Chemical Linkage of Nucleic Acids to Neutral and Phosphorylated Cellulose Powders and Isolation of Specific Sequences by Affinity Chromatography†

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ABSTRACT: DNA and RNA were covalently attached to cellulose and used in a continuous hybridization system to isolate complementary nucleic acid sequences. Simian virus (SV) 40 DNA fragments were stably and efficiently (45–78%) linked to neutral cellulose powder with a water-soluble carbodiimide. SV40 complementary RNA, synthesized *in vitro* with *Escherichia coli* RNA polymerase, was securely immobilized onto phosphocellulose powder activated by 1,1′-carbonyldiimidazole. Approximately 60% of input ^{3}H-labeled SV40 cRNA (1 μg), present in a mixture containing 10 mg of unrelated yeast RNA, was selectively isolated using SV40 DNA cellulose. The continuous hybridization system consisted of a SV40 DNA column maintained at 30° and a circulant mixture (50% formamide (v/v), 0.3 M NaCl, 1 mM EDTA, and 0.03 M sodium phosphate (pH 7.0)) cooled to 4°. Complementary ^{3}H-labeled SV40 DNA could be quantitatively isolated in the presence of an excess of unrelated *Micrococcus lysodeikticus* DNA utilizing a phosphocellulose column, containing unlabeled SV40 cRNA, connected in series to a denaturation column (70°). The thermal stability of labeled SV40 cRNA or DNA hybrid structures with the immobilized species was also evaluated; the T_m values obtained (55–57°) were similar to that determined directly in the circulant mixture for native mouse cell DNA which has a similar G + C content.

Affinity chromatography utilizing specific biochemical interactions has been successfully employed for the purification of macromolecules, several of which have biologic activity. In practice, one of the interacting components is immobilized on a solid supporting substance in a suitable chromatographic system to isolate and purify the other component from a complex mixture (Cuatrecasas and Anfinsen, 1971). Analytic nucleic acid hybridization techniques have been employed to study polynucleotide sequence homology in both prokaryotic and eukaryotic systems. We have developed an affinity chromatography method based on specific nucleic acid interaction to isolate and purify a specific DNA or RNA sequence. This method is of particular relevance in virus research in which a copy of the genome is packaged in virus particles and readily available for study. To overcome the competing DNA–DNA reassociation reaction, which would interfere with the efficient isolation of a specific DNA sequence present in double-stranded DNA molecules, a continuous hybridization system, involving a reassociation column containing immobilized RNA and a denaturation column connected in series, has been described (Shih and Martin, 1973).

Chemical and physical methods for coupling synthetic or natural polynucleotides to cellulose and its derivatives (Gilham, 1968, 1971; Litman, 1968; Merriam *et al.*, 1970; Astell and Smith, 1971; Smith *et al.*, 1972; Saxinger *et al.*, 1972; Shih and Martin, 1973) and to agarose (Poonian *et al.*, 1971; Wagner *et al.*, 1971; Robberson and Davidson, 1972; Rickwood, 1972) have been described. However, the efficient and stable covalent linkage of naturally occurring DNA or RNA to a solid supporting substance suitable for the isolation and purification of a specific RNA or DNA sequence has not yet been realized. RNA–DNA hybridization as well as DNA–DNA reassociation follow second-order kinetics; their second-order rate constants are inversely proportional to complexities of involved nucleic acid sequences (Britten and Kohne, 1968; Wetmur and Davidson, 1968). Frequently the particular nucleic acid sequence to be isolated is present in low concentrations. In addition, it is desirable to keep the concentration of the immobilized species as low as possible to minimize any reaction external to the matrix. These requirements, together with the considerable degree of complexity of the nucleic acid sequences involved, make the length of time required for the affinity chromatography procedure relatively long. Therefore, stable covalent linkage of the immobilized nucleic acid to the supporting medium is essential and nonspecific adsorption of nucleic acid molecules must be avoided if meaningful purification is to occur.

In this paper, methods for coupling double-stranded DNA to neutral cellulose powder by a water-soluble carbodiimide and for immobilizing single-stranded RNA to phosphocellulose powder by carbonyldiimidazole will be described in depth. Simian virus 40 (SV40)[1] DNA or its complementary RNA, transcribed *in vitro* by *Escherichia coli* RNA polymerase, has been used in suitable chromatographic systems to isolate complementary nucleic acid sequences.

Materials and Methods

Immobilization of DNA to Neutral Cellulose Powder by the Carbodiimide Method. Neutral cellulose powder, Cellex N-1 (Bio-Rad Laboratories, Richmond, Calif.), was autoclaved in 1 M sodium bisulfite to remove possible residual lignin impurities (DeLarco and Guroff, 1973). Following a rinse with hot distilled water, the powder was successively washed with 1 N NaOH, 1 N HCl, and sufficient amounts of distilled water to restore the pH to neutrality and then was dried with methanol. The powder was stored at room temperature for 3 days in absolute methanol containing 1% concentrated HCl to methylate free carboxyl groups. The cellulose was then extensively

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[1] Abbreviations used are: SV40, simian virus 40; Mes, sodium 2-(*N*-morpholino)ethanesulfonate; CMC, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate.

washed with distilled water, and fine particles were removed by decantation. The powder was dried with acetone. A thin layer of cellulose powder was prepared by pouring a suspension (0.1–0.2 g/ml of water) onto a Chromaflex channel layer chromatography plate (Kontes Glass Co., Vineland, N. J.) with channels measuring 2 mm deep and 10 mm wide, and air-dried. In a typical experiment, 273 μg of fragmented SV40 DNA I (3×10^5 daltons) in 1.2 ml of 0.015 M NaCl was mixed with 0.3 ml of 0.2 M sodium 2-(*N*-morpholino)ethanesulfonate (Mes) at pH 6.0. 1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (150 mg) (CMC) was then dissolved and the entire mixture was immediately applied in a dropwise fashion to the thin layer of cellulose equivalent to approximately 1.5 g of dry powder. At 2, 5, and 8 hr after application of the reaction mixture, the glass plate was removed from the laboratory atmosphere of 58–66% relative humidity and 20–24°, and was placed in a desiccator saturated with water vapor for 1 hr. The procedure followed is essentially that described for applying RNA to chromatography paper (Shih and Martin, 1973; Gilham, 1971). The cellulose powder was scraped off the glass plate 24 hr following the application of the DNA mixture. The reagents employed to link DNA to cellulose as well as noncovalently adsorbed DNA were removed by exhaustive washing with concentrated ammonia, formamide, and finally with distilled water as will be outlined in the Results section. The cellulose powder was collected on a sintered-glass funnel, dried *in vacuo* over Drierite, and stored at 4°.

