-isonicotinoyl hydrazone and UpA-isonicotinoyl hydrazone were not separable under the conditions of chromatography. ApG- and UpG-isonicotinoyl hydrazones could be distinguished by the use of pancreatic ribonuclease, which digests UpG-isonicotinoyl hydrazone. Hence, although it is not possible to separate all the possible dinucleoside monophosphate isonicotinoyl hydrazones obtainable by ribonuclease T_1 digestion of isonicotinoyl RNA, the only two that cannot be distinguished from one another under the present conditions are UpA- and UpU-isonicotinoyl hydrazones. It may well be possible to separate these hydrazones by changing the flow rate of the column to get better resolution.

Two possible breakdown products of dinucleoside monophosphate isonicotinoyl hydrazones may be found, namely the 5′-nucleotide isonicotinoyl hydrazone or the nucleoside isonicotinoyl hydrazone. Since nucleoside isonicotinoyl hydrazones are not retained by the column, only the 5′-nucleotide isonicotinoyl hydrazones would be expected to interfere with the identification of the dinucleoside monophosphate hydrazones. Fig. 3 shows a chromatogram of ^{14}C-labelled 5′-cytidylic and 5′-guanylic isonicotinoyl hydrazones and ^{3}H-labelled 5′-adenylic and 5′-uridylic isonicotinoyl hydrazones. The relative order of the 5′-adenylic and 5′-uridylic isonicotinoyl hydrazones was determined in a separate experiment with ^{14}C- and ^{3}H-labelled hydrazones. 5′-Adenylic isonicotinoyl hydrazone is eluted close to UpG- and ApG-isonicotinoyl hydrazones and 5′-uridylic isonicotinoyl hydrazone close to GpU-isonicotinoyl hydrazone. These were the only overlaps seen.

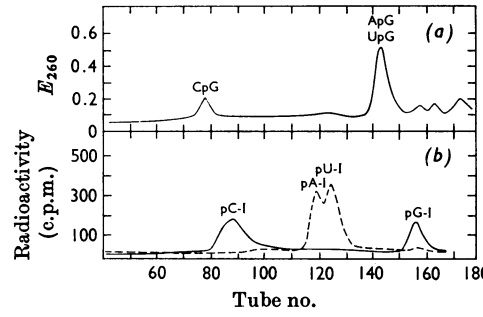

Fig. 3. Chromatography of 5′-nucleotide isonicotinoyl hydrazones with a ribonuclease T_1 digest of RNA dephosphorylated with alkaline phosphatase. (*a*) ——, E_{260} of the carrier ribonuclease T_1-digested RNA (2.4mg). (*b*) ——, radioactivity of ^{14}C-labelled pC- and pG-isonicotinoyl hydrazones; ----, radioactivity of ^{3}H-labelled pA- and pU-isonicotinoyl hydrazones. The relative position of pA- and pU-isonicotinoyl hydrazones was established in a separate experiment. Conditions of chromatography were as for Fig. 1. The flow rate was 30.5ml/h, 3.5ml/tube.

Table 1. *Determinations of the sequences of trinucleoside diphosphates from pancreatic ribonuclease digests*

Electrophoretic mobility is given as the distance migrated from ApC, the distance from ApC to GpU being taken as 1.0. Paper electrophoresis was in 0.1M-ammonium formate, pH3.5. The results from two experiments are shown. The nucleosides and 5′-nucleotides were separated by paper electrophoresis and estimated by their E_{260} and E_{280} after elution in 0.1M-HCl. Values in parentheses are relative molar yields.

Sequence	Electrophoretic mobility	Snake-venom phosphodiesterase digestion products
ApApC	0.41, 0.40	A (1.07), pA (0.88), pC (1.05)
GpApC	0.61, 0.62	G (1.03), pA (0.99), pC (0.99)
ApGpC	0.55, 0.55	A (0.9), pG (0.8), pC (1.3)
ApApU	0.85, 0.76	A (0.9), pA (1.0), pU (1.1)
GpGpC	0.77, 0.76	G (0.92), pG (0.95), pC (1.12)
GpApU	1.07, 1.10	G (0.99), pA (0.99), pU (1.02)
ApGpU	1.02, 0.99	A (1.03), pG (1.05), pU (0.92)
GpGpU	1.20, 1.30	G (1.03), pG (1.04), pU (0.94)

