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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PURIFICATION OF DEOXYNUCLEOSIDE 5'-TRIPHOSPHATES AND THEIR USE IN A SENSITIVE ELECTROPHORETIC ASSAY OF MISINCORPORATION DURING DNA SYNTHESIS

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SUMMARY

This paper describes techniques and strategies for semi-preparative high-performance liquid chromatographic (HPLC) purification of 2'-deoxynucleoside 5'-triphosphates (dNTPs). The procedure yields dNTPs that are sufficiently pure for use in a sensitive electrophoretic assay of misincorporation during DNA synthesis. Anion-exchange HPLC was used to purify the four normal dNTPs (dATP, dGTP, dCTP and dTTP), plus the chemically modified analogues, 5-BrdUTP, 5-IodUTP and $1,N^6$-etheno-dATP (εdATP). Baseline separations were achieved by isocratic elution of dNTPs with potassium dihydrogen phosphate mobile phase. In general, the resolution of dNTPs was highly dependent on pH, although the influence of mobile phase composition on separation of dNTPs was not the same for all three HPLC packing materials used. A Hewlett-Packard diode array detector was extremely valuable in the identification of contaminating peaks and in the development of optimal mobile phase conditions for dNTP purification. The pure dNTPs were used in the electrophoretic assay of misincorporation, yielding information about the mispairing potential of the modified dNTPs. BrdUMP and IodUMP were misincorporated in place of dCMP during chain elongation catalyzed by purified DNA polymerase I of *Escherichia coli*. εdAMP was incorporated into DNA in place of dAMP, although at much lower efficiency than dAMP.

INTRODUCTION

Our laboratory has recently developed a sensitive electrophoretic assay to detect misincorporation during DNA synthesis with purified DNA polymerases. Fig. 1 depicts the rationale of this assay. Briefly, elongation of a [5'-^{32}P]primer, annealed to a bacteriophage DNA template, is catalyzed by purified DNA polymerase and monitored by gel electrophoresis–autoradiography. When polymerization is carried

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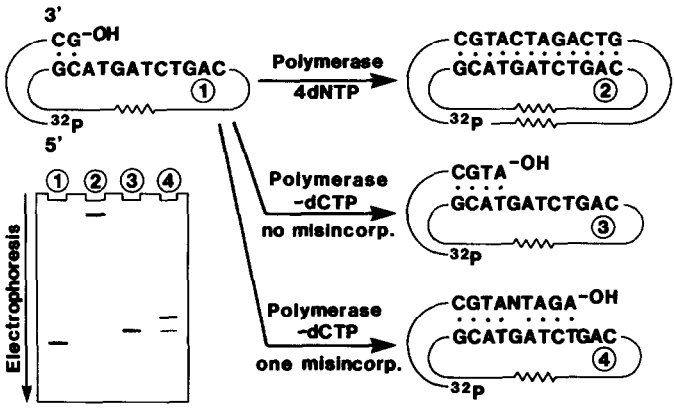


Fig. 1. Schematic representation of the electrophoretic assay of misincorporation. The primer-template used in this assay consists of a disrete [5'-^{32}P]primer, annealed to a circular bacteriophage template strand (1 in upper left-hand corner). A hypothetical nucleotide sequence, downstream from the 3'-OH terminus of the primer, is shown. When this primer-template is incubated with all four dNTPs and a purified DNA polymerase, the primer is rapidly extended around the entire template to give the product, 2. If one of the dNTPs is omitted from the reaction (dCTP in the example shown), then in the absence of misincorporation, the primer is extended only up to the point where the missing nucleotide is needed (the first G in the template in the example shown), yielding the product, 3. However, if a noncomplementary nucleotide (which could be a modified nucleotide added to the *minus* reaction) is incorporated in place of dCMP at this position (depicted by the letter N in 4), then the primer can be elongated to the next position on the template at which the missing nucleotide is needed (in the example, the second G in the template) to yield the product, 4. If these hypothetical products were heated to dissociate the primer from the template, then subjected to electrophoresis in a polyacrylamide gel under denaturing conditions, the pattern of autoradiographic bands should appear as shown in the lower left-hand corner. As misincorporation occurred, the band in lane 3 (corresponding in this case to the primer, elongated by two nucleotides) would gradually disappear and the new (upper) band would appear, corresponding to primers elongated up to where the next dCTP is required (lane 4). If additional misincorporations occurred, other bands would appear further up the gel, corresponding to primers elongated to points at which successive dCMPs (in this case) would normally be incorporated.

out in the presence of only three of the four 2'-deoxynucleoside 5'-triphosphates (dNTPs), elongation of primer (past template positions complementary to the missing dNTP) is dependent on misincorporation at these sites. The use of this procedure has revealed that the propensity for misincorporation varies considerably at different positions along the template[1]. In addition, the base-pairing specificity of any given modified dNTP during DNA synthesis can be tested with this method, by examining whether inclusion of the modified dNTP in each of the four *minus* reactions leads to increased elongation (above that which occurs by misincorporation of the normal dNMPs).

To permit valid conclusions from the data obtained by this electrophoretic assay of misincorporation, the dNTPs used (both unmodified and chemically modified) must be of the highest purity. Otherwise, some or all of the chain elongation observed by electrophoresis–autoradiography may be due to slow incorporation of the "missing" nucleotide, contaminating the other dNTPs present in the polymerase reaction. Multiple cycles of high-performance liquid chromatography (HPLC) are therefore used to prepare ultrapure dNTPs for use in the electrophoretic assay of misincorporation.

This report describes the techniques and strategies that we have used in the preparation of highly pure dNTPs, which include the four normal dNTPs, plus chemically modified dNTPs that we wanted to test for possible mispairing during DNA synthesis. The advantages of a diode array HPLC detector in the preparation of dNTPs suitable for use in the electrophoretic assay of misincorporation is demonstrated. In addition, we report the application of the electrophoretic assay of misincorporation to examine the mispairing potential of 5-halogeno analogues of dTTP (BrdUTP and IodUTP) and the 1,N^6-etheno analogue of dATP (εdATP).

MATERIALS AND METHODS

5-Bromo-2′-deoxyuridine 5′-triphosphate (BrdUTP), 5-Iodo-2′-deoxyuridine 5′-triphosphate (IodUTP) and 1,N^6-etheno-2′-deoxyadenosine 5′-triphosphate (εdATP) were purchased from P-L Biochemicals (Milwaukee, WI, U.S.A.). Commercially HPLC-purified 2′-deoxynucleoside 5′-triphosphates (dATP, dGTP, dCTP and dTTP) were from P-L Biochemicals and ICN Pharmaceuticals (Plainview, NY, U.S.A.). The 2′,3′-dideoxynucleoside 5′-triphosphates (ddNTPs) were purchased from P-L Biochemicals.

DEAE-Sephacel was from Pharmacia. Potassium dihydrogen phosphate (KH_2PO_4) and phosphoric acid (80%, HPLC-grade) were from Fisher Scientific and dipotassium hydrogen phosphate (K_2HPO_4) was from J. T. Baker. *Escherichia coli* DNA polymerase I "large fragment" was purchased from P-L Biochemicals and Bethesda Research Labs. (Bethesda, MD, U.S.A.). Synthetic oligonucleotide primers (15-mer and 17-mer) for preparation of primer-templates with bacteriophage M13mp9 DNA were obtained from P-L Biochemicals. Primer-templates used in the electrophoretic assays of misincorporation are described in a previous paper[1].

