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COLUMN CHROMATOGRAPHIC SEPARATION OF DEOXYNUCLEOTIDE MONOMETHYL ESTERS AND RELATED PRODUCTS OF THE REACTION OF N-METHYL-N-NITROSOUREA WITH DEOXYNUCLEOSIDE MONOPHOSPHATES

P. J. O'CONNOR and J. A. BAILEY

Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester M20 9BX (Great Britain)

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SUMMARY

Dowex 1 ($HCOO^-$) column chromatographic procedures are described for the resolution of deoxynucleoside monophosphate monomethyl esters and other related products of the reaction of the carcinogen N-methyl-N-nitrosourea with deoxynucleoside monophosphates. These procedures provide convenient methods for the isolation and estimation of the products of these reactions.

INTRODUCTION

Alkylation of phosphate as well as the bases is now known to comprise a significant part of the spectrum of products arising from both the *in vitro*[1–3] and *in vivo*[4,5] reactions of carcinogenic methylating and ethylating agents with DNA. However, an assessment of the importance of these reactions with a view to the better understanding of the induction of the carcinogenic process will depend ultimately upon the availability of suitable methods for the analysis of these products. Column chromatographic procedures capable of dealing with milligram quantities of purified nucleic acid are already available for the separation of methylated or ethylated bases on materials such as Sephadex G-10, Dowex 50 (NH_4^+, or H^+)[6], cellulose phosphate[7] or Aminex 6[8], but so far only partial separation of individual phosphotriesters has been achieved. Using columns of Dowex 50 (NH_4^+) and DNA that had been prelabelled with [^{32}P]phosphate or [^{3}H]thymine[2,9] to provide increased sensitivity of detection one of these compounds has been identified as the methyl ester of thymidylyl-(3′-5′)-thymidine after reaction of DNA with N-methyl-N-nitrosourea[10]. Other procedures for analysis of these products have been concerned principally with the total phosphotriester content of DNA and have utilised their degradation under alkaline conditions[1,4,5,11].

The methyl phosphotriester thymidylyl(3′-5′)thymidine, obtained by enzymic hydrolysis of methylated DNA, has been degraded in alkali to yield thymidine and the monomethyl esters of 3′- and 5′-thymidine monophosphate to permit confirmation

of its structure by resolution of these products on MN-cellulose using thin-layer chromatography[10]. Paper chromatography has been employed for the analysis of the monomethyl ester of 7-methylguanosine 5'-monophosphate and its imidazole ring fission product[12] and also for the monomethyl esters of pyrimidine mononucleotides[13]. The development of procedures for the separation and identification of the monomethyl esters of deoxyribonucleotides are therefore of current interest and in the following report column chromatographic procedures are described which allow the resolution of these esters and enable measurements of their formation as a proportion of the parent nucleotide.

MATERIALS AND METHODS

DEAE-Cellulose (DE-52, microgranular) was obtained from H. Reeve Angel & Co. (London, Great Britain) and the Dowex 1 exchange resin AG1-X8 (200–400 mesh) (Cl^-) came from Bio-Rad Labs. (Richmond, Calif., U.S.A.). Ammonium formate (AnalaR grade, 97%; BDH, Poole, Great Britain) was stored over a desiccant; solutions for chromatography were prepared from the dried salt and adjusted to the appropriate pH by the addition of formic acid. N-Methyl-N-nitrosourea (MNU) was prepared from methylurea by nitrosation in the presence of acidified sodium nitrite.

The 2'-deoxynucleoside 3'-monophosphates of guanine, cytosine, thymine and adenine were from P-L Biochemicals (Milwaukee, Wisc., U.S.A.). Authentic marker compounds, 7-methylguanine, 3-methyl-2'-deoxycytidine, 1-methyl-2'-deoxyadenosine and the 2'-deoxynucleoside-5'-monophosphates of guanine, cytosine, thymine and adenine were obtained from Sigma (London, Great Britain), O^6-methyl-2'-deoxyguanosine and 7-methyl-2'-deoxyguanosine were prepared by the reaction of diazomethane with 2'-deoxyguanosine[14]. Products of this reaction were isolated by chromatography on Sephadex G-10, the relative mobility values being 1.0 for 2'-deoxyguanosine; 0.8 for 7-methyl-2'-deoxyguanosine and 1.5 for O^6-methyl-2'-deoxyguanosine at pH 5.9, for columns (85 × 1.5 cm) developed with 1 m*M* potassium phosphate buffer (pH 5.9). The peaks were pooled, reduced to dryness by freeze drying and the products were dissolved up in methanol for recovery as the salt-free nucleoside. [^{14}C]-Methyl phosphate was obtained as described earlier[5].

