PREPARATION OF (^{125}I)-dCTP AND ITS USE AS A SUBSTRATE FOR RNA- AND DNA-DIRECTED DNA SYNTHESIS

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SUMMARY

A method is described for the synthesis and subsequent purification of radioiodinated deoxycytidine triphosphate (^{125}I-dCTP). The resultant preparations were found to be free of mono- or diphosphorylated nucleosides as well as unlabelled dCTP. As a substrate for DNA synthesis (^{125}I)-dCTP showed incorporation into DNA equal to that of its tritiated counterpart in the endogenous RNA-directed DNA synthesizing system catalysed by avian myeloblastosis virus reverse transcriptase, and is also effectively utilized by Escherichia coli DNA polymerase I. Therefore, (^{125}I)-dCTP prepared in this manner may be used to produce cDNA transcripts of viral or mRNA.

The synthesis of DNA complementary to messenger or viral RNA by reverse transcriptase has provided molecular biology with new and valuable probes for specific sequence analysis and quantitation (1,2) as well as possible sources of selected "genes" (3). Recent reports have indicated that high concentrations of DNA precursors are required for obtaining a significant percentage of full-length cDNA transcripts of globin mRNA (4), and avian (5) or mammalian (6) retroviral genomic RNA. The large quantities of conventionally radiolabelled precursors required to maintain high product specific activity at high substrate concentrations may therefore become prohibitively expensive. In this communication, we report a rapid method for the synthesis and purification of high-specific activity (^{125}I)-dCTP. We also show for the first time that the iodinated derivative is an efficient substrate for both RNA- and DNA-directed DNA synthesis, using reverse transcriptase as well as E. coli DNA polymerase I.

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MATERIALS AND METHODS

Radioisotopes and Reagents. Carrier-free Na(^{125}I) and (^{3}H)-dCTP were obtained from New England Nuclear, Inc. Unlabelled deoxynucleoside triphosphates were obtained from P-L Biochemicals, Inc. Disposable columns were purchased from Bio-Rad Laboratories, Inc. Poly (ethyleneimine) (PEI) plates were purchased from Brinkmann Instruments. All other chemicals were of reagent grade.

Virus Isolation and Disruption. Avian myeloblastosis virus (AMV) was supplied as plasma from infected chickens by Life Sciences, Inc., St. Petersburg, Fla., prepared under Contract No. NOICP33291, Virus Cancer Program, NCI. Virus was purified from plasma as previously described (7). The purified virus was suspended in 0.01M Tris-HCl, pH 7.8, 0.15M NaCl and 0.01M EDTA (TNE buffer) containing 50% glycerol. Protein determinations were carried out according to the method of Lowry et al (8) using bovine serum albumin (Fraction V) as a standard. For studies on endogenous DNA synthesis, AMV at a protein concentration of 2 mg/ml was disrupted by the addition of Nonidet P-40 to a final concentration of 0.02% and dithiothreitol (DTT) to a final concentration of 2 mM.

Enzymes. AMV DNA polymerase was purified using polycytidylate-agarose affinity chromatography according to Marcus et al (7). E. coli DNA polymerase I (Fraction VII, Jovin procedure) was purchased from Boehringer-Mannheim, Inc. DNAase I and pancreatic RNAase were purchased from Worthington Biochemicals, Inc.

DNA Polymerase Assays. All assays were carried out in a total volume of 0.1 ml. Reactions in which synthesis was directed by activated calf thymus DNA and catalysed by E. coli DNA polymerase I consisted of 50mM Tris-HCl, pH 7.8, 2mM $MgCl_2$, 1mM DTT, 20 µg bovine serum albumin, 100µM each dATP, dGTP, and dTTP, 20µM dCTP, and sufficient (^{3}H)- or (^{125}I)-dCTP to yield a final specific activity of 1000 to 4000 cpm/picomole. Calf thymus DNA activated by the procedure of Aposhian and Kornberg (9) was used at 5 µg per assay. Three nanograms of E. coli DNA polymerase I were used per assay. Reactions measuring endogenous DNA-synthesizing capacity of disrupted preparations of AMV were similar except that concentrations of unlabelled triphosphates were 200µM, and 10mM $MgCl_2$ and 50mM KCl were also used. Approximately 10 µg of detergent-disrupted AMV protein was used per assay in the absence of exogenous template-primers. Reaction mixtures containing purified AMV reverse transcriptase (approximately 20 nanograms/assay) and activated DNA as template-primer were essentially the same as those in which E. coli DNA polymerase was used, except that 60mM $MgCl_2$ was present. Assays were incubated at 37° C for various times and were terminated by the addition of 5% (wt/vol) trichloroacetic acid solution containing 0.01M Na pyrophosphate. Acid insoluble material was collected onto glass fiber filters and radioactivity was measured in a liquid scintillation counter for (^{3}H) or using a gamma counter for (^{125}I).