Several different anion-exchange HPLC columns were used for purification of dNTPs. All were 250 × 4.6 mm I.D. and were fitted with a 20 × 4.1 mm I.D. Valco guard column (from Custom LC, Houston, TX, U.S.A.), dry-packed with Vydac SC-30-40 Micron pellicular anion-exchange material. Columns of DuPont Zorbax SAX (8 μm) were obtained from Fisher Scientific and HPLC Technology (Palos Verdes Estates, CA, U.S.A.). The Whatman Partisil SAX 10-μm column was obtained from Custom LC. The Techsphere SAX 5-μm column was purchased from HPLC Technology.

dNTPs were purified by anion-exchange HPLC at room temperature, using a Perkin-Elmer Series 4 liquid chromatograph and a Hewlett Packard HP1040A diode array detection system. During chromatographic separations spectrophotometric data were collected, analyzed, and plotted, as described in a previous paper[1]. For preparative purifications, approximately 10 μmol dNTP (in 0.1 ml water or mobile phase) was injected onto the column. Isocratic elution was carried out with KH_2PO_4 mobile phase (350–750 m*M*, pH 3.1–4.7, depending on identity of column packing and desired separation). The precise mobile phase composition is specified in the figure legend for each experiment. During exit of the main peak from the detector, 15-sec fractions were collected. The first 1.5–2.0 ml of the main peak (containing approximately 75% of the peak material) was directly reinjected into the column to achieve additional purification.

Immediately after the second cycle of preparative HPLC, fractions were neu-

tralized by addition of appropriate quantities of 1 *M* K_2HPO_4. The dNTPs were desalted by DEAE-Sephacel chromatography using the volatile buffer, triethylammonium bicarbonate[2], as follows. Pooled and neutralized fractions from the second preparative HPLC were diluted with water to $\leqslant$400 m*M* phosphate, then applied to a 7.0 × 1.0 cm I.D. column of DEAE-Sephacel, equilibrated with 0.1 *M* triethylammonium bicarbonate, pH 7.5. (prepared by bubbling carbon dioxide through an aqueous solution of redistilled triethylamine.) The column was washed with 50 ml 0.1 *M* buffer (mono- and diphosphates were not retained), then the triphosphate was eluted with 0.4 *M* buffer. It was crucial to use a different DEAE-Sephacel column for each dNTP, to avoid cross-contamination. Pooled fractions were evaporated to dryness under reduced pressure. The residue was dissolved in water or 50% methanol, then repeatedly evaporated and dissolved, until no triethylamine could be detected. The dNTP was dissolved in water or 10 m*M* Tris–HCl, pH 7.5 and stored at −20°C. Final preparations were checked for purity by analytical HPLC.

A previous paper describes in detail the electrophoretic assay of misincorporation, including preparation of primer-templates and conditions for DNA synthesis and electrophoresis[1].

RESULTS AND DISCUSSION

HPLC purification of unmodified dNTPs

The influence of pH on retention times of dNTPs varied somewhat with anion exchangers obtained from different manufacturers. The data shown in Fig. 2 illustrate the influence of pH on resolution of dNTPs on the Techsphere 5-SAX column. Relative retention times were very sensitive to slight changes in pH, as has been previously noted for separation of nucleic acid constituents by conventional and HPLC anion-exchange chromatography[3–6]. The diode array detection system greatly facilitated identification of peaks. For example, at pH 4.7 (top panel in Fig. 2) dCTP and dTTP were eluted together in a sharp peak. However, examination of spectral data collected at the upslope, apex and downslope revealed that the front and back edges of this peak consisted of nearly pure dCTP and dTTP, respectively.

As discussed previously[5], the decreased retention of dGTP, dATP and dCTP, relative to that of dTTP at lower pH, can be attributed to protonation of the heterocyclic ring (pK_a = 2.3 for G, 3.7 for A and 4.4 for C). The tendency of purine nucleotides to be eluted after pyrimidine nucleotides (evident in Fig. 2 and well-known for traditional anion exchangers[3,4]) indicates some non-ionic interaction of dNTPs with the HPLC matrix. The chromatographic behavior of dNTPs on DuPont Zorbax SAX closely resembled that on Techsphere SAX (data not shown). With Whatman Partisil SAX, however, we found that higher KH_2PO_4 concentrations were required for elution of dNTPs than with the above supports. Furthermore, relative retention of dNTPs was less sensitive to changes in pH with the Whatman Partisil SAX column than with the other two columns (elution order was dCTP, dTTP, dATP, dGTP at pH values 3.1–4.0 on the Partisil SAX column).

Before dNTPs were purified on a semi-preparative scale (5–10 mg) for use in the electrophoretic assay of misincorporation, experiments like that shown in Fig. 2 had to be conducted with each SAX column. For purification of each dNTP we chose

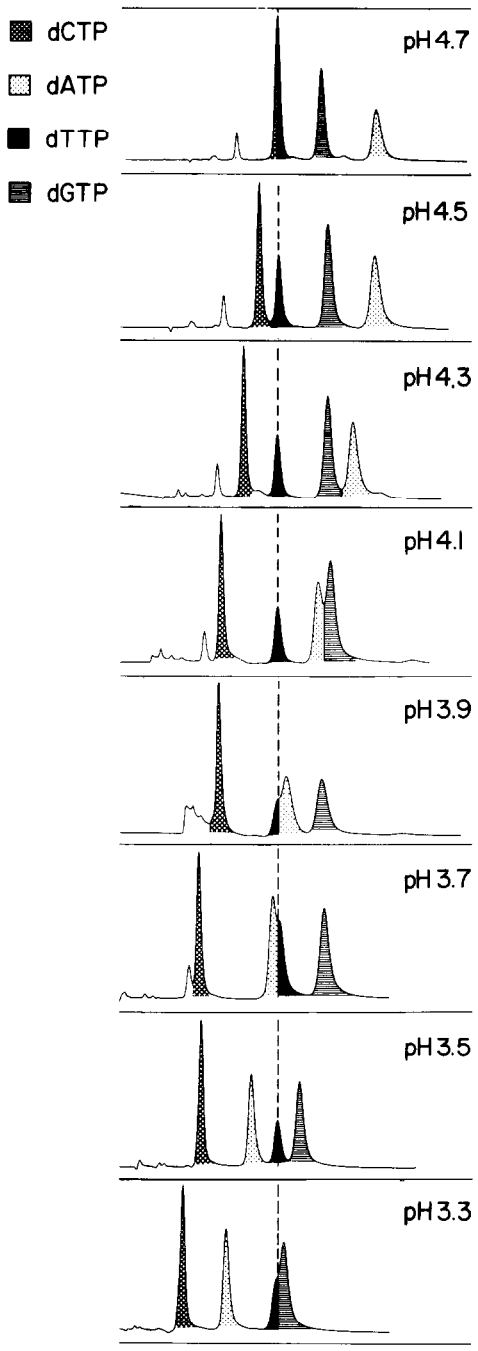
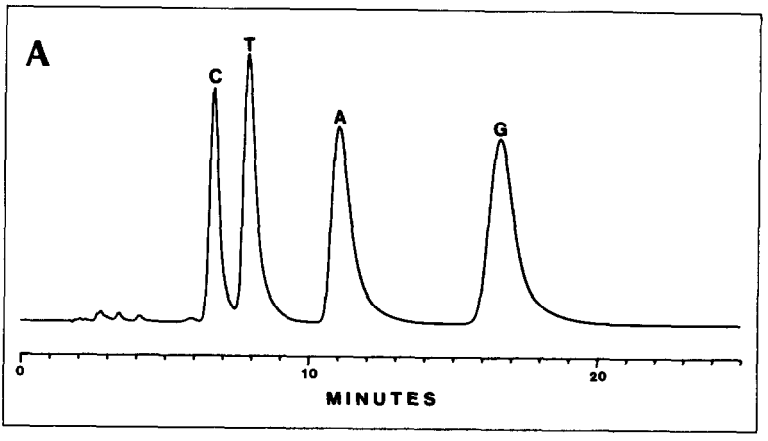