Alkaline phosphatase (*E. coli*) Type III S and venom exonuclease (*Crotalus adamanteus*) Type II were supplied by Sigma; spleen exonuclease was prepared from fresh spleens by the method of Bernardi[15] and assay procedures for the latter two enzymes were as described previously[16].

Chromatography

Dowex 1-X8 (200–400 mesh) resin was prepared in the formate form by repeated suspension in 3 *M* ammonium formate. The resin was washed with 20% formic acid and then again with water to neutrality. Columns (26 × 1.5 cm) were packed, washed with 1.75 *M* ammonium formate–0.5 *M* formic acid (pH 4.2) and then with 0.02 *M* ammonium formate (pH 4.2) until the UV baseline was stable. The ammonium formate–formic acid wash was used for regeneration of columns.

DEAE-Cellulose was exchanged and columns (20 × 1 cm) were prepared for equilibration with 0.01 *M* ammonium hydrogen carbonate buffer (pH 8.5). Thin-layer chromatography (TLC) was carried out on strips of Polygram CEL 300 UV

(Macherey, Nagel & Co., Düren, G.F.R.) developed in 2-propanol–aqueous ammonia (sp.gr. 0.880)–water (7:1:2, v/v/v). Paper chromatography (on Whatman No. 1) was carried out in a descending direction with the same solvent system.

Chromatography on Sephadex G-10 for the analysis of the products of reaction of MNU with deoxyribonucleotides was carried out on a 60 × 1 cm column equilibrated with 50 m*M* potassium phosphate buffer (pH 5.9). Samples for analysis were hydrolysed in 0.1 *M* hydrochloric acid for 30 min at 70° and then adjusted to pH 5.9 for application to the column.

Distillation of methanol

Samples (2–4 ml) containing radioactively labelled methanol were placed in a small round-bottomed flask for distillation at 68° using a fractionation column (10 cm) of single-turn glass helices. Routinely, three separate additions of carrier methanol (2 ml) were made and under these conditions the distillate was approximately 98% methanol; total recovery of labelled methanol from these distillates was >98%.

Preparation of deoxynucleoside monophosphate monomethyl esters

The four common deoxyribonucleoside 5′-monophosphates were reacted with MNU under the following conditions.

(a) Nucleotides (30 μmoles each) were dissolved separately in 0.2 *M* Tris–HCl pH 7.5 (1 ml) in the presence of N-[^{14}C]methyl-N-nitrosourea (0.12 mmoles; sp.act. 0.47 μCi/mmole) and maintained at room temperature until the evolution of nitrogen had ceased. Mixtures, were adjusted to pH 8.9 with sodium hydroxide and incubated overnight at 37° in the presence of alkaline phosphatase (3 I.U.). Checks made by TLC then showed that the final products were deoxynucleoside and a compound with the expected R_F of a single net negatively charged species. After dilution (to 10 ml) the mixtures were applied individually to a column (20 × 1.5 cm) of DEAE-cellulose stabilised with 0.01 *M* ammonium hydrogen carbonate and then washed with the same buffer to elute the nucleoside; residual products were eluted with a linear gradient 0.05–0.7 *M* ammonium hydrogen carbonate (0.1 l of each). The major salt-eluted peak of UV-absorbing material was coincident with 95% or more of the radioactivity, except in the case of guanine when other minor products were observed; under these conditions about 10–13% of the nucleotide reacted with the nitrosamide. The products contained in the major peaks were concentrated by freeze drying. A sample of thymidine 3′-monophosphate was treated in a similar way to prepare the corresponding 3′-phosphate monomethyl ester.

(b) Unlabelled deoxynucleoside monophosphate monomethyl esters were prepared by reaction of either 5′- or 3′-monophosphates (approx. 40 μmoles each) with MNU (0.5 mmoles) under pH-stat conditions (7.0) with 0.4 *M* sodium hydroxide as titrant. By this method yields were increased to between 30 and 50% (see Fig. 1). The products obtained from these reactions were separated by the Dowex 1 ($HCOO^-$) procedure described in this report.