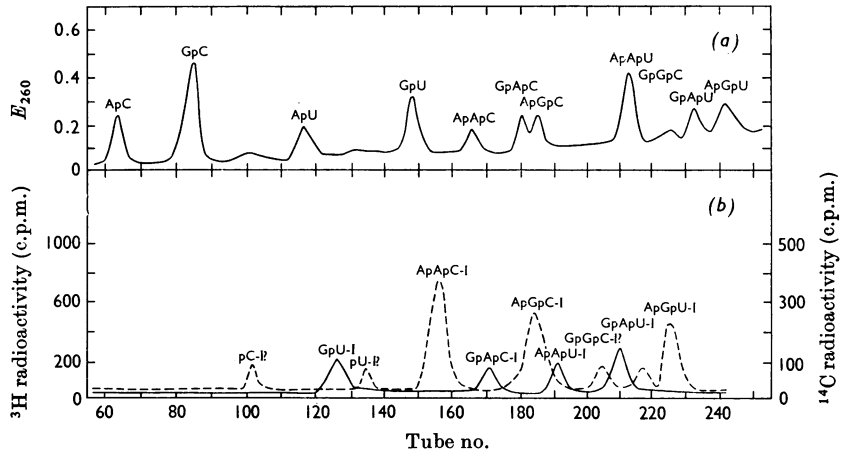

Fig. 4. Chromatography of trinucleoside diphosphate isonicotinoyl hydrazones with a pancreatic ribonuclease digest of RNA dephosphorylated with alkaline phosphatase. (*a*) ——, E_{260} of the pancreatic ribonuclease digest of RNA (4.5mg). (*b*) ——, radioactivity of ^{14}C-labelled GpU-, GpApC-, ApApU- and GpApU-isonicotinoyl hydrazones; ----, radioactivity of ^{3}H-labelled ApApC-, ApGpC-, GpGpC- and ApGpU-isonicotinoyl hydrazones. In this experiment ^{14}C breakthrough in the ^{3}H channel obscured the exact position of the ^{3}H peaks, which were established by running a separate experiment with the ^{3}H-labelled trinucleoside diphosphate isonicotinoyl hydrazones alone. Conditions of chromatography were as for Fig. 1. The flow rate was 34.5ml/h, 3.5ml/tube.

Table 2. *Determinations of the sequences of trinucleoside diphosphates and tetranucleoside triphosphates from ribonuclease T_1 digests*

Paper electrophoresis was in 0.1M-ammonium acetate, pH3.5. Conditions for base analysis were the same as in Table 1, except that mobilities were measured relative to UpG.

Sequence	Electrophoretic mobility	Snake-venom phosphodiesterase digestion products
CpCpG	0.30	C (0.9), pC (1.0), pG (1.2)
ApCpG	0.41	A (1.0), pC (1.1), pG (0.8)
UpCpG	0.80	U (0.9), pC (0.8), pG (1.3)
CpUpG	0.78	C (1.0), pU (0.9), pG (1.0)
ApApG	0.49	A (1.04), pA (0.96), pG 1.00)
CpCpCpG(?)	0.36	C (0.8), pC (1.7), pG (1.5)
A(pApU)pG	0.93	A (1.1), pA (0.7), pU (0.9), pG (1.3)
A(pApC)pG	0.51	A (0.9), pA (0.8), pC (0.9), pG (1.4)
ApCpCpG(?)	0.85	A (0.6), pC (2.0), pG (1.4)

Separation of the trinucleoside diphosphate isonicotinoyl hydrazones and higher oligonucleotide isonicotinoyl hydrazones. Of the possible 64 trinucleoside diphosphate isonicotinoyl hydrazones, only those that could be readily isolated from ribonuclease digests of RNA have so far been synthesized and characterized. From pancreatic ribonuclease digests ApApC, GpApC, ApGpC, ApApU, GpGpC, ApGpU and GpGpU have been isolated (Table 1). Fig. 4 shows a composite chromatogram of isonicotinoyl hydrazones of all of these oligonucleotides except GpGpU, which shows an excellent separation between each of them. GpU-isonicotinoyl hydrazone was added as a dinucleoside monophosphate isonicotinoyl hydrazone marker.

Only these seven of the 16 possible trinucleoside diphosphate isonicotinoyl hydrazones obtainable from a pancreatic ribonuclease digest of isonicotinoyl RNA have been chromatographed, and it is to be expected that there will be some overlap with the other trinucleoside diphosphate isonicotinoyl hydrazones. Better separation should also be obtainable by the use of a shallower gradient in this region.