Fig. 2. Influence of pH on separation of dNTPs on Techsphere 5-SAX. dNTPs (5 μl of a mixture of dATP, dGTP, dCTP and dTTP at 1.25 m*M* each) were injected onto the column and eluted at 1.5 ml/min with 400 m*M* KH_2PO_4 (pH 3.3–4.7). Spectrophotometric data were collected and analyzed as described in Materials and methods, to identify peaks. To permit assessment of the effect of changes in net charge on retention, all absorption profiles were positioned to line up the peaks of dTTP (the only dNTP that does not undergo protonation of base at low pH).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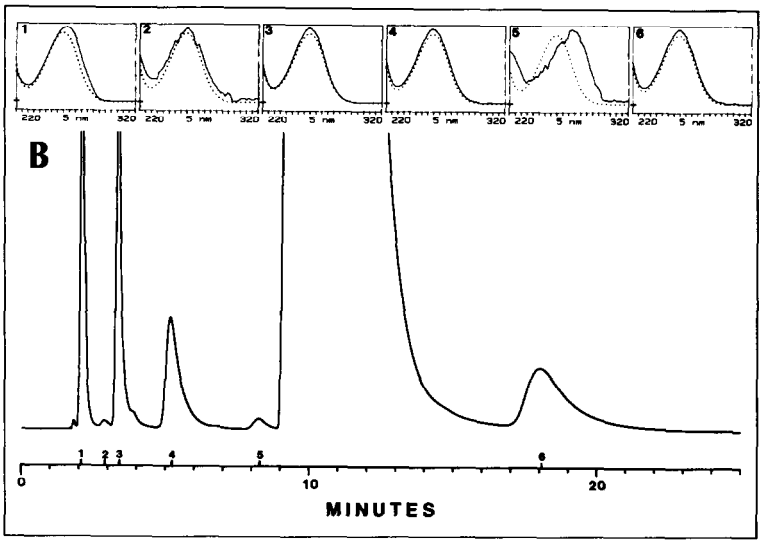
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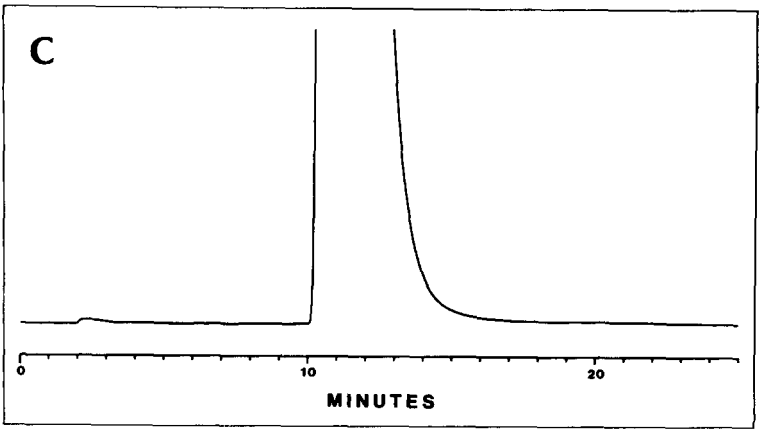
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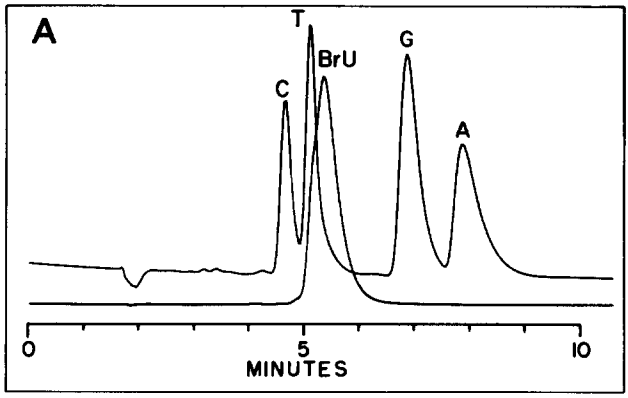
a pH that gave maximal separation from any of the other three dNTPs, regardless of whether the possible contaminants were separated from each other. In addition, we chose a pH at which deamination products would be removed (dITP from dATP and dUTP from dCTP). Artifactual chain elongation would occur if these products were present in the *minus* reactions (dITP efficiently replaces dGTP and dUTP efficiently replaces dTTP). In the case of the Techsphere column, 400 m*M* KH_2PO_4 at pH 4.1 was chosen for preparative purification of both dCTP and dTTP (dUTP elutes after dCTP), whereas 400 m*M* KH_2PO_4 at pH 4.7 was chosen for preparative purification of both dATP and dGTP (dITP is eluted well ahead of dATP).

Fig. 3 represents a semi-preparative HPLC purification of a dNTP (10 μmol HPLC-pure dATP from P-L Biochemicals). Elution was carried out under conditions that separated dATP from the other dNTPs, as seen in panel A. Post-run analyses in the purification of dATP yielded the plots shown in panels B and C. The large quantities of dATP injected in the first cycle of purification permitted detection of small quantities of contaminating species, the peaks of which are greatly exaggerated, compared with analyses performed at low dNTP concentrations.

The contaminating species were essentially eliminated by a single pass through the column, as seen by comparison of absorption profiles obtained during the first (panel B) and second (panel C) separations. In repurification of the commercial HPLC-purified dNTPs, nearly all of the contaminating peaks had UV absorption spectra that resembled those of the main peaks. This suggests that the major sources of "contamination" are spontaneous hydrolysis and disproportionation reactions occurring during storage, yielding free bases and nucleosides, mono- and diphosphates, and tetraphosphate species (the latter eluted after dNTP). In no case were deamination products detected in the dNTP preparations. In only two cases there was indication of minor cross-contamination of nucleotide species. There was a minor peak in the dTTP preparation with the absorption spectrum and retention time expected form dGDP. This would pose no problem in the electrophoretic assay of misincorporation, since it would not act as substrate for the polymerase. In the preparation of dATP, however, we detected a very small peak during the first separation (peak 5, Fig. 3B), having the retention time and absorption spectrum expected for dTTP. This result was obtained in two independent preparative purifications of the dATP supplied by P-L Biochemicals. From spectrophotometric data we estimate that the level of contamination of the commercially purified dATP by dTTP was on the order of 0.01%. As reported previously[1], commercial preparations of HPLC-purified dNTPs may be sufficiently pure for use in the electrophoretic assay of misincorporation with certain primer-templates. However, as will be demonstrated below, dNTP contamination at the level seen in the commercial dATP preparation can cause increased chain elongation in *minus* reactions in certain experiments.