RESULTS

Preparation and Purification of (^{125}I)-dCTP. A standard reaction for the iodination of dCTP contained, in a final volume of 100 µl, 8 µl Na (^{125}I) (4.8 mCi, or approximately 2.5×10^{-9} moles), 8 µl KI (1.2×10^{-9} moles), 50 µg dCTP, 4 µl of 5mM $TlCl_3$ and 1.2 µl of 0.075M HNO_3. The reaction was carried out in a sealed vial at 60° C for 10 minutes. After the addition of 0.2 ml of 2M ammonium acetate, pH 8.0, the vial was again incubated at 60° C for an addi-

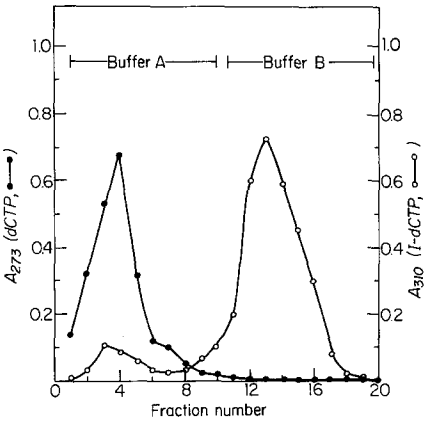

Figure 1. Separation of iodinated from non-iodinated deoxycytidine nucleotides by Dowex AglX10 column chromatography. Buffer A = 4M formic acid and 0.3M ammonium formate. Buffer B = 4M formic acid and 1M ammonium formate. Fraction volume = 2 mls. The standard reaction mixture described in Materials and Methods was scaled up for this experiment, and only nonradioactive KI was used.

tional 15 minutes. The samples were then applied to a 0.5 x 3 cm colum of Dowex AglX10 resin which had previously been equilibrated with distilled water. After application of the samples, the AglX10 column was washed with a quantity of buffer containing 4M formic acid and 0.3M ammonium formate sufficient to remove all mono- and diphosphorylated iodinated cytidine derivatives as well as uniodinated dCTP. The (^{125}I)-dCTP remaining on the column was eluted using buffer containing 4M formic acid and 1M ammonium formate. Representative column profiles are illustrated in Figure 1, in which the elution of non-iodinated deoxycytidine derivatives was monitored by measuring A_{273}, while elution of iodinated deoxycytidine derivatives was followed by their characteristic absorbance at 310 nm. Thin-layer chromatography of (^{125}I)-labelled material in column eluate fractions on PEI-cellulose plates as described by Randerath and Randerath (10) with subsequent autoradiography was used to determine the relative quantities of mono- di- and triphosphorylated nucleosides in eluate fractions (Fig. 2). Our results indicate that the

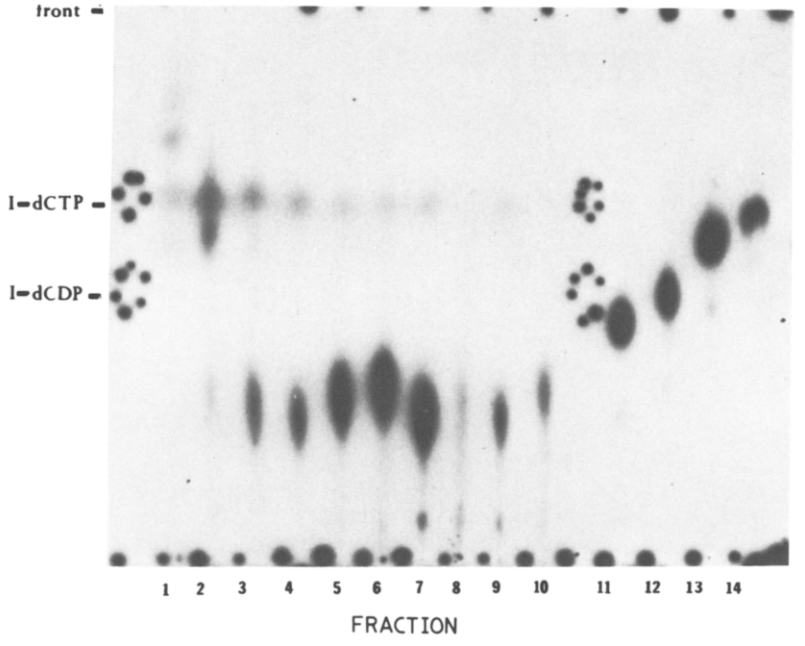

Figure 2. Autoradiograph of PEI-cellulose thin-layer chromatogram run to determine the purity of (^{125}I)-deoxycytidine nucleotides obtained via AglX10 column chromatography. The numbers below sample origins correspond to similar fraction numbers in Figure 1. Approximately 50,000 (^{125}I)-cpm was spotted for each fraction. The chromatogram was developed using 1M LiCl. Positions of unlabelled di- and triphosphorylated standards were outlined with (^{32}P)-containing ink. R_f values for I-dCTP and I-dCDP determined in this chromatography system were 0.69 and 0.53, respectively.