Identification of the deoxynucleoside monophosphate methyl esters

Confirmation of the structure of these compounds was obtained in the following way. Samples of the methylated derivatives of the four common deoxyribonucleoside 5′-monophosphates were tested for sensitivity to the venom exonuclease

by overnight incubation in the presence of excess enzyme followed by chromatography of a sample of the products on cellulose TLC plates. The hydrolysis product in each case ran in the position of the corresponding marker nucleoside 5'-monophosphate while the compound in the control sample occupied a position intermediate between the nucleoside and the monophosphate. For example, in the case of the thymidine compound R_F values were, thymidine, 0.93; thymidine 5'-monophosphate methyl ester, 0.58 and thymidine monophosphate, 0.22. Similar analyses were carried out on paper and assay of radioactivity showed that virtually all (>95%) of the label was rendered volatile by incubation in the presence of the exonuclease. From the established mechanism of action of this enzyme[17] it was concluded that the methyl group was attached to the phosphate part of the nucleotide to form a phosphodiester link. Incubation of each of these compounds in the presence of limiting amounts of the exonuclease showed that they were equally susceptible to degradation by the enzyme.

By contrast the spleen exonuclease did not degrade the deoxyribonucleoside 5'-monophosphate monomethyl compounds, although as expected[17] the radioactive label was released readily from the thymidine 3'-monophosphate monomethyl ester. The identity of the 3'-compound was checked further by hydrolysis for 1 h in 72% perchloric acid which rendered 44% of the radioactivity volatile. This could be distilled off and after the addition of carrier methanol and the radioactivity in the residue eluted from a column of DEAE-cellulose in the position of authentic methyl phosphate. Finally, incubation of the residue with alkaline phosphatase rendered a further 54% volatile as methanol leaving only 2% as a non-volatile material in the residue.

RESULTS AND DISCUSSION

Chromatography of deoxynucleotide monophosphate–MNU reaction products

The solvent system for the elution of the products of reaction of deoxynucleotides with MNU was as follows: (a) linear gradient 0.01 *M* ammonium hydroxide (pH 9.9)–0.02 *M* ammonium formate; (b) 0.02 *M* ammonium formate wash; (c) linear gradient 0.02 *M*–0.4 *M* ammonium formate and (d) a further wash with 0.4 *M* ammonium formate. Reaction mixtures were dephosphorylated before application to columns of Dowex 1 ($HCOO^-$) and deoxynucleosides were eluted in the order dC, dA, dT by gradient (a) and dG was eluted by wash (b). Negatively charged species (containing phosphate) were then removed by the second linear gradient (c) in the order dC, dT, dA, and dG was eluted by the second wash (d); the linearity of gradient (c) was determined from measurements of buffer concentration at 235 nm.

Fig. 1 shows the four elution profiles for the dephosphorylated reaction products of each of the deoxynucleoside 5'-monophosphates; the major product in each case was the deoxynucleoside monophosphate monomethyl ester. Essentially similar profiles were obtained following methylation of the corresponding 3'-nucleotides but there were some differences in the relative yields. With the guanine nucleotides some N-7 methylated products were obtained also, but owing to exposure to the mildly alkaline conditions used for the enzymic dephosphorylation these were converted by fission of the imidazole ring to a pyrimidine product[12]. These latter were identified from their known UV-absorption data[12], and in the case of the nucleoside the elution-point corresponded to that of the alkaline hydrolysis product of authentic 7-methyl-2'-

deoxyguanosine. Conversion to the ring fission product was probably not complete at pH 8.9, as is the case at higher pH[12], since some 7-methylguanine (about 2%) was detected (Fig. 1B). On the other hand, if prior to dephosphorylation the reaction mixture was heated under conditions expected to release the labile 3- and 7-methyl purines (see ref. 5), these fission products were undetectable and there was a corresponding increase in the amount of 7-methylguanine.