Fig. 3. HPLC purification of dATP. Separations were achieved on a Whatman Partisil SAX column, eluted with 750 m*M* KH_2PO_4 (pH 3.7) at 2 ml/min. A, UV absorption profile obtained during analytical chromatography of a mixture of the four normal dNTPs. B, UV absorption profile (100 ma.u.f.s.) obtained during first preparative chromatography of dATP (10 μmoles). UV absorption spectra (220–320 nm) of contaminating peaks are plotted at the top of panel B (solid lines), along with the absorption spectrum of dATP (dotted lines). Peak fractions (74% of material in main peak) were directly reinjected onto the column. C, UV absorption profile (74 ma.u.f.s.) obtained during the second preparative purification.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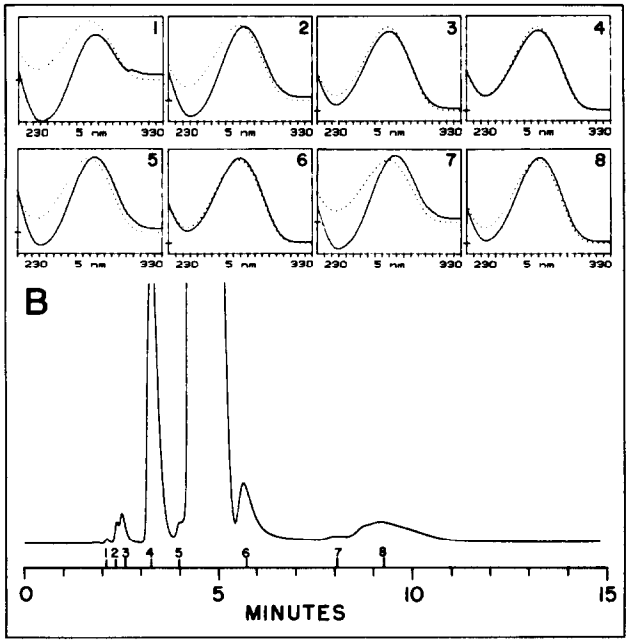
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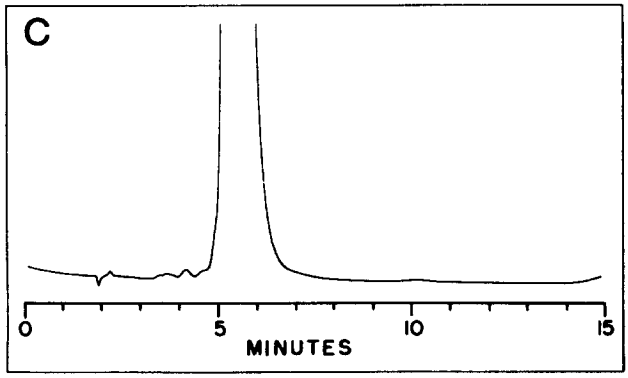
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HPLC purification of BrdUTP and IodUTP

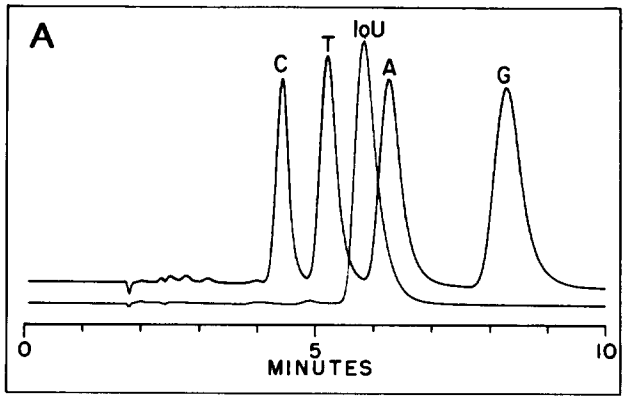
Fig. 4 shows data on the purification of BrdUTP. As seen in panel A, under conditions chosen for preparative purification, BrdUTP was eluted from the column at a position between dTTP and dGTP, separated from dATP and dCTP (the nucleotides for which BrdUTP substituted during DNA synthesis, as determined by the electrophoretic assay of misincorporation).

The contaminating peaks seen in the first preparative HPLC (panel B) were vastly reduced in the subsequent chromatographic separation (panel C). As could be concluded from the analysis of spectra (panel B, upper part), there were two kinds of contaminants in the initial sample of BrdUTP. Peaks 3, 4, 6 and 8 showed spectra absolutely identical to that of BrdUTP (dotted line), with maximum absorption at 280 nm, while peaks 1, 2, 5 and 7 appeared to represent another absorbing species, having a maximum at 282 nm and a lower minimum (at 245 nm) than that of the BrU peaks. Peaks 3, 4, 6 and 8 are most likely BrdUMP, BrdUDP and BrdU-polyphosphates, respectively. Peaks 1, 2, 5 and 7 may represent the ribo derivatives or may differ in the position of bromination. Although BrdUTP and dCTP have very similar UV absorption spectra, they can be reproducibly distinguished from one another on the basis of their relative absorption at 210 nm *vs.* 280 nm (for dCTP, $A_{210} < A_{280}$, whereas for BrdUTP, $A_{210} > A_{280}$). Comparison of spectra of all contaminating peaks of panel B with that of dCTP from 210–320 nm (data not shown) revealed that none of the observed contaminants were deoxycytosine species.

The method used in the commercial preparation of BrdUTP and IodUTP involves halogenation of dUMP or dUDP and subsequent phosphorylation to the triphosphate (personal communication with P-L Biochemicals). With this preparative procedure it is highly unlikely that significant quantities of dATP, dGTP or dCTP are present in the commercial preparations of BrdUTP and IodUTP. None of the UV spectra presented in panel B even remotely resembled spectra of dATP or dGTP. The minor contaminants remaining after the first preparative chromatography (panel C) were at concentrations too low to permit spectral plots. Only the main peak in panel C was used in the electrophoretic assay of misincorporation.

Fig. 5 shows spectrophotometric data collected during purification of IodUTP. The analytical separations illustrated in panel A show that the retention time of IodUTP was intermediate between those of dTTP and dATP, and far removed from those of dCTP and dGTP (important in the interpretation of chain elongation experiments with IodUTP replacing dCTP and dGTP). Panel B represents the initial preparative purification of IodUTP. The spectra of peaks 2–5, 7 and 8 have the same shapes and maxima of absorption (288 nm) as IodUTP (dotted line) and propably represent the mono-, di- and polyphosphates of IodU or IoU. Peak 6, however, had a retention time and spectral properties similar to those of dGTP. Such contamination would cause artifactual chain elongation in a "-G" DNA synthesis reaction.

