Separation of Ribonucleotides and Deoxyribonucleotides on Columns of Borate Covalently Linked to Cellulose. Application to the Assay of Ribonucleoside Diphosphate Reductase†

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ABSTRACT: Deoxyribonucleotides were separated from ribonucleotides by chromatography on columns of *N*-[*N'*-(*m*-dihydroxyborylphenyl)succinamyl]aminoethylcellulose in a buffer containing magnesium, and from free bases by chromatography on very small columns of Dowex 1-formate. Deoxyadenosine and deoxyguanosine mono-, di-, and triphosphates, or deoxycytidine and deoxyuridine mono-, di-, and triphosphates, were collected in a fraction of 6 or 8 ml of 2 M ammonium formate; total time required was under 5 hr. Assays by this procedure of ribonucleoside-diphosphate reductase (EC 1.17.4.1) activity in high-speed supernatants from Novikoff rat tumor, cultured Chinese hamster cells, and human leukemic cells gave results which agreed with those obtained by standard assay methods, using ADP or CDP as substrate.

The assay of ribonucleotide reductases (ribonucleoside-diphosphate reductase, EC 1.17.4.1, or ribonucleoside-triphosphate reductase, EC 1.17.4.2) has presented a number of problems, especially with crude extracts of most tissues. The chemical detection of deoxyribonucleotides is not sensitive enough for assaying most enzyme preparations, especially when pyrimidine deoxynucleotides are to be determined.

The original procedure of Reichard (1958), using Dowex 50 columns to separate CMP and dCMP, is probably capable of the greatest accuracy. It permits use of ^{14}C-, ^{3}H-, or ^{32}P-labeled substrate, and allows correction for carrier recovery, but it is limited to the cytidine nucleotides and requires more than a normal working day to complete. The paper chromatographic methods (Reichard, 1958; Moore, 1967; Fujioka and Silber, 1970) are also slow. While they are useful with any substrate, they require an enzymatic dephosphorylation which is an additional source of errors, and they do not allow easy correction for recovery. They are also subject to interference by degradation products such as uracil, adenine, or hypoxanthine when unpurified enzymes are used. A thin-layer chromatographic method has been described which we have not tested, since we became aware of it after the completion of this work (Yeh and Tessman, 1972). It appears to offer several advantages, but still requires the spotting of sample and is limited by sample size. The spectrophotometric assay (Holmgren *et al.*, 1965) is limited to highly purified enzymes.

The Dowex 1-borate procedure of Steeper and Steuart (1970) is rapid, but is limited to the cytidine nucleotides and requires dephosphorylation. Cory *et al.* (1973) have very recently (since this work was completed) published an adaptation of this method to the adenine nucleotides.

The reports by Weith *et al.* (1970) of the synthesis of a borate-cellulose material and by Rosenberg *et al.* (1972) of its nucleotide-binding properties prompted us to investigate this material for the assay of ribonucleotide reductase. We have developed methods for the assay of reduction of either purine or pyrimidine ribonucleotides, labeled with ^{14}C, without hydrolysis or dephosphorylation. All the deoxynucleotide products are obtained in a single fraction within a few hours.

Experimental Section

Buffers used for the elution were the following: (1) 0.05 M Tris-Cl buffer (pH 8.45) with 0.05 M $MgAc_2$ and 4 μg/ml of NaN_3 (Tris-Mg); (2) 0.05M sodium citrate (pH 5.9) with 4 μg/ml of NaN_3; (3) ammonium formate (pH 5, 0.02, 0.1, and 2.0 M) with NaN_3 added to the two lower concentrations.

The *N*-[*N'*-(*m*-dihydroxyborylphenyl)succinamyl]aminoethylcellulose was synthesized as described by Weith *et al.* (1970) except that the amount of aminoethylcellulose was increased to compensate for a lower content of amine groups in the batch we used. Three preparations were combined after their capacity was found to be similar. Approximately 0.15 μmol of boronic acid was bound per g of cellulose. We shall refer to this material as borate-cellulose.

The enzyme extracts were: a 100,000*g* supernatant from a 40% homogenate of Novikoff rat ascites hepatoma (Moore, 1967); the supernatant from a 20% sonicate of Chinese hamster cells (DON strain) centrifuged in a clinical centrifuge; and a 100,000*g* supernatant from a 40% sonicate of human leukemic cells. The assay mixes and procedures were those previously described (Moore, 1967) unless otherwise stated, except that GTP (2.1 mM) was used instead of dGTP as activator for reduction of ADP.