peak of iodinated material in the 4M formic acid, 1M ammonium formate eluate is essentially pure (^{125}I)-dCTP. Desalting of such fractions was carried out at 4° C using a Sephadex G-10 column (0.5 x 12 cm.) with water as the developing solvent. Samples obtained and desalted in this manner were passed through an identical column as a second desalting step. The (^{125}I)-dCTP thus obtained was used immediately or lyophilized and stored at -20° C until used. Only those fractions containing the triphosphate were used for desalting, and no breakdown of the product was found to occur as a result of the desalting step.

(^{125}I)-dCTP as a Substrate for DNA Synthesis. Figure 2 shows the use of

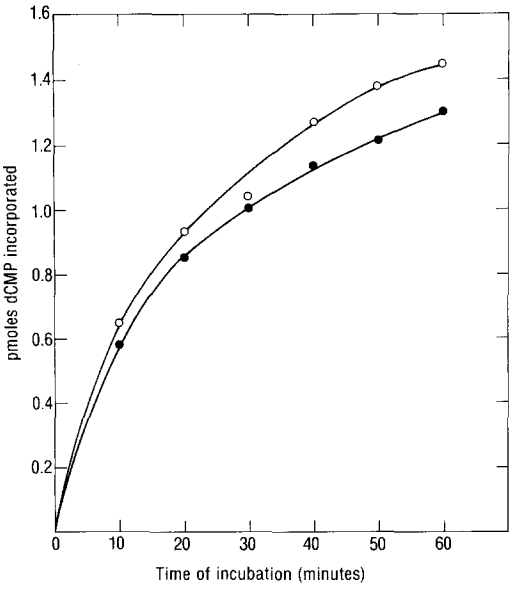

Figure 3. Kinetics of DNA synthesis directed by the endogenous RNA template present in detergent-disrupted AMV, measured by the incorporation of (^{3}H)- and (^{125}I)-dCTP into trichloroacetic acid-insoluble product. Assays were carried out as described in Materials and Methods.

(^{125}I)-dCTP to monitor endogenous DNA synthesis catalysed by disrupted AMV compared with the commercially available (^{3}H)-dCTP compound. No significant difference in the time course of synthesis was noted. Therefore, (^{125}I)-dCTP appears to be an effective substrate for retroviral DNA polymerase. In an effort to insure that what is shown in Figure 3 represents DNA synthesis rather than non-specific incorporation of labelled material into another acid-insoluble form with time, a series of control experiments were carried out using purified AMV reverse transcriptase as well as E. coli DNA polymerase I. The results of these studies are summarized in Table I. Endogenous DNA synthesis catalysed by detergent-disrupted AMV was dependent upon the presence of all four deoxynucleoside triphosphates and, in addition, was abolished by pre-incubation of disrupted virions with high concentrations of RNase. Activated DNA-dependent DNA synthesis catalysed by purified AMV or E. coli DNA polymerase also required the presence of all four DNA precursors, but was insensitive to

TABLE I

(^{125}I)-dCTP as a Substrate for RNA- and DNA-directed DNA Synthesis

Enzyme	Template	Reaction	pmoles Incorporated (^{125}I)-dCTP	(^{3}H)-dCTP
AMV DNA Polymerase	Endogenous	Complete	1.5	1.35
"	"	-dGTP	0.2	0.2
"	"	+RNAase[a]	< 0.05	< 0.05
"	Activated DNA	Complete	88	80
"	" "	+Actinomycin D (100μg/ml)	20	19
"	" "	-dATP, dGTP	9	8
"	" "	+RNAase[a]	86	80
E. coli DNA Polymerase I	Activated DNA	Complete	100	95
" "	" "	-dGTP, dATP	10	9
" "	" "	+DNAase[b]	< 0.1	< 0.1
" "	" "	+Alkali[c]	90	85

Assays were performed as described in Materials and Methods. Incubation was carried out at 37°C for 60 minutes in all cases.

[a]Pancreatic ribonuclease at a final concentration of 50μg/ml was preincubated with disrupted virions or DNA template prior to addition of reaction mixture.