Fig. 4. HPLC of BrdUTP. Separations were achieved on a DuPont Zorbax SAX column, eluted with 500 m*M* KH_2PO_4 (pH 4.25) at 2 ml/min. A, Analytical chromatography of dCTP, dTTP, dGTP and dATP (upper baseline) and BrdUTP (lower baseline). B, Preparative chromatography of 10 μmoles BrdUTP. Above the plot of absorption *vs.* time are displayed spectral data (230–330 nm) for each peak (continuous line), superimposed on the UV spectrum of a highly purified preparation of BrdUTP (dotted line). C, Rechromatography of peak fractions from the first preparative purification.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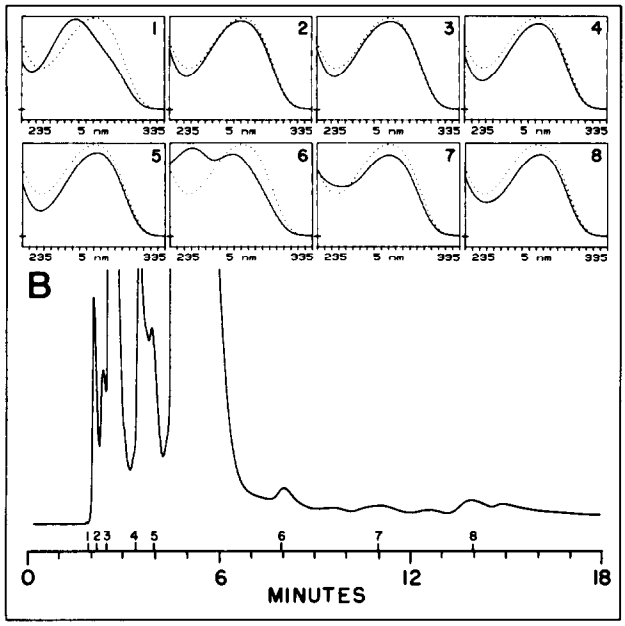
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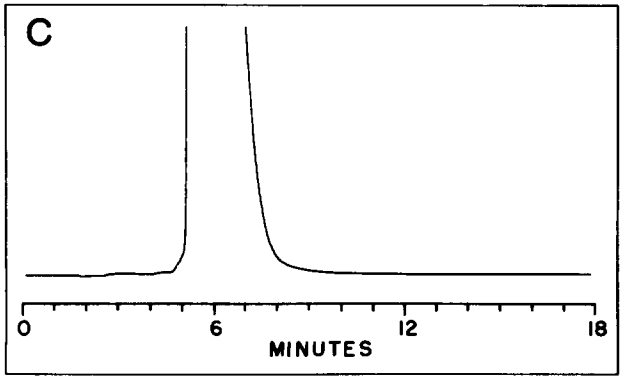
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However, the contaminants were efficiently removed during the chromatography, and only the peak fractions from the second preparative chromatography (panel C) were used in the electrophoretic assay of misincorporation.

HPLC purification of εdATP

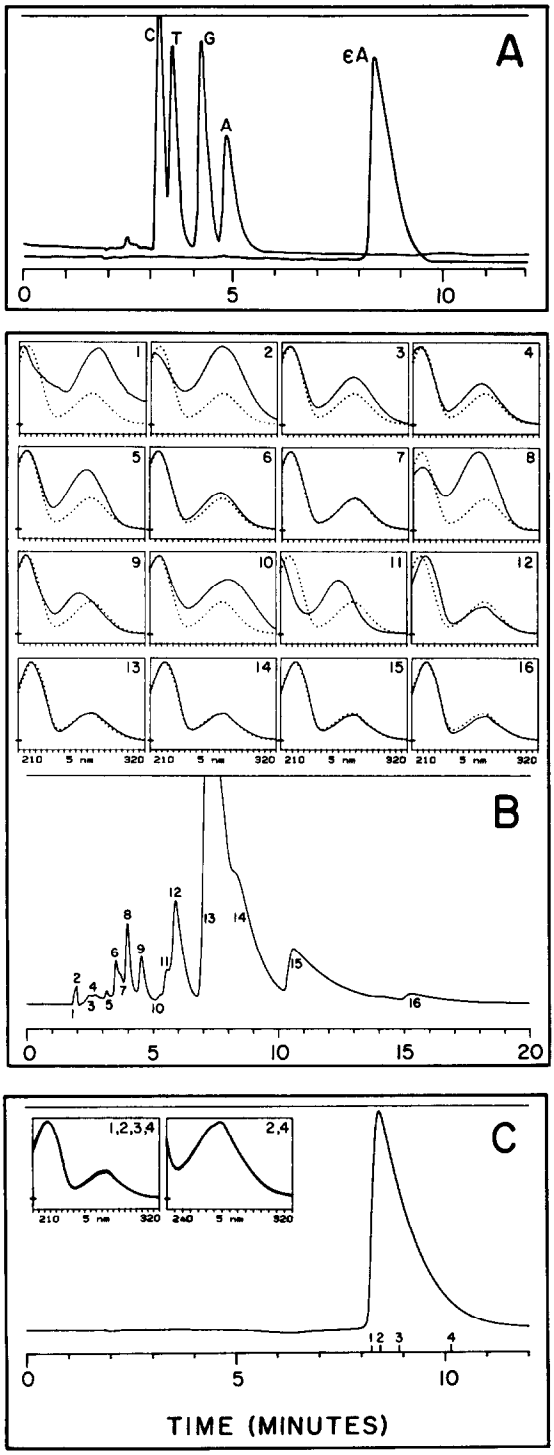
Fig. 6A and B show data obtained during purification of the chemically modified nucleotide, 1,N^6-etheno-dATP (εdATP). As seen in panel A, εdATP was very well separated from any other dNTP. The commercial preparation of εdATP was contaminated by many additional species (panel B). Not all of them could be recognized from their individual spectra, although most of them appeared to be εA adducts. We were more concerned with removing these contaminants from εdATP than with identifying them, since our main purpose was to insure that εdATP was the only modified dNTP present during the subsequent analysis of its base-pairing potential during DNA synthesis.

In general, our data on spectral properties of ε adducts are in agreement with recent reviews on the subject[7,8]. The pure εdA products, according to our numerous observations, have very characteristic absorption spectra, taken from 205 (or 210) nm to 320 nm. The first absorption maximum occurs in the range 218–228 nm, depending on the pH and the number of phosphate groups on the ε adduct. The magnitude of this first absorption maximum is always greater (by a constant factor) than that of the second maximum, which occurs at 274 nm. Thus, peaks 6, 7 and 12–15 are undoubtedly εA compounds, whereas peaks 1, 2, 5 and 8–10 represent either unidentified products with unique spectra or unresolved compounds. Peak 11 is without question dATP (which must be removed to permit valid conclusions from the electrophoretic analysis of base-pairing specificity of εdATP during DNA synthesis). As seen in panel C, the first preparative separation effectively removed the contaminants. Only the peak fractions from the second HPLC purification were used in the subsequent electrophoretic assay of misincorporation.

Electrophoretic assay of the mispairing potential of modified dNTPs during DNA synthesis

BrdUTP and IodUTP. Fig. 7 shows the extent of primer elongation obtained when each of the four dNTPs was replaced by BrdUTP or IodUTP during synthesis catalyzed by DNA polymerase I of *Escherichia coli.* The concentration of unmodified dNTPs was held at 10 μM; nucleotide analogues were added at 10 and 50 μM. Increased chain elongation in the presence of analogue was taken as evidence for replacement of the missing dNTP by analogue. The nucleotide sequence of the DNA template in the region of chain elongation (ten base pairs at the 3'-OH end of the primer, plus 50 additional template residues) is displayed along the right edge, with lines connecting bands in the ddATP sequencing lane with positions in the template at which dAMP would normally be inserted into the elongating RA50 primer.