Unlabeled nucleotides were measured spectrophotometrically at 260 nm, which is sufficiently near the peak absorbance for all the compounds at the pH of the buffers. Labeled compounds were counted in Aquasol (4-ml sample to 15 ml of Aquasol) in a Beckman (room temperature) or Packard (refrigerated) scintillation counter. The counts were corrected for quenching by the external standard channel ratio method, corrected for carrier recovery, and converted to nanomoles according to the specific activity of the substrates.

[2-^{14}C]CDP (Schwarz BioResearch) was purified by adsorption and elution from a borate-cellulose column (Table I) followed by adsorption and elution from a 0.7 × 25 cm column

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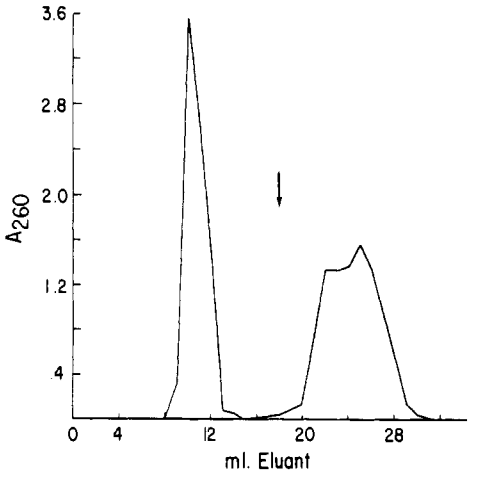

FIGURE 1: Elution of pyrimidine nucleotides and bases from borate-cellulose. A mixture containing uracil, cytosine, dCMP, dCTP, dUMP, dUTP, CMP, CDP, CTP, UMP, UDP, and UTP, in amounts from 0.74 to 5.1 A_{260} units, was applied in 1.2 ml of Tris-Mg to a 1 × 14 cm column of borate-cellulose. The column was eluted with Tris–Mg buffer (18 ml) followed (arrow) by citrate buffer. Fractions of 1 ml were collected and A_{260} was determined. The A_{260} of the corresponding fraction from a blank column (no sample) was subtracted from each reading. The total recovery (sum of all fractions) was 91.1% of the A_{260} applied to the column.

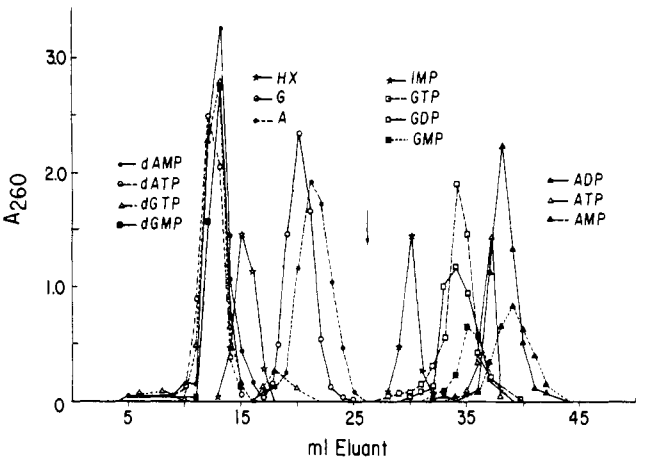

FIGURE 2: Elution of purine nucleotides and bases from borate-cellulose. To individual 1 × 14 cm columns of borate-cellulose, each purine nucleotide or base was applied in 1.2 ml of Tris–Mg buffer and eluted with the same buffer (25 ml) followed (at arrow) by citrate buffer. Fractions of 1 ml were collected and the A_{260} was determined. The A_{260} of corresponding fractions of a blank column was subtracted. Adenosine was eluted from a similar column in a fraction from 46 to 56 ml, xanthosine at from 50 to 60 ml, and inosine at from 64 to 74 ml. Abbreviations used are: A, adenine, G, guanine, HX, hypoxanthine.

of Dowex 1-formate with a gradient approaching 2 M ammonium formate (pH 4.5). About 0.8% deoxynucleotides, 0.4% UMP, and 1.2% UDP were removed.