Immobilization of RNA to Phosphocellulose Powders by the Carbonyldiimidazole Method. Cellex P powder (exchange capacity, 0.92 mequiv/g) (Bio-Rad Laboratories, Richmond, Calif.) and Whatman Cellulose Phosphate Powder P11 (exchange capacity, 3.7 mequiv/g) (Reeve Angel, Clifton, N. J.) were exhaustively washed with distilled water. Each was then washed successively with 0.25 M NaCl–0.25 M HCl, distilled water, 0.25 M NaOH, and distilled water, and converted to the tributylammonium salt after several resuspensions in a large volume of 0.1 M $(Bu)_3N{\cdot}HCl$ (pH 6). Following filtration and drying *in vacuo* over P_2O_5–KOH, 5 g of phosphocellulose powder was activated by treatment with 50 ml of 1,1′-carbonyldiimidazole (100 mg/ml) in dimethylformamide at room temperature for 16 hr as described by Saxinger *et al.* (1972). The reagents were removed by washing the phosphocellulose with dimethylformamide and acetone. The dried, activated powders were stored in a desiccator over Drierite at −20°. In a typical experiment, 350 μg of SV40 cRNA in 0.5 ml of distilled water was applied to 1 g of activated phosphocellulose. The powder was then dried *in vacuo* for 16 hr over Drierite and mixed with 50 ml of dehydrated pyridine at 50° for 24 hr. Less than 1% of the input RNA was solubilized into the pyridine during this reaction. The cellulose powder was then deactivated with 15% ammonia solution at 4° for 20 min, and washed with sodium phosphate buffer (0.1 M, pH 5) until the pH reached 7.0. The phosphocellulose powder was then extensively washed by shaking with 50% formamide (pH 7) for 3 days followed by several changes of distilled water for another 2 days at 4°. The RNA cellulose was then dried and stored at 4°.

Preparation of SV40 DNA. Primary African green monkey kidney cells were infected with the small plaque variant of SV40 at a multiplicity of approximately 0.05 pfu/cell. Virus, propagated in the presence of [^{3}H]thymidine (20 Ci/mmol) (20 μCi/ml) was isolated 9 days following infection, and was purified by CsCl density gradient centrifugation (Yoshiike, 1968). SV40 DNA I was prepared from purified virions following treatment with 1% sodium dodecyl sulfate and isopycnic centrifugation in CsCl-containing ethidium bromide (300 μg/ml) as previously described (Radloff *et al.*, 1967). Viral DNA (1.1×10^5 cpm/μg) was fragmented in a Ribi cell fractionator at 50,000 psi to a mean molecular size of 3×10^5 daltons (Gelb *et al.*, 1971).

Unlabeled SV40 DNA was prepared from infected green monkey kidney cells by differential salt precipitation (Hirt, 1967), isopycnic centrifugation in CsCl containing ethidium bromide, and rate zonal sedimentation through a 5–30% (w/v) sucrose containing 0.1 M NaCl, 0.01 M Tris (pH 7.5), and 0.002 M EDTA in a SW41 rotor at 27,000 rpm for 16 hr at 10°.

Preparation of SV40 cRNA. SV40 cRNA was prepared *in vitro* using a SV40 DNA I template and *E. coli* RNA polymerase as previously described (Shih and Martin, 1973). The final RNA preparation was dialyzed against deionized water and was concentrated by vacuum dialysis in a collodion bag (Schleicher and Schuell, No. 100, mol wt cutoff of 25,000). ^{3}H-Labeled SV40 cRNA was synthesized in a reaction mixture containing [^{3}H]UTP (specific activity of 250 μCi/μmol). Low molecular weight RNA was removed from the final preparation with Sephadex G50 equilibrated with 0.1 M NaCl. The RNA in the excluded fraction was collected and dialyzed against distilled water. Its specific radioactivity was 1.7×10^5 cpm/μg.

Chemicals and Other Materials. Commercial high grade formamide (*e.g.*, reagent grade from Fisher Scientific Co., Fair Lawn, N. J.) was routinely purified with Amberlite MB-1 and Norit A (Tibbetts *et al.*, 1973) followed by crystallization (Robberson *et al.*, 1971). The purified formamide used in all experiments possessed an A_{270} = 0.175, and was not hydrolyzed following a 3-hr incubation at 90°. CMC and 1,1′-carbonyldiimidazole were obtained from Aldrich Chemical Co., Milwaukee, Wis. Pyridine and *N,N*-dimethylformamide (reagent grade, Matheson Coleman and Bell, Norwood, Ohio) were dehydrated by passage through a neutral alumina column and were stored over a molecular sieve (activated type 4A from J. T. Baker Chemical Co., Phillipsburg, N. J.). All other chemicals and solvents were obtained from ordinary commercial sources and were of reagent grade, if available.

Yeast RNA (highly polymerized, A grade) was purchased from Calbiochem, San Diego, Calif. *Micrococcus lysodeikticus* DNA was obtained from Sigma Chemical Co., St. Louis, Mo., and was sheared in Ribi Cell Fractionator as described above for SV40 DNA.

Analytic Methods. Radioactivity was determined with a Beckman liquid scintillation spectrometer employing a toluene based counting medium containing Triton X. The cellulose-bound [^{3}H]RNA was released by 1 N KOH hydrolysis (30 min in boiling water) or by 0.3 N KOH hydrolysis (18 hr at 37°). The bound [^{3}H]DNA was released by pancreatic DNase I digestion (0.3 mg/ml, 37°, overnight) or by hydrolysis with 10% trichloroacetic acid (60 min, 100°) or 1 N $HClO_4$ (10 min at 100°). Radioactivity was determined after neutralization with acid or alkali. As will be discussed in the Results section, none of the above procedures completely releases all of the chemically linked DNA or RNA for accurate counting without some degree of quenching. The concentration of SV40 DNA and RNA was determined by ultraviolet (uv) adsorption at 260 nm. A_{260} values of 20 and 24 (1 mg/ml) were used to calculate the concentrations of SV40 DNA and SV40 RNA, respectively. Optical thermal denaturation was performed in a Gilford recording spectrophotometer.