Fig. 5. HPLC of IodUTP. Separations were achieved on a Whatman Partisil SAX column at pH 3.45. See legend to Fig. 3 for details. A, Analytical chromatography of a mixture of the four normal dNTPs (upper baseline) and IodUTP (lower baseline). B, First preparative chromatography of IodUTP (10 μmol). Spectral data (235–335 nm) for each peak (continuous line) are displayed above the plot of absorption *vs.* time, along with the spectrum of purified IodUTP (dotted line). C, Rechromatography of peak fractions from the first preparative purification.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10
TIME (MINUTES)

Control reactions, represented by lanes 2–4, demonstrated that the nucleotide analogues, even at 50 μM, did not inhibit chain elongation in reaction mixtures containing all four normal dNTPs. Although these data were obtained with a long (1 h) incubation time (at which small differences in elongation rate would not be detected), separate comparisons at short (5–10 min) reaction times confirmed that the HPLC-purified analogues did not inhibit polymerization (data not shown). As shown by lanes 6–9, both nucleotide analogues, at 10 and 50 μM, were efficiently utilized in place of dTTP, as expected from their chemical structures (having the same potential for hydrogen-bonded base pairing as dTTP). Again, data obtained at short reaction times (not shown) showed essentially equal elongation when dTTP was replaced by BrdUTP or IodUTP, compared with reactions containing only the four normal dNTPs. Lanes 12–16 depict elongation of the RA50 primer by *E. coli* DNA polymerase I in the absence of dCTP (compare with the ddC reaction, lane 11). Chain elongation in the absence of dCTP is shown in lane 12. During the 1-h period of reaction most of the primers had undergone one misincorporation (indicated by elongation past the first position at which dCMP would normally be incorporated). As discussed in previous papers[1,9], double (consecutive) misincorporations generally occur more slowly than single misincorporations. An example of this is seen in the "−C" reaction (lane 12), in which elongation of most primers ceased at the first GG doublet on the template. Some of the primers were elongated past this site, but none were elongated past the second GG doublet on the template. As seen in previous work[1,9], chain elongation paused not only at positions at which the missing dNTP was required, but sometimes after addition of one, and occasionally two, additional nucleotides (possibly because mismatched primer termini are poor substrates for subsequent elongation).

Addition of BrdUTP or IodUTP to polymerase reactions lacking dCTP gave rise to increased elongation of the RA50 primer, as evidenced by the appearance of bands further up the gel in lanes 13–16, and disappearance of bands corresponding to the first ddC band. Elongation was greater with 50 μM analogue than with 10 μM, and misincorporation in place of dCTP was greater with IodUTP than with BrdUTP. In the reaction containing 50 μM IodUTP in place of dCTP, some primers underwent at least 20 misincorporations. This result, which we also found with a different primer-template[1], indicates that BrdUTP and IodUTP can mispair with G residues in the template during DNA synthesis. Our findings support the mechanism of BU-induced mutagenesis first forwarded by Freese[10]. Additional support for BU·G mispairing during synthesis with purified DNA polymerase of bacteriophage T4 was recently reported by Lasken and Goodman[11].

Examination of reaction products formed in the absence of dGTP (lanes 18–22) reveals that little or no detectable misincorporation of either analogue occurred

Fig. 6. HPLC of εdATP. Separations were performed on a DuPont Zorbax SAX column, eluted with 500 mM KH_2PO_4 (pH 4.0) at 2 ml/min. A, Analytical chromatography of dCTP, dTTP, dGTP and dATP (upper baseline) and purified εdATP (lower baseline). B, Preparative HPLC of 6 mg εdATP. Above the plot of absorption *vs.* time are displayed the UV spectra (210–320 nm) for each peak (continuous line), superimposed on spectra (dotted line) of εdAdo (peak 3) or εdADP (peak 12). C, Rechromatography of the main peak fractions obtained during the first preparative purification. Above the plot of absorption *vs.* time are displayed spectral data (210–320 nm and 240–320 nm) for the single peak of highly purified εdATP.

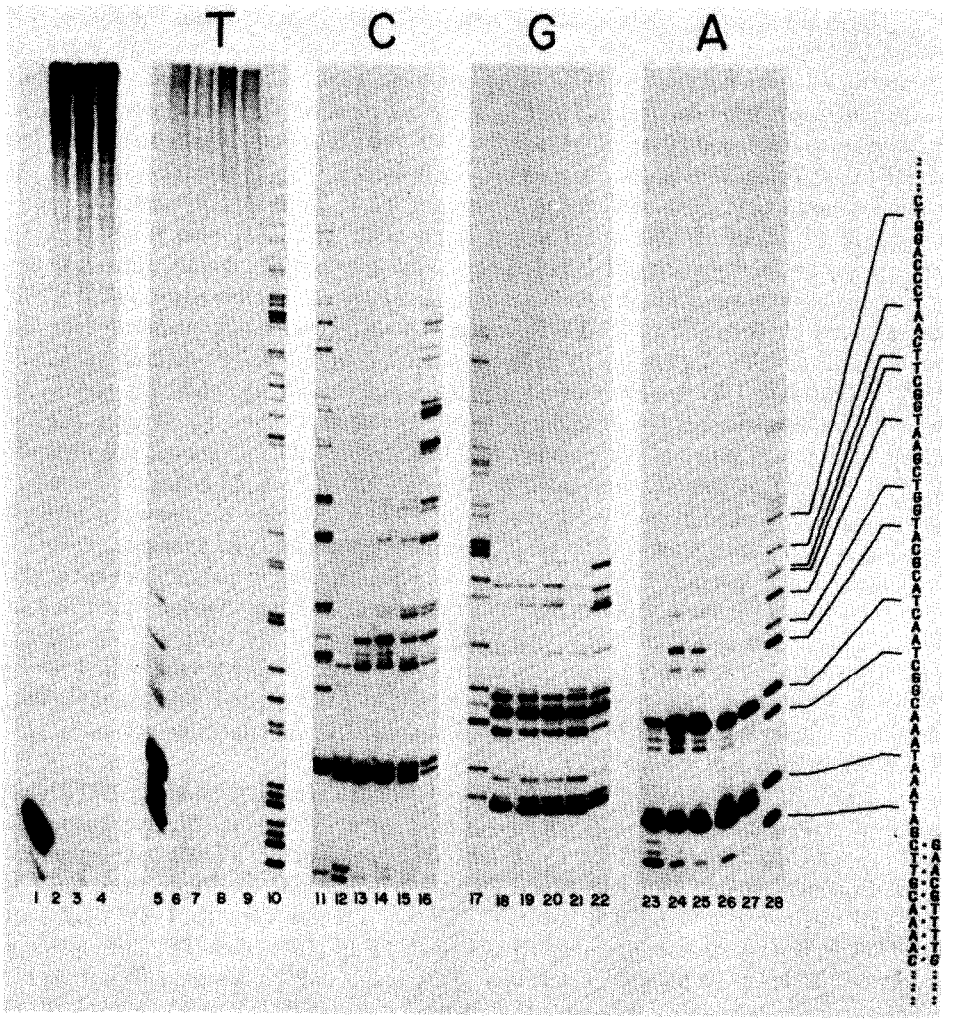

Fig. 7. Electrophoretic analysis of the mispairing potential of BrdUTP and IodUTP during elongation of primer RA50 by DNA polymerase I of *E. coli*. Polymerization was carried out at 30°C for 1 h in the presence of 10 μM unmodified dNTPs. Details of the reaction conditions and electrophoretic method are given in Ref. 1. Lane 1, no polymerase. Lanes 2–4, complete reactions (containing all four normal dNTPs), with addition of no analogue (lane 2), 50 μM BrdUTP (lane 3), or 50 μM IodUTP (lane 4). Lanes 5–9, −T reactions with addition of no analogue (lane 5), 10 μM (lane 6) and 50 μM (lane 7) BrdUTP, or 10 μM (lane 8) and 50 μM (lane 9) IodUTP. Lane 10, ddT. Lane 11, ddC. Lanes 12–16, −C reactions with addition of no analogue (lane 12), 10 μM (lane 13) and 50 μM (lane 14) BrdUTP, or 10 μM (lane 15) and 50 μM (lane 16) IodUTP. Lane 17, ddG. Lanes 18–22, −G reactions with addition of no analogue (lane 18), 10 μM (lane 19) and 50 μM (lane 20) BrdUTP, or 10 μM (lane 21) and 50 μM (lane 22) IodUTP. Lanes 23–27, −A reactions with addition of no analogue (lane 23), 10 μM (lane 24) and 50 μM (lane 25) BrdUTP, or 10 μM (lane 26) and 50 μM (lane 27) IodUTP. Lane 28, ddA. The nucleotide sequence of the template in the region of the 3′-OH terminus of the RA50 primer is displayed at the right.