[b]DNase I at a concentration of 20μg/ml was preincubated with template.

[c]Reaction was terminated by addition of 10μl of 6N NaOH and incubated at 37°C for an additional 15 mins. after which the pH was brought to neutrality by the addition of 6N HCl. Product was then precipitated and acid-insoluble radioactivity determined as described in Materials and Methods.

RNase treatment. Addition of actinomycin D to DNA-dependent reactions inhibited synthesis; the product of such reactions was degraded by DNase I but resistant to alkali. Incorporation of (^{125}I)-dCTP and (^{3}H)-dCTP were nearly identical for all systems tested, with the iodinated derivative showing slightly

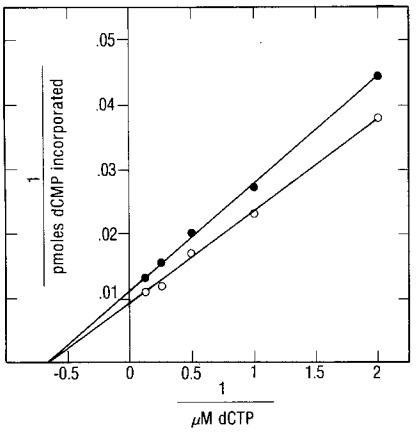

Figure 4. Determination of the apparent Km for dCTP using E. coli DNA polymerase I, with activated DNA as the template-primer. Units of velocity are expressed as picomoles (^{3}H)-dCMP (●—●) or (^{125}I)-dCMP (o——o) incorporated per 30-minute interval under standard assay conditions (see Materials and Methods). The concentration of dCTP was varied while the other three precursors were kept at saturating levels.

better incorporation in all systems than the tritiated dCTP.

In order to determine whether iodination of dCTP might affect the affinity of DNA polymerase for this substrate, an experiment measuring the velocity of DNA synthesis as a function of (^{125}I)- and (^{3}H)-dCTP concentration was carried out. The results of this study carried out using E. coli DNA polymerase I are shown, in the form of a double-reciprocal plot, in Figure 4. The apparent Km values obtained are identical for both (^{125}IO- and (^{3}H)-dCTP. The Vmax value obtained using the iodinated substrate is approximately 10% higher than that of its tritiated counterpart. The apparent Km value for both (^{125}I)- and (^{3}H)-dCTP was 1.5μM.

The (^{125}I)-dCTP prepared in the above manner could be stored as lyophilized material for periods of up to one month without loss of activity other than that which could be accounted for by the half-life correction for radioactive decay (data not shown).

DISCUSSION

We have described a rapid method for the preparation and subsequent purification of (^{125}I)-dCTP. Material prepared in this manner was found to be free of mono- or diphosphorylated nucleosides as well as unlabelled dCTP (Figs. 1 and 2). As a substrate for DNA synthesis in endogenous RNA-directed reactions catalysed by avian myeloblastosis virus DNA polymerase, (^{125}I)-dCTP was incorporated into DNA as well as or slightly better than the tritiated compound (Fig. 3). Analysis of the product indicated that it was DNA rather than some other acid-insoluble material (Table 1), and that E. coli DNA polymerase I was also capable of utilizing (^{125}I)-dCTP as substrate. Apparent Km values for both tritiated and iodinated substrates were identical; a slightly increased Vmax observed for the iodinated precursor may be due to the possible presence of impurities in the commercially obtained (^{3}H)-dCTP preparations.

Although the preparation of (^{125}I)-dCTP has previously been reported (11, 12), such preparations either required time-consuming laborious methodology (11) or resulted in preparations which lost over 90% of substrate activity upon storage (12). The rapid procedure we have described results in a product which can be used for both RNA- and DNA-directed DNA synthesis and which is stable when stored lyophilized at -20°C. The high concentrations of DNA precursors which have been recently found to be required in order to obtain high percentages of full-sized cDNA with retroviral (5,6) or messenger RNA (2-4) as templates necessitate the use of large quantities of tritiated or other labelled precursors in order to obtain products with reasonably high specific activity. Although we have used up to 4000 cpm/picomole specific activities in this study with (^{125}I)-dCTP, we have obtained specific activities five-fold higher at substrate concentrations up to 0.1mM (data not shown). The use of (^{125}I)-dCTP for labelling of cDNA may allow the synthesis of high-specific activity product at substrate concentrations allowing full-length transcripts to be consistently produced at low cost to the laboratory. Since the specific activity of a singly substituted iodine molecule approaches 2200

Ci/millimole, a completely (^{125}I)-dCTP containing reaction mixture lacking unlabelled dCTP could ideally produce a product 75-fold greater in specific activity than a similarly labelled (^{3}H)-dCTP-containing product.

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