Results and Discussion

Preliminary tests with a 1 × 10 cm column of borate-cellulose, connected to an ultraviolet (uv) monitor and fraction collector, showed that although CMP and dCMP were separated in 0.1 M phosphate buffer or 0.05 M Tris at pH 7 with 0.1 M $MgAc_2$, the separation was not adequate for a routine assay. The separation was considerably better with 0.05 M Tris (pH 8.45) and $MgAc_2$ at either 0.1 or 0.05 M. The latter concentration was chosen. All of the cytidine and deoxycytidine nucleotides were separated under these conditions, as were the adenosine and deoxyadenosine nucleotides. When this procedure was applied to actual incubation mixtures, however, very high radioactivity was found in the "deoxynucleotide" fraction and was found to be in the free bases. To separate these from the deoxynucleotide fraction, a small column of Dowex 1-formate was used (Hurlbert *et al.*, 1954). In the procedure finally adopted, the appropriate part of the eluate from the borate-cellulose columns was allowed to drip directly onto the Dowex 1-formate column.

Because the purine nucleotides are bound more tightly than the pyrimidine nucleotides, both to the borate-cellulose (Rosenberg *et al.*, 1972) and to Dowex 1 (Hurlbert *et al.*, 1954), an elution scheme was not found which could be used for both the purine and pyrimidine series. However, we devised schemes by which all purine, or all pyrimidine, deoxynucleotides were collected together in a single fraction of 6 or 8 ml.

Columns (1 × 14 cm) of borate-cellulose were packed and washed with 22 ml of Tris-Mg buffer. Samples of about 0.5 μmol of single purine compounds or mixed pyrimidine compounds, in 1.2 ml of Tris-Mg, were applied to the columns and eluted with Tris-Mg. Fractions of one ml were collected and the absorbance was read to establish the peak position. After a sufficient volume of Tris-Mg to elute all the deoxynucleotides was applied, the ribonucleotides were eluted with citrate buffer. Figures 1 and 2 show that the deoxynucleotides were eluted, together with some of the bases, in a fraction from 8 to 14 ml (pyrimidines) or from 10 to 15 ml (purines).

Similar samples were applied to 0.7 × 1.4 cm columns of Dowex 1-formate in 6 or 9 ml of Tris-Mg to simulate effluents containing deoxynucleotides from the borate-cellulose columns. Columns with cytosine, uracil, deoxycytidine, deoxyuridine, dCMP, dCTP, dUMP, and dUTP were eluted with 7 ml of 0.02 M and 7 ml of 2 M ammonium formate. Fractions of 1 ml were collected and the A_{260} was determined, with subtraction of the A_{260} of fractions from a blank column. Cytosine was not bound by the column, and all uracil was eluted in the first three ml of 0.02 M ammonium formate. The deoxynucleosides behaved similarly. Based on the sum of all fractions, 95% of the dCMP, 97% of dCTP and dUMP, and 98% of dUTP were found in the first 4 ml of 2 M ammonium formate. The A_{260} had dropped to negligible levels by the fourth milliliter for the monophosphates, the fifth for dCTP, and the seventh for dUTP. Only traces of the monophosphates were found in the 0.02 M ammonium formate wash.

In a similar manner, columns containing the purine bases and deoxynucleosides and their mono- and triphosphates were eluted with 10 ml of 0.1 M and 8 ml of 2 M ammonium formate. Hypoxanthine and deoxyadenosine were almost all eluted in the first 3 ml of 0.1 M ammonium formate. Deoxyguanosine, adenine, and guanine followed with peaks at 3, 4, and 5 ml, respectively, and the A_{260} dropping to less than 5% of the maximum at 8, 8, and 9 ml, respectively. A trace of material from the purine deoxynucleotides was eluted at the position of the bases, probably due to some degradation, but the A_{260} was very low in the latter part of the 0.1 M ammonium formate wash. It rose in the first milliliter of 2 M ammonium formate and reached its maximum in the second milliliter for dAMP, dATP, and dGMP and in the third for dGTP. By the sixth milliliter, dATP was completely eluted, but the other three still showed about 5% of their maximum A_{260} at the eighth milliliter. Higher concentrations of ammonium formate could not be used because of separation of phases in the scintillation counting cocktail.

To determine whether ^{32}P-labeled substrates could be used, P_i was liberated by incubating a sample of [α-^{32}P]CDP with alkaline phosphatase. The incubation mixture was put through

TABLE I: Standard Elution Schemes.