in place of dGMP in this region of the G4 template. As reported previously[1], misincorporation (particularly of IodUMP) in place of dGMP is clearly detectable with certain template sequences. Comparison of lanes 23–25 reveals a slight misincorporation of BrdUMP in place of dAMP in this region of the template. Again, this type of misincorporation occurs in some regions of natural DNA templates, but not in others. These results bolster the emerging view that the sequence of nucleotide residues on the template influences the type of mispairing that occurs during DNA synthesis.

εdATP. In the experiment represented by Fig. 8 we examined the specificity of incorporation of 1,N^6-etheno-dATP during DNA synthesis. This product is formed by treatment of dATP with chloroacetaldehyde[12], a metabolite of the environmental carcinogen, vinyl chloride. The controls represented in lanes 2–4 (Fig. 8) demonstrate that the analogue did not inhibit polymerization when added (at 10 μM or 50 μM) to a reaction containing all four normal dNTPs. As seen in lanes 6–8, addition of εdATP to a −A reaction resulted in substantial chain elongation, indicating that εdAMP was incorporated in place of dAMP during DNA synthesis catalyzed by *E. coli* polymerase I, even though the etheno ring would prevent normal Watson-Crick pairing of εA with T. Significant utilization of εdATP in place of dGTP also occurred during chain elongation (lanes 10–12), although at a much lower efficiency than replacement of dATP. No detectable substitution of εdATP for dCTP or dTTP was seen in this experiment. These results represent the first indication that εdATP can mispair during DNA synthesis, although evidence exists[13,14] for mispairing during *in vitro* replication past εA residues in the template. More data on the mispairing properties of εdATP will be presented elsewhere[15].

In order to measure the efficiency of utilization of εdATP by polymerase I, relative to utilization of dATP, more precisely, we carried out on a series of −A reactions, with addition of various concentrations of εdATP or dATP. Reaction products were visualized at short times so that the data would reflect initial rates of incorporation. As seen in Fig. 9, the rate of chain elongation which occurred at 10 μM εdATP was achieved at a 500- to 1000-fold lower concentration of dATP. Thus, the etheno-A derivative is utilized as substrate for chain elongation, but at a much lower efficiency than the unmodified nucleotide. Also seen in Fig. 9 is the level of intentional dNTP contamination required to produce additional elongation in a *minus* reaction. Addition of 0.001 μM dATP to the −A reaction (lane 7) caused detectable chain elongation above that seen in the total absence of dATP (lane 1). This level of contamination (one part in ten thousand) is approximately equal to the quantity of dTTP seen with the diode array detector during the further purification of commercially supplied HPLC-pure dATP (Fig. 3). Thus, although commercially HPLC-purified dNTPs may yield valid results with the electrophoretic assay of misincorporation in some experiments[1], the trace contamination present in some commercial dNTP preparations may cause artifactual chain elongation when the electrophoretic assay of misincorporation is used with certain primer-templates.

From the degree of attenuation of signal required to produce contaminant peaks of equal size in the first and second preparative HPLC purifications of dNTPs, we estimate that each cycle of HPLC achieved at least 100-fold purification from adjacent contaminating peaks. Thus, if one started with a commercial preparation of dNTP that contained cross-contamination at a level of one part per thousand, the