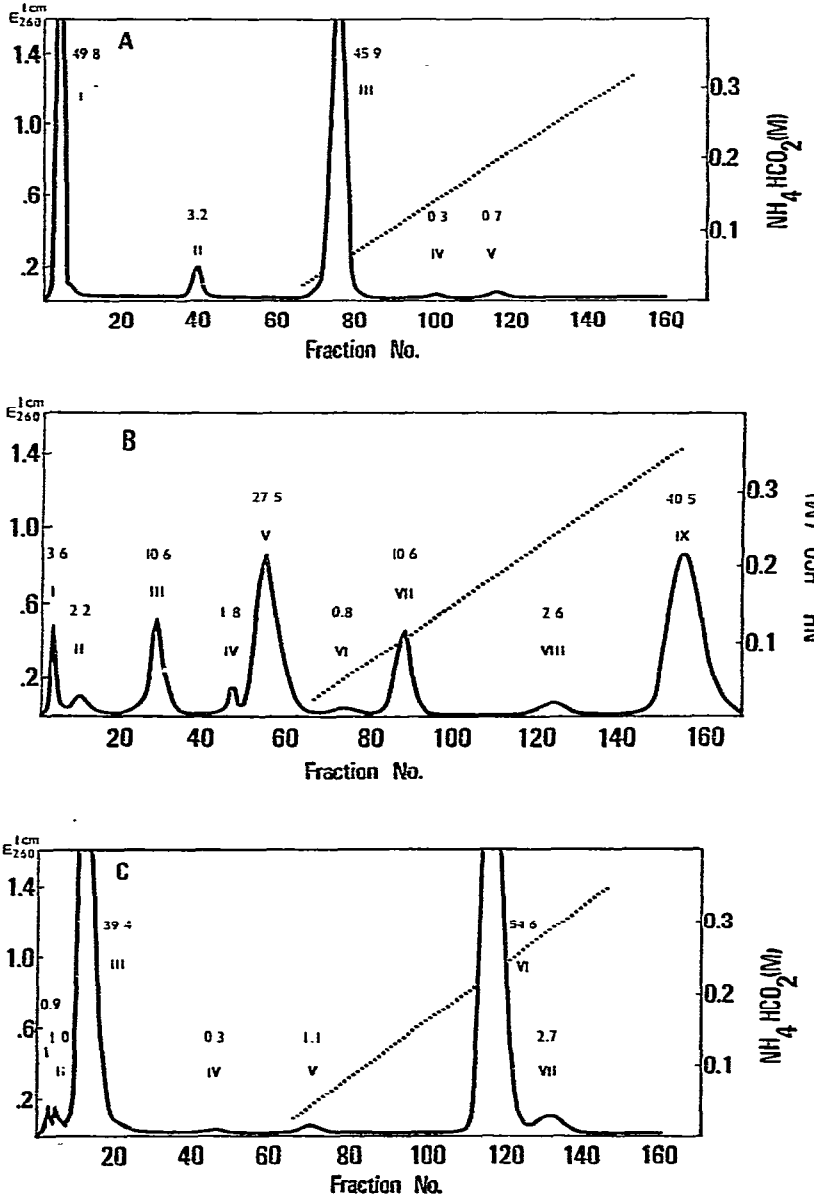

Fig. 1

(Continued on p. 132)