Results

Chemical Linkage of DNA to Neutral Cellulose Powder by the Carbodiimide Method. In double-stranded DNA, the bases in one strand are specifically hydrogen bonded to complemen-

TABLE I: CMC-Dependent Binding of ^{3}H-Labeled SV40 DNA Fragments to Neutral Cellulose Powder.

Expt	Conditions of Binding	Conditions of Washings	Amt of DNA[c] Applied (cpm)	% Radioact. Recovered in Washings	% Radioact. Recovered in Cellulose	Total
I	400 mg of cellulose powder in thin layer, 25 mg of CMC	PO_4–NH_3 (15%)–PO_4–$HCONH_2$ (50%)–H_2O (5 days, 22°)	440,000	22	44[b]	66
	Minus CMC, plus 25 mg of GuCl	Same	440,000	85	3[b]	88
IIa	150 mg of cellulose powder in thin layer, 15 mg of CMC	PO_4–H_2O (6 days, 4°)	293,000	28	34[a]	62
	Minus CMC	Same	293,000	90	12[a]	102
IIb	Same as IIa	PO_4–$HCONH_2$ (50%)–H_2O (6 days, 4°)	293,000	25	25[a]	50
	Minus CMC	Same	293,000	86	5[a]	91
IIc	Same as IIa	PO_4–$HCONH_2$ (100%)–H_2O (6 days, 4°)	293,000	26	31[a]	57
	Minus CMC	Same	293,000	82	7[a]	89
III	200 mg of cellulose powder 10 mg CMC	PO_4-H_2O-$HCONH_2$ (100%) (11 days, 4°)	190,000	67	17[a]	84
	Minus CMC	Same	190,000	105	4[a]	109

[a] Bound DNA was released by DNase I digestion for counting. [b] Bound DNA was released by 10% trichloroacetic acid hydrolysis for counting. [c] The specific radioactivity of sheared ^{3}H-labeled SV40 DNA was 1.15×10^5 cpm/μg.

tary counterparts on the other strand and are stacked in the core of a double helix. As a result, the functional groups of the bases (*e.g.*, the N-1 ring nitrogen of guanine and N-3 ring nitrogen of thymine), which are readily modified by CMC in slightly alkaline solution, are protected by the double helical structure (Kochetkov and Budowsky, 1969). This modification, however, can be suppressed by carrying out the reaction in Mes buffer at pH 6.0 (Naylor and Gilham, 1966). The 3′- or 5′-terminal monoester of phosphoric acid is the most readily activated group by nucleophilic substitution with CMC. Such an activated terminal phosphate could then be coupled with the nucleophilic hydroxyl group of cellulose. The coupling reaction is best carried out in the solid state with controlled moisture to effect high local concentrations of DNA on cellulose fibers and minimizing competition by H_2O (Gilham, 1968). A difficulty of this "dry-down" method of applying DNA to cellulose is that noncovalently linked DNA is very difficult to remove (Alberts and Herrick, 1971). For this reason, the chemical linkage of DNA to cellulose powder and the optimal conditions for removing the noncovalently linked DNA were studied in detail.

Since high molecular weight DNA adsorbs more strongly to cellulose than fragmented preparations, SV40 DNA was mechanically sheared in a Ribi Cell Fractionator to a molecular size of about 300,000 daltons (Gelb *et al.*, 1971). Table I shows CMC-dependent binding of ^{3}H-labeled SV40 DNA fragments to neutral cellulose powder. In experiment I, 4.4×10^5 cpm of sheared ^{3}H-labeled SV40 DNA (4 μg) in 0.2 ml of 0.015 M NaCl was mixed with 0.05 ml of Mes buffer (0.2 M, pH 6.0) and 25 mg of CMC. The mixture was applied to a thin layer (about 400 mg) of cellulose powder. In a control experiment, the CMC was replaced by an equivalent amount of guanidine-HCl (an inert quaternary ammonium salt similar to CMC). The cellulose powder was washed with 15% ammonia solution (w/v) (50% saturated NH_3 solution) at 37° for about 15 min as described in Materials and Methods to deactivate any residual CMC and to remove any modification of DNA bases. During this procedure the double-stranded DNA is denatured. Any remaining nonspecifically adsorbed DNA was removed by extensively washing with 50% formamide solution in 1 mM phosphate buffer (pH 7) for 2 days and in deionized water for 3 days. This procedure is accelerated if carried out at room temperature with gentle shaking. Experiment 1, Table I, indicates that 44% of the ^{3}H-labeled SV40 DNA bound to cellulose in the presence of CMC; only 3% of the input counts were retained in the absence of CMC. Table II shows the fate of ^{3}H-labeled SV40 DNA following its addition to cellulose powder. In the absence of CMC most of the adsorbed DNA was removed with the 15% NH_3 solution (Table II, experiments Ib and IIb). In the complete system (Table II, experiments Ia and IIa) only small amounts of the viral DNA are eluted at this step even though these conditions favor alkaline denaturation. This suggests that both DNA strands are linked to the cellulose.

Conditions to remove nonspecifically adsorbed DNA were evaluated in experiment II of Table I. After a brief wash with dilute phosphate (0.05 M, pH 7.0), the DNA cellulose was heated at 70° in water for 3 min. The cellulose powders were then subjected to extensive washings with distilled water (experiment IIa), 50% formamide (experiment IIb), or 100% formamide (experiment IIc) for 6 days with constant shaking and finally rinsed with distilled water. Only 5% of the input radioactivity remained attached to the cellulose powder following the 50% formamide wash (Table I, experiment IIb); distilled water elution (Table I, experiment IIa) was not as effective. Experiment III of Table I shows that application of the DNA reaction mixture directly to cellulose powder that is not spread out in a thin layer resulted in poorer efficiency of binding, presumably due to uneven application of the reaction mixture under these conditions.