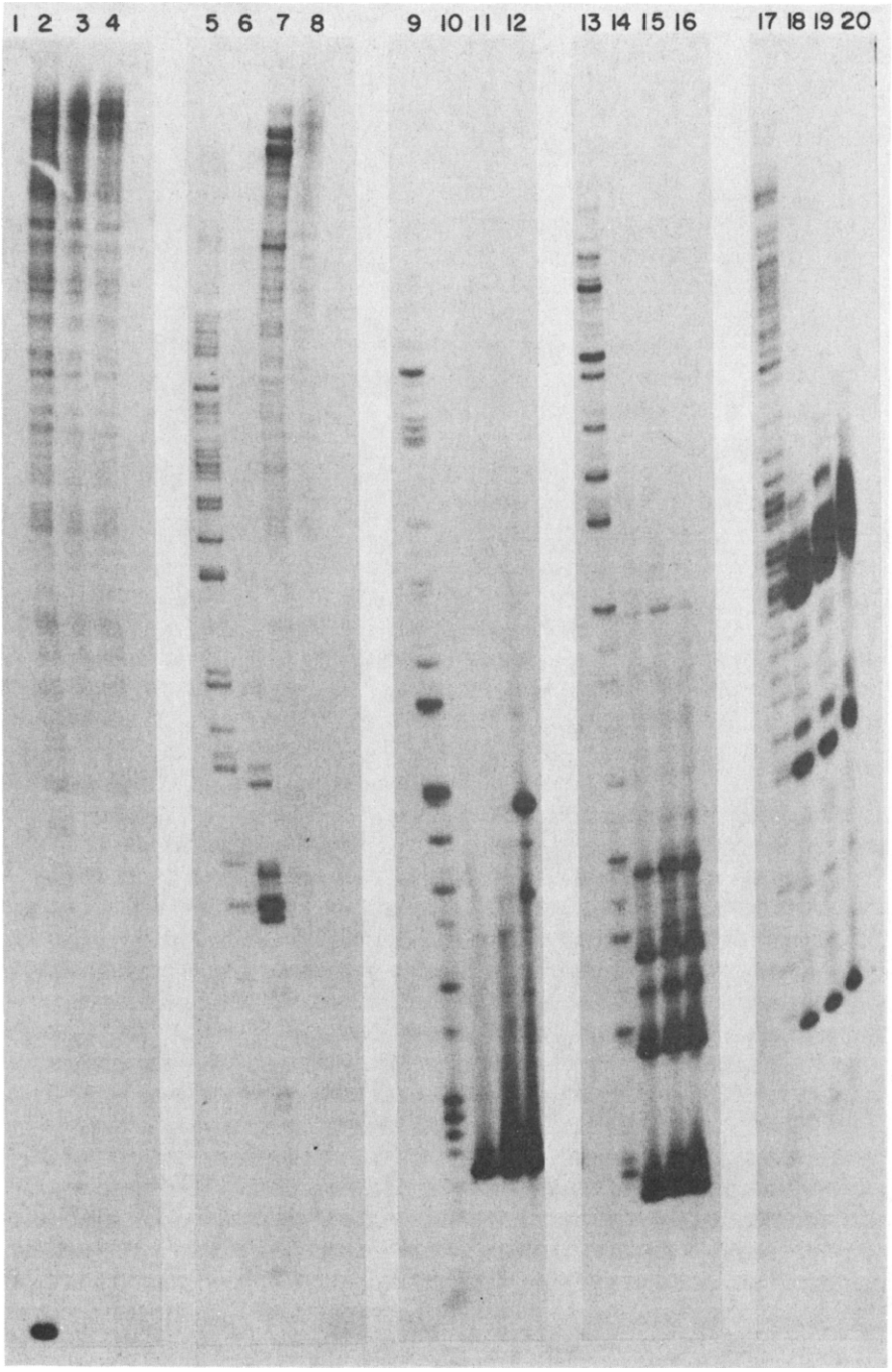

Fig. 8. Electrophoretic analysis of the mispairing potential of εdATP during chain elongation catalyzed by *E. coli* DNA polymerase I. Primer-template S17-M13mp9 was elongated for 30 min at 30°C in the presence of 10 μM normal dNTPs. Lane 1, no polymerase. Lanes 2–4, all four normal dNTPs present, plus 0, 10 and 50 μM εdATP, respectively. Lane 4, ddA sequencing reaction. Lanes 6–8, −A reactions with addition of 0, 10 and 50 μM εdATP, respectively. Lane 9, ddG sequencing reaction. Lanes 10–12, −G reactions with addition of 0, 10 and 50 μM εdATP, respectively. Lane 13, ddC sequencing reaction. Lanes 14–16, −C reactions with addition of 0, 10 and 50 μM εdATP, respectively. Lane 17, ddT sequencing reaction. Lanes 18–20, −T reactions with addition of 0, 10 and 50 μM εdATP, respectively.

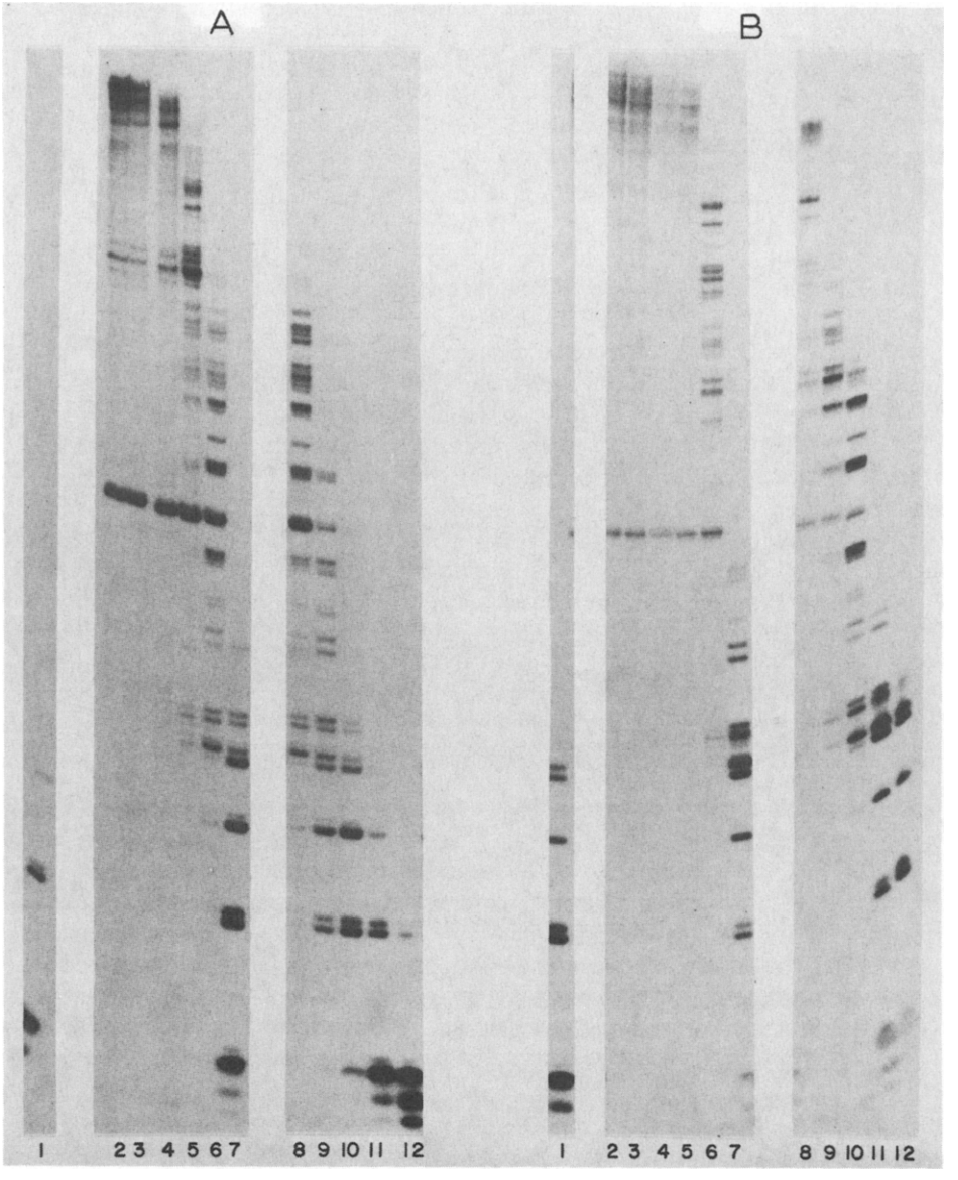

Fig. 9. Electrophoretic analysis of the efficiency of utilization of εdATP in place of dATP during chain elongation catalyzed by *E. coli* DNA polymerase I. In all reactions S15-M13mp9 primer-template was elongated in the presence of 10 μM each of dGTP, dCTP and dTTP, with various concentrations of dATP (lanes 2–7) or εdATP (lanes 8–12) for 1 min (panel A) or 5 min (panel B). To each −A reaction was added dATP at 0 μM (lane 1), 10 μM (lane 2), 5 μM (lane 3), 1 μM (lane 4), 0.10 μM (lane 5), 0.01 μM (lane 6) or 0.001 μM (lane 7), or εdATP at 10 μM (lane 8), 5 μM (lane 9), 1 μM (lane 10), 0.10 μM (lane 11) or 0.01 μM (lane 12).

procedure outlined here would result in reduction of such contamination to a level of at most, one part per ten million, which is surely adequate for use in any electrophoretic assay of misincorporation. The semi-preparative dNTP purification described here (10-μmol scale) yields sufficient dNTP for the conduct of 5000–10,000 DNA polymerase reactions.

We conclude with the following comments regarding the use of HPLC-purified dNTPs in the electrophoretic assay of mispairing of modified dNTPs during DNA synthesis. (a) Commercially HPLC-purified unmodified dNTPs can be used for this purpose, although without further purification of dNTPs, the background against which analogue incorporation must be measured may be artificially high in many experiments. (b) After evidence is obtained for the mispairing of a modified dNTP during DNA synthesis, it is necessary to insure that the normal dNTP for which substitution appeared to occur was efficiently resolved from the modified dNTP during purification. In some cases this may require repurification of the analogue with a different mobile phase, followed by repeat of the electrophoretic assay of misincorporation. (c) In order to use a chemically modified dNTP in the electrophoretic assay, it must be sufficiently stable so as not to be transformed into a form with altered base-pairing potential (stability can be tested by HPLC analysis). (d) Finally, whenever possible, the DNA synthesized in the presence of modified dNTP should be digested by the gentlest means possible and the products should be analyzed by HPLC to obtain confirmatory evidence for incorporation of the analogue. Items (c) and (d) are dealt with in a subsequent paper[15] with regard to εdAMP incorporation.

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