Columns	Pyrimidine System (ml)	Purine System (ml)
Borate-cellulose (1 × 14 cm)		
Apply sample in Tris-Mg	1.12	1.12
Wash in, Tris-Mg	1.0	1.0
Add, discarding effluent, Tris-Mg	6.0	6.0
Add, effluent to Dowex, Tris-Mg	6.0	9.0
Add Tris-Mg, collect fractions	4.0	4.0
Add Tris-Mg (if bases are desired)		5.0
Elute ribonucleotides Citrate	12.0 (or 60)[a]	60.0
Dowex 1-formate (0.7 × 1.4 cm)		
Elute bases, 0.02 M formate	7.0	
Elute bases, 0.1 M formate		9.0
Elute deoxynucleotides, 2 M formate	6.0	8.0

[a] If the reaction mixture contained ATP or other purine nucleotides.

borate-cellulose and Dowex 1-formate columns in accordance with the pyrimidine procedure of Table I. The Dowex column was washed first with 0.02 M ammonium formate, then with 0.1 M, and eluted with 2 M. Of the radioactivity due to P_i, 90% was found in the 0.1 M ammonium formate wash and 1.4% in the 2 M ammonium formate eluate.

The schemes we adopted are shown in Table I. We collect four 1-ml fractions of Tris-Mg following the elution of the deoxynucleotides from the borate-cellulose columns, which may be counted to check for separation. We wash both types of Dowex 1 columns with extra 2 M ammonium formate to ensure that any contaminating purine deoxynucleotides are removed. Columns can be reused several times if they are carefully washed and prevented from drying out. They are stored in the cold with the tips immersed in water when not being used. The borate-cellulose compressed slightly during the first few runs, but then stabilized. The exact volumes required for elution from borate-cellulose may vary from one batch to another; our experience is not sufficient to determine this. The useful life of the material has not been completely determined; after about 7 months of frequent use, the batch used for these experiments still separated adenine nucleotides satisfactorily but no longer gave clean separation of cytidine nucleotides.

The choice of carriers to be added after the incubation presents some theoretical difficulty, since the product from a crude enzme preparation would be expected to occur as a mixture of mono-, di-, and triphosphates, and the recovery of the three is not necessarily identical in a finite fraction. However, the volumes collected in our standard procedure did not omit a significant amount of any of the deoxynucleoside mono- or triphosphate test compounds. We have used both monophosphate and triphosphate carriers with products from crude enzymes, and have not observed any significant difference in recovery between dAMP and dATP (varying from 80 to 90% for both) and dCMP and dCTP (90–95% with fresh borate-cellulose, 80–90% with older material when the volume collected from the borate-cellulose column was reduced to 5.5 ml to avoid contamination by ribonucleotides). For enzymes free of kinases and phosphatases the expected product would be preferable.

Comparative assays for reduction of CDP were run on extracts from three types of cells, namely, the Novikoff rat hepatoma, the DON strain of cultured Chinese hamster cells, and human leukemic cells. Duplicate incubation tubes were analyzed by the borate-cellulose technique and by standard techniques. The results are shown in Table II. Samples to be analyzed by the new method were heated in boiling water for 3 min, 1 ml of Tris-Mg and carrier were added, and the samples were placed (without hydrolysis) on the borate-cellulose columns. The samples were counted for radioactivity the same day. The standard procedure using acid hydrolysis and Dowex 50 columns (Reichard, 1958) was used for comparison with the new method. The elution of the Dowex 50 columns was extended to elute deoxycytidine, cytidine, and cytosine. To establish the level of uridine nucleotides in this case, the first 10 ml of effluent from the Dowex 50 column was dried and chromato-