These experiments indicate that DNA can be chemically immobilized on neutral cellulose powder. The percentage of input radioactivity retained on cellulose ranges from 30 to 45% when

TABLE II: Elution Pattern of Adsorbed DNA from Dried-Down Celluloses.[a]

Washing Conditions	% Input [^{3}H]DNA Eluted			
	Complete Reaction Mixt.		Minus CMC Plus GuCl as Control	
	Expt Ia	Expt IIa	Expt Ib	Expt IIb
(a) Sodium phosphate (0.05 M, pH 7.0), 4°, 20 min	0.4	3.1	2.9	9.0
(b) 15% NH_3 solution, 37°, 15 min	0.4	7.0	65.2	70.9
(c) Sodium phosphate (0.1 M, pH 5), 22°, 4 hr	1.2	2.6	9.9	8.5
(d) 50% formamide plus 1 mM phosphate (pH 7), 22°, 2 days	14.0	7.8	6.0	2.4
(e) Water, 22°, 3 days	5.7	2.0	1.2	1.5
(f) Total bound DNA after washings	44.3	42.9	2.9	5.4
Released by acid hydrolysis	41.5	38.3	2.7	4.6
Remained on cellulose residue	2.8	4.6	0.2	0.8
Total recovered radioact.	66.0	66.1	88.1	97.9

[a] In experiment I, 440,000 cpm of sheared ^{3}H-labeled SV40 DNA I (3.8 μg) was applied to 400 mg of cellulose thin layer in the presence or absence of CMC. In experiment II, 220,000 cpm of DNA (1.9 μg) was applied to 120 mg of cellulose. The [^{3}H]-DNA was eluted, in all cases, with a mechanical shaker. The radioactivity remaining attached to the cellulose was released by hydrolysis in 10% trichloroacetic acid (expt I) or 1 N $HClO_4$ (expt II), and the supernatant and residue were counted separately.

TABLE III: Chemical Linkage of ^{3}H-Labeled SV40 cRNA to Phosphocelluloses.[a]

Phosphocellulose	Amt Applied (cpm)	% Input Radioact. in All Washing Solutions	% Input Radioact. in Phosphocellulose
Cellex P	155,000 (27 μg)	ND[b]	2
Activated Cellex P	Same	ND	54
Activated Cellex P	Same	32	53
Whatman Powder P11	155,000 (27 μg)	ND	0.4
Activated P11	Same	ND	23
Activated P11	Same	81	19

[a] Bio-Rad Cellex P and Whatman P11 Cellulose Phosphate powder were washed, and the phosphate group was activated with 1,1′-carbonyldiimidazole as described under Materials and Methods. Portions (100 mg) of phosphocelluloses were used in the above experiments. Radioactivity of [^{3}H]RNA was determined after 1 N NaOH hydrolysis (boiling water for 30 min and neutralization with HCl). [b] Not determined.

the procedure outline in Table II is followed. The lower recovery of input radioactivity under conditions favoring extensive binding of SV40 DNA to cellulose powder (experiments Ia and IIa, Table II) may reflect quenching by cellulose and suggests that a larger proportion of the viral DNA becomes covalently attached. The true coupling efficiency may well be in the range of 70–78% as evaluated by the radioactivity recovered in all eluates. Large scale preparations of unlabeled DNA cellulose for use in the affinity chromatography system were always run in parallel with labeled DNA cellulose in order to monitor the efficiency of coupling and removal of nonspecifically adsorbed material.

Chemical Linkage of RNA to Phosphocellulose Powder by the Carbonyldiimidazole Method. Carbonyldiimidazole has been employed to link RNA to phosphocellulose filter disks by a two-step reaction (Saxinger *et al.*, 1972). The two-step process was chosen to minimize the possible modification of the bases of single-stranded RNA by first preparing activated phosphocellulose and then allowing RNA to react with the activated phosphocellulose. We have modified this procedure to covalently couple RNA to phosphocellulose powder suitable for affinity chromatography. The activated group in the phosphocellulose, the imidazolide of the phosphoric acid ester of cellulose, is stable at −20°. It must be stressed that small volumes (0.5–1.0 ml) of RNA must be applied to the phosphocellulose powder to ensure high local concentrations of nucleic acid and to avoid hydrolytic deactivation of phosphocellulose.

Table III shows the efficiency of coupling ^{3}H-labeled SV40 cRNA, synthesized *in vitro*, to phosphocellulose powder obtained from two commercial sources. The immobilization of the labeled RNA is dependent upon the activation of phosphocellulose by carbonyldiimidazole. Virtually none of the [^{3}H]RNA applied to the inactivated phosphocellulose powder, prepared under similar conditions with the omission of carbonyldiimidazole, was immobilized. The applied [^{3}H]RNA (54%) (or 68% as estimated from radioactivity recovered in the washings) was chemically linked to activated Cellex P obtained from Bio-Rad, and about 20% was immobilized onto activated Whatman Powder P11. Although the coupling efficiency was reproducible for a given activated phosphocellulose, considerable variation was noted from preparation to preparation. Immobilization of RNA on phosphocellulose is stable to the concentrated NH_3 employed during the washing procedure (15% NH_3 solution, 20 min, 4°). This is in contrast to RNA immobilized by the CMC method previously described (Shih and Martin, 1973) which is removed under alkaline conditions. This suggests that the RNA chain may be attached at multiple points through phosphoester bonds involving ribose hydroxyl groups rather than at a single terminal group as may be the case with the carbodiimide method. Cleavage of the RNA chain at a few sites would, therefore, not solubilize the linked RNA.