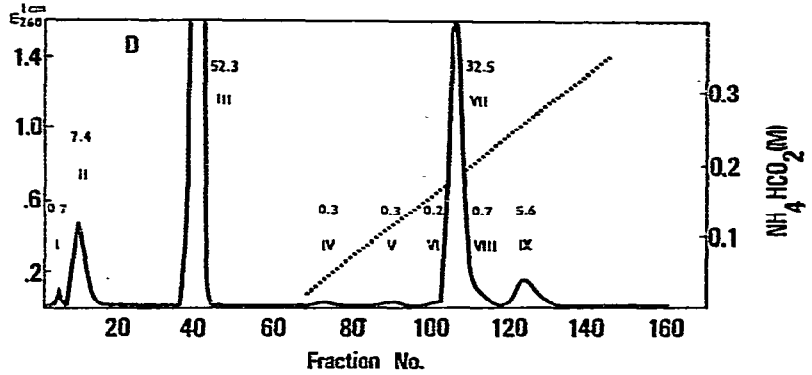

Fig. 1. Dowex 1 ($HCOO^-$) elution profiles for products of reaction of deoxynucleoside 5′-monophosphates with MNU after dephosphorylation of residual nucleotide material. The columns (26 × 1.5 cm) were developed with buffer systems (see *Chromatography of deoxynucleotide monophosphate–MNU reaction products*) in the following proportions: (a) 300 ml + 300 ml (linear); (b) *ca.* 250 ml wash; (c) 600 ml + 600 ml (linear) and (d) *ca.* 250 ml wash; 12.4-ml fractions were collected at a flow-rate of *ca.* 50 ml/h. The upper numbers show the percentage yield of each product in each of the four reaction mixtures as determined from measurements made at 260 nm. Peaks are as follows. (A) For deoxycytidine 5′-monophosphate: I, deoxycytidine; III, deoxycytidine 5′-monophosphate monomethyl ester. (B) For deoxyguanosine 5′-monophosphate: III, 7-methyldeoxyguanosine (ring fission product); IV, 7-methylguanine; V, deoxyguanosine; VIII, 7-methyldeoxyguanosine 5′-monophosphate monomethyl ester (ring fission product) and IX, deoxyguanosine 5′-monophosphate monomethyl ester. (C) For deoxyadenosine 5′-monophosphate: III, deoxyadenosine; VI, deoxyadenosine 5′-monophosphate monomethyl ester. (D) For deoxythymidine 5′-monophosphate: III, deoxythymidine; VIII, deoxythymidine 5′-monophosphate monomethyl ester.

Some monomethyl ester of O^6-methylguanine nucleotide was observed also. These products were eluted with the imidazole ring-fission product of the corresponding 5′- or 3′-nucleotide; their presence, at 1% or less of the total reaction, was detected by analysis of mild acid hydrolysates of material from that region of the column on Sephadex G-10.

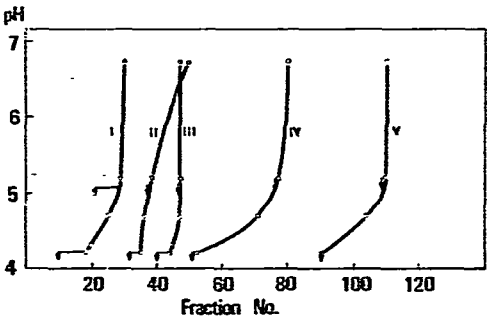

Fig. 2. Changes in Dowex 1 ($HCOO^-$) elution positions for deoxynucleoside 5′-monophosphate monomethyl esters and of methyl phosphate in relation to the pH of the eluting buffer. The columns (26 × 1.5 cm) were developed as a linear gradient 0.02 *M*–0.4 *M* ammonium formate (600 ml of each) and finally washed with 0.4 *M* ammonium formate; the pH of the eluting buffer is shown in the figure. The arrowed lines show the effect of developing the columns with solvent systems a and b (see text) for the separation of nucleosides prior to elution of the monomethyl esters of dCMP (I), dTMP (III), dAMP (IV) and dGMP (V) and of methyl phosphate (II).