TABLE II: Assay of Reduction of [2-^{14}C]CDP by Two Methods.[a]

	nmol/Incubation Tube			
	No Enzyme	Novikoff Tumor Extract	DON Cell Extract	Human Leukemic Cell Extract
By borate-cellulose method				
Total deoxynucleotides	0.012, 0.011	1.43, 1.46	0.56, 0.55	0.33, 0.34
By Dowex 50 and paper chromatography				
dCMP + dUMP	0.05, 0.04	1.49, 1.47	0.62, 0.62	0.36, 0.43
dCMP	0.003, 0.008	1.17, 1.20	0.50, 0.49	0.23, 0.20
dUMP	0.04, 0.04	0.29, 0.30	0.12, 0.13	0.16, 0.16
Deoxycytidine	0.002, 0.005	0.02, 0.02	0.02, 0.02	0.006, 0.005
Deoxyuridine	0.04, 0.04	0.04, 0.04	0.04, 0.04	0.05, 0.05
Cytidine + cytosine	0.09, 0.07	0.14, 0.13	0.30, 0.35	0.11, 0.10
UMP + uridine + uracil	0.06, 0.04	0.14, 0.12	0.13, 0.11	0.19, 0.18

[a] Replicate samples were prepared by incubation of [2-^{14}C]CDP (40 nmol; 2228 cpm/nmol) with extracts of three cell types; two of each were analyzed by the new method and two by our previous methods. Novikoff tumor extract (50 μl) and DON-cell extract (60 μl) were incubated for 30 min; human leukemic cell extract (40 μl) was incubated 30 min.

graphed on paper. Spots were eluted and counted, and carrier recovery was determined. The entire procedure required 10 days.

It may be noted that up to one-half the product from reduction of CDP was recovered as dUMP, although only small amounts of other uracil derivatives were found. This probably results from the action of dCMP-aminohydrolase (EC 3.5.4.5) which is usually found in the rapidly growing tissues containing ribonucleotide reductase (Weber *et al.*, 1971). The ability to detect both deoxyuridine and deoxycytidine nucleotides is a useful feature of the new method.

The results of comparative assays for reduction of ADP are shown in Table III. The standard technique was enzymatic dephosphorylation and paper chromatography (Fujioka and Silber, 1970). The paper system we have previously used (Moore, 1967) was not satisfactory with these enzymes because hypoxanthine was not completely separated from deoxyadenosine.

Table III shows that the borate-cellulose assay gave results quite comparable to the other procedure. We suspect that the slightly higher results with the new method may be due to the presence of deoxyinosine nucleotides, but we have not yet been able to separate deoxyinosine clearly enough to be certain. The time required to collect the deoxynucleotide fraction from the borate-cellulose and Dowex 1-formate columns was about 4 hr. The ribonucleotides could be washed off overnight.

Tritium-labeled substrates could be used in this procedure provided chemiluminescence of the 2 M formate buffer was avoided by counting at low temperatures. Quench corrections are probably necessary with either ^{14}C or ^{3}H. ^{32}P cannot be used with the present procedure with crude enzymes because P_i would be eluted with pyrimidine deoxynucleotides and would not be completely separated from purine deoxynucleotides. If P_i was not formed, however, the method would be very rapid since the separation of free bases would be unnecessary.

The new method will not detect any product which may be present as deoxynucleosides. However, Table II shows that no significant amount of deoxyuridine or deoxycytidine was found after incubation with any of these three unpurified extracts. We would not expect any greater conversion to purine deoxynucleoside. Although deoxycytidine can be isolated by the Dowex 50 method, if elution is carried far enough, we do not routinely determine this compound.

This procedure offers a simple, rapid assay for ribonucleotide reductase which is suitable for all four substrates of interest and which can be used for enzymes at any stage of purification, without the necessity of hydrolysis or dephosphorylation of the product. It could easily be adapted to double-label experiments with two substrates. In the case of enzymes purified to the extent that free bases are not formed, omission of the Dowex 1 column would further simplify the procedure.

TABLE III: Assay of Reduction of [8-^{14}C]ADP by Two Methods.[a]

Enzyme Source	Deoxyadenosine Nucleotides Found: Borate-cellulose Method	Deoxyadenosine Nucleotides Found: Dephosphorylation and Paper Chromatography
None	0.09	0.05
DON cells	0.28, 0.27	0.20, 0.21, 0.22
DON cells[b]	0.69, 0.70	0.75, 0.71, 0.53
Novikoff tumor (partially purified)	1.23, 1.37	1.00, 0.90, 0.90, 1.02

[a] Replicate samples were prepared by incubation of [8-^{14}C]-ADP with three enzyme preparations; two of each were assayed by the new method and others by the method of Fujioka and Silber (1970). Results are in nanomoles per incubation tube. [b] Endogenous nucleotides were removed from this preparation by passage over Dowex 1-Cl.

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