Isolation of Complementary RNA Sequences with DNA Cellulose Powder. The use of DNA cellulose powder, prepared by the carbodiimide method, for the purification of complementary RNA is shown in Figure 1. The chromatographic system employed is a modification of our previous system for the isolation of specific DNA sequences with RNA cellulose (Shih

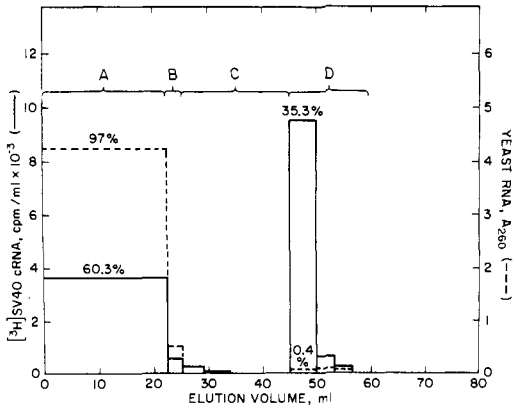

FIGURE 1: Isolation of ^{3}H-labeled SV40 cRNA in the presence of a large excess of yeast RNA using SV40 DNA cellulose. Cellulose powder (100 mg) containing about 10 μg of covalently linked fragmented SV40 DNA was packed in the reassociation column immediately after 30-sec exposure to 100° in formamide (pH 7). Approximately 1 μg of ^{3}H-labeled SV40 cRNA (151,000 cpm/μg) was mixed with 95 A_{260} units of unlabeled yeast RNA in 15 ml of circulant which was pumped through the reassociation column (37°) and a second reservoir column maintained at 4° at a flow rate of about 0.5 ml/min. After 27 hr of incubation, the circulant was removed and the entire system was purged with another 10 ml of fresh circulant mixture at 22° which was combined with the former and represented the first fraction in A. The column was further washed with 2.7 ml of 2 × SSC (B) and 20 ml of 0.2 × SSC (C) at 3°. The hybridized RNA was eluted with deionized water at 80° (D). The solid line indicates the elution profile of ^{3}H-labeled SV40 cRNA and the dashed line that of yeast RNA as determined by A_{260} in various fractions; 95% of both input radioactivity and A_{260} was recovered in all fractions.

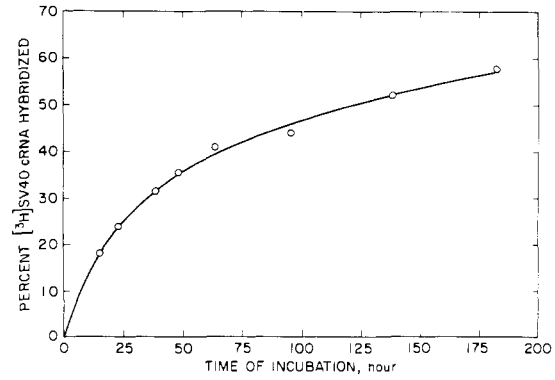

FIGURE 2: The time course of hybridization of ^{3}H-labeled SV40 cRNA to SV40 DNA cellulose. ^{3}H-Labeled SV40 cRNA (150,000 cpm, 0.9 μg), predenatured in formamide at 37° for 10 min, was mixed with 300 A_{260} units of yeast RNA in a circulant mixture of 30 ml. The solution was incubated with 200 mg of SV40 DNA cellulose powder containing about 20 μg of immobilized SV40 DNA as described in Figure 1. At the indicated times, 0.5-ml portions of circulant were withdrawn and assayed for radioactivity. The percentage of SV40 cRNA hybridized was determined from the fraction of radioactivity remaining in the circulant. At the conclusion of the experiment the column was eluted as described in Figure 1. The 65% of the ^{3}H-labeled SV40 cRNA recovered in the hybridized fraction was associated with only 0.3% of input unlabeled yeast RNA.

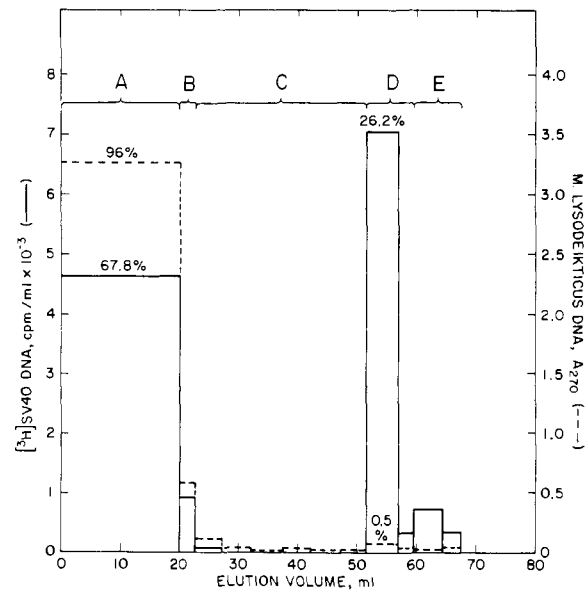

FIGURE 3: Isolation of ^{3}H-labeled SV40 DNA in the presence of a large excess of *M. lysodeikticus* DNA using SV40 cRNA phosphocellulose. SV40 cRNA Cellex P powder (100 mg), containing about 20 μg of covalently linked SV40 cRNA, was packed in the reassociation column. Sheared ^{3}H-labeled SV40 DNA I (137,000 cpm; 1.1 μg) was mixed with 60 A_{260} units of sheared *M. lysodeikticus* DNA (see text) in 15 ml of circulant mixture which was pumped through the reassociation column (37°), a reservoir column (room temperature), and a denaturation column (80°) at a flow rate of about 0.5 ml/min. Following a 24-hr incubation, the column was eluted as described in Figure 1. The solid line indicates distribution of ^{3}H-labeled SV40 DNA and the dashed line, that of *M. lysodeikticus* DNA, as measured by A_{270}. Total input of ^{3}H-labeled SV40 DNA (99%) was recovered in all fractions. Elution conditions are: (A) circulant mixture at 22°; (B) 2 × SSC at 4°; (C) 0.2 × SSC at 4°; (D) H_2O at 60°; (E) H_2O at 80°.

and Martin, 1973). The DNA cellulose is packed in a small jacketed column (0.6 × 5 cm) maintained at a temperature optimal for nucleic acid reassociation (*i.e.*, at 30 or 37° for a reaction mixture containing 0.3 M NaCl, 1 mM EDTA, 0.03 M sodium phosphate (pH 7.0), and 50% formamide (v/v)). Since RNA is single stranded, the denaturation column, previously described, was eliminated from the system. In order to minimize any degradation of the RNA during the prolonged period of incubation, the circulant solution not in contact with the DNA cellulose was maintained at 4° in a large jacketed column. In the experiment shown in Figure 1, 1 μg of ^{3}H-labeled SV40 cRNA was mixed with 3.2 mg of highly polymerized yeast RNA in a volume of approximately 15 ml. This circulant mixture was incubated for 27 hr in a continuous hybridization system containing about 10 μg of denatured SV40 DNA immobilized on cellulose. Most of the yeast RNA (97%) was recovered in the circulant mixture while about 35% of the labeled SV40 cRNA remained on the SV40 DNA cellulose. This [^{3}H]RNA could be eluted by dissociating the RNA–DNA hybrid in a small volume of deionized water suggesting a substantial purification of the viral RNA from the unrelated yeast RNA. The elution of the ^{3}H-labeled SV40 cRNA was almost quantitative even after a brief exposure (2–5 min) of the column to 80 or 60°; at 37°, 83% of the hybridized RNA was recovered.