Only five of the nine trinucleoside diphosphates that can be isolated from ribonuclease T_1 digests have

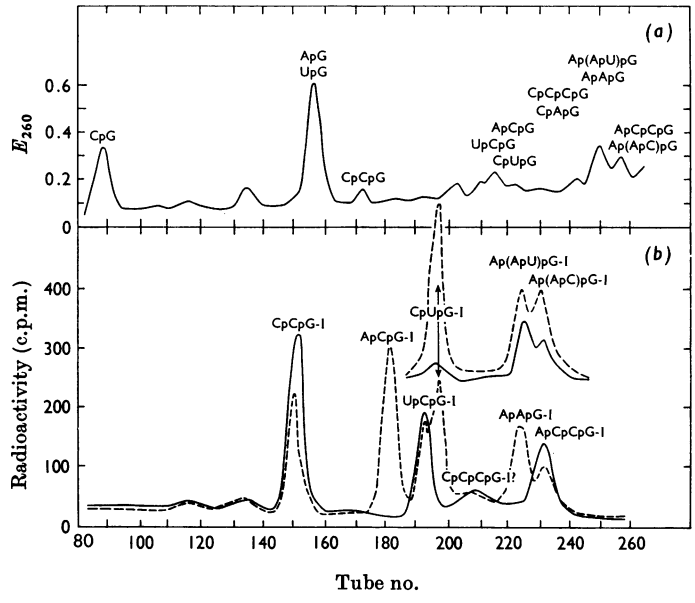

Fig. 5. Chromatography of trinucleoside diphosphate and tetranucleoside triphosphate isonicotinoyl hydrazones with a ribonucleose T_1 digest of RNA dephosphorylated with alkaline phosphatase. (*a*) ——, E_{260} of the ribonuclease T_1 digest of RNA (2.4 mg). (*b*) ——, radioactivity of ^{14}C-labelled CpCpG-, UpCpG-, CpCpCpG- and ApCpCpG-isonicotinoyl hydrazones, and, in the inset, of Ap(ApU)pG-isonicotinoyl hydrazones; ----, radioactivity of ^{3}H-labelled ApCpG-, CpUpG- and ApApG-isonicotinoyl hydrazones; and in the inset CpUpG- and Ap(ApC)pC-isonicotinoyl hydrazones. Conditions of chromatography were as for Fig. 1. The flow rate was 38.7 ml/g, 3.5 ml/tube.

been isolated and fully characterized (Table 2). Some tetranucleoside triphosphates are also eluted in the same area as the trinucleoside diphosphates, and cause some confusion in sequence determination. Four tetranucleoside triphosphates were tentatively identified, although they were only isolated in low amounts (Table 2). The confusion of the trinucleoside diphosphates and tetranucleoside triphosphates can be overcome by chromatography at pH 6–7 in 7 M-urea (Tener, 1967), but this method has not been applied in this work to the isolation of any further ribonuclease T_1 digestion products. Fig. 5 shows the separation of the trinucleoside diphosphate and tetranucleoside triphosphate isonicotinoyl hydrazones of the compounds characterized in Table 2. There will be a great deal of overlap, not only between the trinucleoside diphosphate isonicotinoyl hydrazones (36 possible trinucleoside diphosphate hydrazones can be obtained from a ribonuclease T_1 digest of isonicotinoyl RNA), but also between trinucleoside diphosphate and tetranucleoside triphosphate isonicotinoyl hydrazones. But useful separations are obtainable, and these can be made better by the use of a shallower gradient.

The use of this chromatographic system is to obtain information for terminal-sequence analysis of RNA from different sources after periodate oxidation and coupling with isoniazid (Hunt, 1965). Since it is most easy to identify specific dinucleoside monophosphate isonicotinoyl hydrazones and some trinucleoside diphosphate isonicotinoyl hydrazones, it being borne in mind that the only identifiable part of the molecules is the radioactivity of the isoniazid, the best scheme for sequence determination of RNA molecules using this method would be as follows:

(*a*) Chromatography of the pancreatic ribonuclease and ribonuclease T_1 digests on DEAE-Sephadex columns in 7 M-urea to determine the size of the oligonucleotide isonicotinoyl hydrazones liberated;

(*b*) Identification of the mononucleoside isonicotinoyl hydrazones by paper electrophoresis (Hunt, 1965);

(*c*) Identification of the di- and tri-nucleoside isonicotinoyl hydrazones by DEAE-cellulose chromatography as described in the present paper;

(*d*) Confirmation of the sequences determined by use of stepwise degradation after periodate oxidation by the use of aniline (Steinschneider & Fraenkel-Conrat, 1966). This last step will produce dinucleoside monophosphate isonicotinoyl hydra-

zones where trinucleoside diphosphate isonicotinoyl hydrazones were previously found, and new oligonucleotide isonicotinoyl hydrazones where mononucleoside hydrazones were found. For longer oligonucleotide isonicotinoyl hydrazones, the stepwise-degradation steps may need to be repeated several times.

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