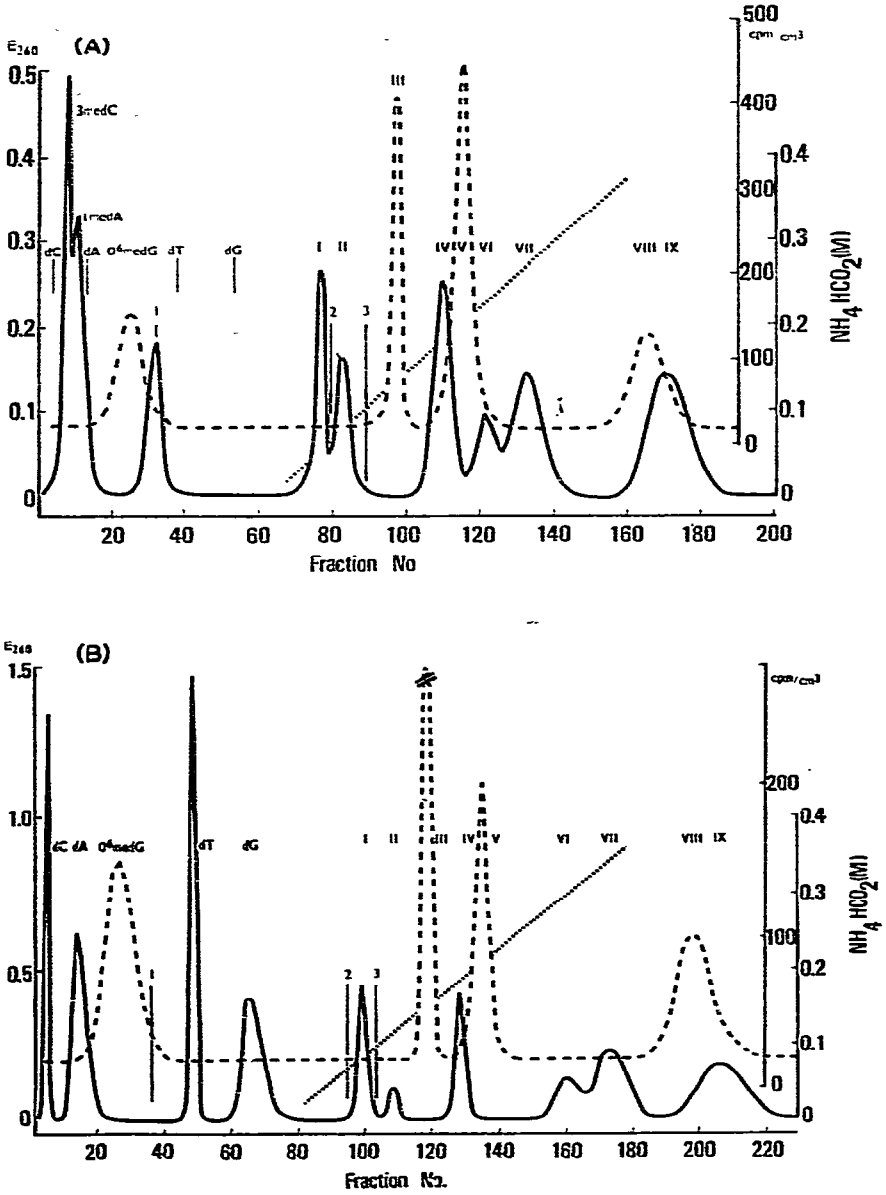

Fig. 3. Separation of deoxynucleoside 5′- (and 3′-) monophosphate monomethyl esters and of some other methylated compounds. The columns (26 × 1.5 cm) were developed with buffer systems a, b, c, and d with ammonium formate, buffer at pH 4.2 as described in the text, and detailed under Fig. 1. (A) or, with the same buffer system at pH 5.1, except that the volume of washes b and d were increased to approx 400 ml (B). In both cases, compounds were eluted in the following order: dC, 3-medC, 1-medA, dA, O^6-medG, dT and dG for the deoxynucleosides of cytosine, 3-methylcytosine, 1-methyl adenine, adenine, O^6-methylguanine, thymine and guanine; 1, 2 and 3 for the imidazole ring fission products as the deoxynucleoside, the nucleoside 3′- or 5′-monophosphate monomethyl ester, respectively; I, IV, VI and VIII for the deoxynucleoside 5′-monophosphate monomethyl esters of cytosine, thymine, adenine and guanine, respectively; II, V, VII and IX for the corresponding derivatives of the 3′-mononucleotides and III, for methyl phosphate.

Modification of degree of resolution of deoxynucleoside monophosphate monomethyl esters

The elution of these compounds from Dowex 1 ($HCOO^-$) can be modified by adjustment of the pH of the eluting buffer. Using the 5'-compounds and monomethyl phosphate as convenient markers the effect of altering the pH was followed to determine optimum conditions for the separation of these compounds. From the pH effect shown in Fig. 2, the choice of eluting buffer will be guided by the region of the column profile to be explored for reaction products, although in most cases it will be an advantage to increase the resolution in the region of the deoxythymidine and deoxyadenosine compounds.

The full elution system is shown in Fig. 3, which compares the effect of using the ammonium formate buffer at pH 4.2 and pH 5.1. As indicated in Fig. 2, the principal difference lies in the degree of separation between the deoxythymidine and the deoxyadenosine compounds. Also, the elution point for the deoxycytidine products is noticeably affected, this is at about 0.05 *M* ammonium formate for deoxycytidine 5'-monophosphate methyl ester at pH 4.2 but delayed until 0.08 *M* at pH 5.1.

The position of the esters derived from the imidazole ring fission compounds does not appear to be altered by these changes of conditions, but in contrast to the other 5'- and 3'-phosphate monomethyl esters the 3'-compound, in this case, is the first to be eluted.

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