The time course of the reaction of labeled SV40 cRNA with DNA cellulose is shown in Figure 2. Since the RNA not hybridized with immobilized DNA remains in the circulant mixture, aliquots were removed at the various times to assess the extent of the reaction. It can be seen in Figure 2 that the hybridization reaction was incomplete even after an incubation period of 183 hr. This is in contrast with the results obtained with RNA cellulose (see below and Figure 4) where nearly all of the complementary DNA was removed from the circulatory buffer. The 60–65% of the labeled SV40 cRNA eluted from the DNA cellulose contained less than 0.07% of the yeast RNA originally added to the system. Thus, the cellulose powder containing covalently linked DNA selectively retains complementary RNA in the presence of a large excess of an unrelated RNA. Although immobilized DNA was prepared and stored in denatured conditions, to avoid any reannealing before use, the DNA

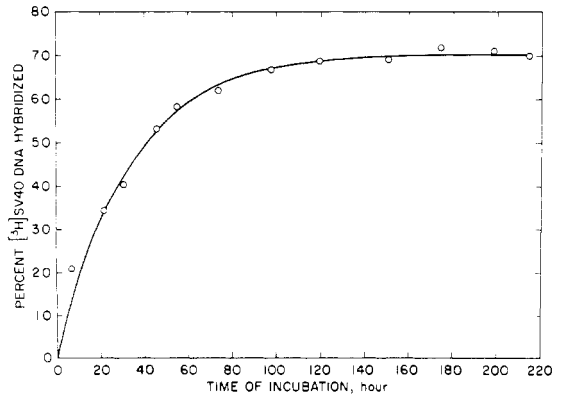

FIGURE 4: The time course of hybridization of ^{3}H-labeled SV40 DNA fragments to SV40 cRNA phosphocellulose. Fragmented ^{3}H-labeled SV40 DNA (318,000 cpm, 2.6 μg) was mixed with 60 A_{260} units of sheared *M. lysodeikticus* DNA in a circulant mixture of 15 ml. The solution was incubated in the system described in Figure 3 with 150 mg of SV40 cRNA Cellex P powder containing approximately 30 μg of immobilized SV40 cRNA. At the indicated times, duplicate aliquots (0.1 ml of circulant) were withdrawn for counting. The percentage of ^{3}H-labeled SV40 DNA fragments hybridized was determined as described in Figure 2. At the end of the incubation, the column was eluted as described in Figure 3. Radioactivity (75%) of ^{3}H-labeled SV40 DNA was recovered in the hybridized fraction in association with 0.4% of the input *M. lysodeikticus* DNA.

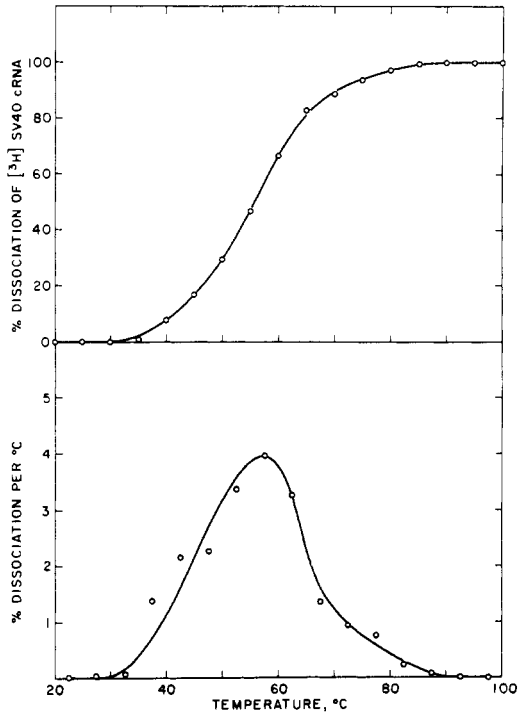

FIGURE 5: The melting profile of ^{3}H-labeled SV40 cRNA hybridized to SV40 DNA cellulose. Predenatured ^{3}H-labeled SV40 cRNA (240,000 cpm, 1.4 μg), in a circulant mixture of 30 ml, was incubated at 30° with 100 mg of SV40 DNA I cellulose containing about 10 μg of immobilized fragmented SV40 DNA as described in Figure 1. After 167 hr of incubation (at which point 64% of ^{3}H-labeled SV40 cRNA had hybridized), the column was washed with 20 ml of fresh circulant at 20° until no residual radioactivity could be eluted. The column was then exposed to 5-ml aliquots of fresh circulant (50% formamide (v/v), 0.3 M NaCl, 0.03 M sodium phosphate, and 1 mM EDTA (pH 7.0)) and the temperature was raised by 5° increments. The temperature was maintained at the indicated temperature for 6 min at which time the circulant was removed. The percentage of total recovered radioactivity in each fraction was divided by five and plotted as a function of the midpoint of each temperature increment (bottom). The accumulated percentage of total recovered radioactivity at each temperature increment is presented in the top panel.

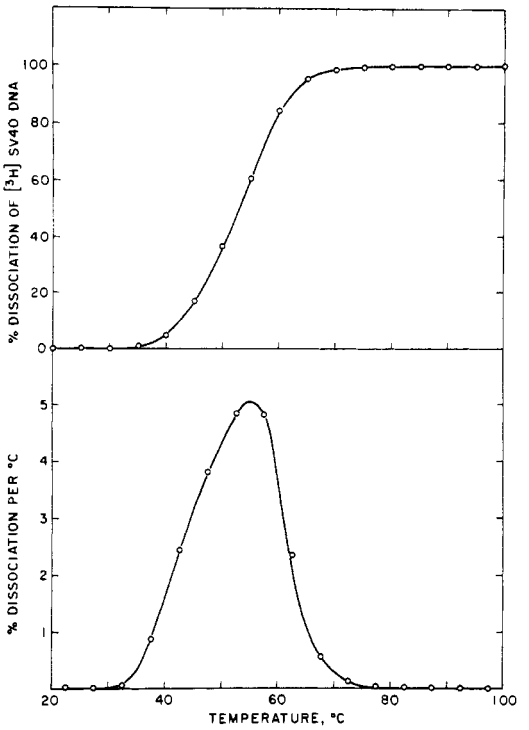

FIGURE 6: The melting profile of ^{3}H-labeled SV40 DNA hybridized to SV40 cRNA phosphocellulose. Fragmented ^{3}H-labeled SV40 DNA (276,000 cpm, 2.3 μg) was mixed with 2.4 mg of sheared *M. lysodeikticus* DNA in a circulant mixture of 30 ml. SV40 cRNA P11 powder (300 mg) containing about 21 μg of immobilized SV40 cRNA was packed in a reassociation column maintained at 30° as described in Figure 3. the temperature of the denaturation column was also lowered to 70°. After 210 hr of incubation (at which point 75% of ^{3}H-labeled SV40 DNA fragments had hybridized), the column was washed with 20 ml of fresh circulant mixture at 20° until no residual radioactivity could be recovered. The column was then eluted with 5-ml portions of circulant mixture and the data were presented as described in Figure 5.

cellulose was often denatured again in 100% formamide (pH 7) (2 min at 50° or 30 sec at 100°) immediately prior to being packed in the column. However, no appreciable difference in its capacity to bind cRNA was observed in our hands.

Isolation of Complementary DNA with RNA Phosphocellulose Powder. In the experiment shown in Figure 3, RNA, chemically linked to phosphocellulose powder, was used to isolate complementary DNA in the presence of a large excess of unrelated DNA. The continuous hybridization system employed is similar to that previously described (Shih and Martin, 1973); a reservoir column, connected in series between the reassociation column and the denaturation column, has been added in order to accommodate the large volume of circulant. In this experiment, 1 μg of ^{3}H-labeled SV40 DNA and 3 mg of *M. lysodeikticus* DNA, similarly sheared to a molecular weight of 3×10^5 daltons, were incubated for 24 hr in a chromatographic system which contained about 20 μg of SV40 cRNA immobilized on phosphocellulose powder. After 24 hr of incubation nearly all of the unlabeled *M. lysodeikticus* DNA was still present in the circulant mixture, while about 30% of the input ^{3}H-labeled SV40 DNA had bound to the SV40 cRNA phosphocellulose. Figure 4 shows the time course of the reaction of sheared ^{3}H-labeled SV40 DNA with SV40 cRNA immobilized on phosphocellulose powder under conditions of continuous hybridization. In contrast to the results obtained with DNA cellulose (Figure 2) the reaction of labeled DNA with RNA phosphocellulose is essentially complete (65–70%) in 120 hr. It has been previously shown that the supercoiled form of SV40 is asymmetrically transcribed *in vitro* (West-

phal, 1970). The RNA synthesized is complementary to one of the two viral DNA strands (Westphal, 1970; Khoury and Martin, 1972). The higher value we observed in this experiment may reflect the presence of small amounts of symmetrically transcribed SV40 cRNA which reacts with sequences on both strands of viral DNA. Similar reaction kinetics were observed when the reassociation column was maintained at 30 or 37°, and the denaturation column at 70, 80, or 90°. In this regard, SV40 DNA (42% G + C content) has a T_m of 57° in the circulant mixture and is completely denatured at 70°.

Thermal Stability of Labeled DNA or RNA Hybridized to Nucleic Acids Immobilized on Cellulose. The thermal dissociation profiles of SV40 cRNA retained by SV40 DNA cellulose or of labeled SV40 DNA bound to SV40 cRNA phosphocellulose are shown in Figures 5 and 6, respectively. Denaturation was carried out in 0.3 M NaCl, 0.03 M sodium phosphate (pH 7.0), 1 mM EDTA, and 50% (v/v) formamide. The melting profiles of labeled SV40 RNA and DNA from the cellulose columns indicate that a cooperative structural transition occurs characteristic of the dissociation of a nucleic acid duplex rather than the dissociation of material nonspecifically adsorbed to the matrix. The product of the reaction between labeled SV40 cRNA and SV40 DNA cellulose had a T_m of 57° as determined from the derivative curve (56° for 50% dissociation) (Figure 5, bottom). A similar analysis of ^{3}H-labeled SV40 DNA bound to SV40 cRNA cellulose indicated a T_m of 55° (53° for 50% dissociation). The T_m values of mouse cellular and calf thymus DNAs, both of which have similar G + C contents as SV40 (42%), were 57 and 59°, respectively, when thermal denaturation was monitored optically under similar experimental conditions. These results suggest that the selective retention of labeled viral DNA or RNA by complementary nucleic acid sequences chemically linked to cellulose involves the formation of base-paired hybrid structures.

Discussion

The advantage of carrying out RNA–DNA hybridization reactions in a closed circulatory system as opposed to the commonly employed static hybridization systems is that competing or undesirable side reactions are physically separated. For example, any DNA–DNA reassociation that occurs during the hybridization can be eliminated during passage through the denaturation column. Upon its reentry into the renaturation column, the DNA, now in the single-stranded form, is again capable of reacting with the immobilized RNA. The degradation of RNA during the prolonged incubation with DNA cellulose can be greatly reduced by maintaining the temperature of the reaction mixture circulating external to the DNA cellulose at 4°. Theoretically, the interaction of nucleic acid in the continuous hybridization system should have reaction kinetics similar to that observed in a static system. Thus this technique, coupled with the use of formamide to lower the incubation temperature, should facilitate the isolation of specific RNA species. The large capacity of the DNA or RNA cellulose columns described above should expedite the preparation of specific DNA or RNA sequences in quantities sufficient for detailed biochemical and biophysical studies.

The choice of a suitable solid matrix for the chromatographic system outlined above is of critical importance. Well-cut natural cellulose powder and its derivatives have better flow-through properties and bind lower amounts of nucleic acid nonspecifically than the pulped chromatography paper we previously used (Shih and Martin, 1973). Cellulose powder, which is stable to both organic solvents and extreme temperatures, would appear to be superior to agarose gels as the matrix. The DNA or RNA immobilized on cellulose is apparently free to interact with complementary sequences in the mobile phase; a gel matrix imposes an additional permeability barrier. Although denatured DNA or mRNA containing poly(A) stretches exhibits strong affinity for cellulose preparations with lignin-like impurities or possessing a microcrystalline structure (Kitos *et al.*, 1972; DeLarco and Guroff, 1973; Larsen *et al.*, 1973; Sullivan and Roberts, 1973), nonspecific binding can be reduced by using highly purified fibrous cellulose powder and extensively washing of the column with a low ionic strength solution (*e.g.*, 0.2 or 0.1 × SSC) at 4° prior to the elution of the retained hybrids. Due to the charge repulsion at low ionic strength, the additional phosphate anions present in phosphocellulose may help in reducing the nonspecific adsorption of polyanionic DNA or RNA to the solid supporting medium.

Although double-stranded DNA fragments can be readily linked to the neutral cellulose powder by the carbodiimide method, enzymatically synthesized RNAs (*e.g.*, SV40 cRNA), with their less nucleophilic triphosphate termini, are more difficult to directly couple to cellulose, especially in the powder form. For natural RNAs, 3′-terminal phosphate groups, generated by partial hydrolytic cleavage during preparation, are rapidly cyclized by carbodiimide and are not linked to cellulose. Therefore, we have employed the two-step carbonyldiimidazole procedure, which does not depend upon the terminal groups of RNA chains, for the immobilization of RNA. The likely generation of multipoint linkages by this method secures the RNA chain to the cellulose during the subsequent prolonged RNA–DNA hybridization reactions.

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Effect of pH on the Reactivity of the Active-Site Sulfhydryl Groups in Yeast Alcohol Dehydrogenase†

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ABSTRACT: The relative reactivity of the two active-site sulfhydryl groups (designated X and Y) in yeast alcohol dehydrogenase is determined by both the nature of the reagent used and the pH at which the reaction is carried out. Iodoacetate modifies 30% of the X-sulfhydryl and 70% of the Y-sulfhydryl at pH 5.7, but is specific for the Y-sulfhydryl at pH 7.5. For the uncharged reagents butyl isocyanate and iodoacetamide the pH effect is less pronounced. The former reagent modifies 55–60% of the X-sulfhydryl and 40–45% of the Y-sulfhydryl at both pH 5.7 and 6.5, and the latter reagent modifies about 40% of the X-sulfhydryl and 60% of the Y-sulfhydryl at pH 5.7 and 6.5 and 25% of X-sulfhydryl and 75% of the Y-sulfhydryl at pH 7.5. The Y-sulfhydryl has now been identified as Cys-43 in the primary sequence of yeast alcohol dehydrogenase (Jörnvall, H. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 2295), but the location of the X-sulfhydryl is still unknown.

Evidence has been presented in previous publications that yeast alcohol dehydrogenase contains two distinct active-site sulfhydryl groups, both of which appear to be required for catalytic activity. One of the sulfhydryl groups (designated X) reacts preferentially with butyl isocyanate at pH 5.7 (Twu and Wold, 1973), and the other (designated Y) reacts exclusively with iodoacetate at pH 7.5 (Harris, 1964). Both sulfhydryl groups react with iodoacetamide at pH 6.5 (Twu *et al.*, 1973). With all three reagents an inactive enzyme derivative is obtained when one sulfhydryl per active site has been modified, and it can be demonstrated that the specific reactivity of either one sulfhydryl group is lost when the other reacts. It was recognized in a previous report (Twu *et al.*, 1973) that the observed difference in the reactivity of the two sulfhydryl groups could reflect either subtle differences in the reaction specificity of iodoacetate, iodoacetamide, and butyl isocyanate, or effects of pH on the relative reactivity of the two sulfhydryl groups toward all three reagents. The purpose of the work reported in the present paper was to try to distinguish between these two possibilities.

Experimental Section

Materials. Yeast alcohol dehydrogenase (crystallized and lyophilized; 400 units/mg) was purchased from Sigma Chemical Co. and was of the same quality as the preparations used in previous work in our laboratory (Twu and Wold, 1973; Twu *et al.*, 1973). [^{14}C]Butyl isocyanate (labeled in the carbonyl carbon, 3.25 Ci/mol), [1-^{14}C]iodoacetamide (12.2 Ci/mol), [1-^{14}C]iodoacetic acid (13.45 Ci/mol), and [^{3}H]iodoacetic acid (67.2 and 357 Ci/mol) were obtained from New England Nuclear. If the radioactive samples were diluted before use, the specific radioactivity of the final reagent sample was determined by preparing the corresponding *S*-cysteine derivative and relating the radioactive counts directly to micromolar concentration of derivative as determined on the long column of the amino acid analyzer.

Assays and Methods. The procedures for enzyme assays, protein assays, and amino acid analyses, as well as the determination of radioactivity, have been described in a previous paper (Twu and Wold, 1973). The modification of dehydrogenase with butyl isocyanate, iodoacetate, and iodoacetamide also followed previously described methods (Twu and Wold, 1973; Twu *et al.*, 1973; Whitehead and Rabin, 1964; Rabin *et al.*, 1964). In our hands, the specificity of the inactivation with the alkylating reagents decreased significantly at low pH, and complete inactivation was often associated with incorporation of more than the expected 3–4 mol of reagent/mol of protein.

Results

To establish whether the two different sulfhydryl groups react to a different extent at different pH, it was first required to ascertain that the same two sulfhydryl groups are involved in the reaction over the whole pH range. To this end two samples of 100 mg each of yeast alcohol dehydrogenase in 20 ml of 0.1 M potassium phosphate buffer, one at pH 5.7 and one at pH 7.5, were treated in parallel with 7 mol/mol of enzyme of [^{14}C]iodoacetate and [^{3}H]iodoacetate, respectively. The course of the inactivation was checked carefully to avoid overreaction. When the activity had dropped to below 10%, the samples were subjected to gel filtration to remove excess reagent and after

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