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Sulfhydrylcellulose: A New Medium for Chromatography of Mercurated Polynucleotides†

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ABSTRACT: We have synthesized a new medium, sulfhydrylcellulose, for affinity chromatography of mercurated polynucleotides. It is the product of reaction between aminoethylcellulose and *N*-acetylhomocysteine thiolactone. Sulfhydrylcellulose carries up to 90 μmol of SH groups/g and is inexpensive, easy to prepare, and stable. Because it binds mercurated RNA specifically and reversibly and exhibits no size discrimination, sulfhydrylcellulose should have wide applications.

Many investigators have used mercurated nucleotide precursors for in vitro transcription of eukaryotic or viral chromatin by either endogenous or exogenous RNA polymerases (Konkel & Ingram, 1977; Mory & Gefter, 1978; Smith & Huang, 1976; Zasloff & Felsenfeld, 1977). To facilitate the separation of mercurated polynucleotides and large complexes from nonmercurated products, we have synthesized a new affinity chromatography medium, sulfhydrylcellulose. Although other sulfhydryl chromatography media are available, they are expensive if purchased or require lengthy preparation if made in the laboratory. Moreover, they carry few sulfhydryl groups per gram of solid support. In contrast, sulfhydrylcellulose is an inexpensive (about 2% of the cost of currently available commercial media), efficient chromatography medium that should be useful for a wide range of investigations that involve mercurated nucleotides or nucleic acids.

We have found sulfhydrylcellulose to be superior to other sulfhydryl media for chromatography of polynucleotides. We can obtain a sulfhydryl activity on cellulose of up to 90 μmol of sulfhydryl groups (SH) per g, in contrast to the 1–6 μmol of SH per g of solid support on currently available media. Sulfhydrylcellulose can be synthesized in 2 h by a procedure similar to but much simpler than that for the preparation of sulfhydryl-Sepharose (Cuatrecasas & Anfinsen, 1971), and it can be readily adapted to a "batch" procedure as well as to column chromatography. Most important is the fact that we have observed neither irreversible binding of mercurated RNA nor nonspecific binding of nonmercurated RNA to sulfhydrylcellulose. We have frequently had these problems with sulfhydryl-Sepharose made in our laboratory or with commercially available sulfhydryl-Agarose.

Fibrous sulfhydrylcellulose should be especially useful for the purification of large mercurated molecules or complexes because it exhibits no size discrimination. In contrast, sulfhydryl media derived from Sepharose, Agarose, or controlled pore glass consist of porous particles. Since most of the surface area, and therefore most of the active sulfhydryl groups, is inside these particles, mercurated molecules must be small enough to penetrate the pores of the matrix in order to bind efficiently.

Experimental Procedures

Preparation of Sulfhydrylcellulose. Our procedure for synthesis of sulfhydrylcellulose involves mixing aminoethylcellulose (Sigma, 0.29 mequiv/g) with a solution of *N*-acetylhomocysteine thiolactone (AHT,[1] ICN Biochemicals) in 0.5 M $NaHCO_3$, adjusted to pH 9.7 with NaOH, stirring the suspension at room temperature, and washing the cellulose with about 60 bed volumes of 0.1 M NaCl. One gram of aminoethylcellulose produces about 6 mL of sulfhydrylcellulose (packed volume). To obtain high specific activity sulfhydrylcellulose (up to 90 μmol of SH per g of aminoethylcellulose), we react an 8% (w/v) suspension of aminoethylcellulose with AHT at a ratio of 1 g of aminoethylcellulose to 1.2 g of AHT for 3 h. Because we varied the incubation time and concentrations of the reagents to characterize the reaction, we present details of the preparation under Results and Discussion.

To determine the concentration of active sulfhydryl groups on the product, we react sulfhydrylcellulose with Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid), in 0.03 M potassium phosphate buffer, pH 8.0] (Ellman, 1959). Briefly, we add an aliquot of a sulfhydrylcellulose suspension to 0.8 mL of 0.03 M potassium phosphate buffer, pH 8.0, adjust the volume to 2.0 mL with distilled water, add 20 μL of 10 mM Ellman's reagent, and incubate the mixture for 15 min at room temperature. We then remove the sulfhydrylcellulose by centrifugation and determine the absorbance at 412 nm of the

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[1] Abbreviations used: AHT, *N*-acetylhomocysteine thiolactone; $NaDodSO_4$, sodium dodecyl sulfate; NP40, Nonidet P40.

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supernatant fluid. We calculate the molar concentration of SH groups per gram of the aminoethylcellulose used for the reaction from the following relationship: [SH]/g = (A_{412}/13 600)/g of cellulose (Ellman, 1959).

Preparation of Mercurated CTP. Before mercurating CTP (Dale et al., 1975), we purify it by ion-exchange column chromatography on DE-52 (Whatman) equilibrated with 0.01 M sodium acetate, pH 6.0. After loading the sample (6 mg/mL in 0.01 M sodium acetate, pH 6.0), we wash the column sequentially with 0.01 M sodium acetate, pH 6.0, and with 0.05 M sodium acetate, pH 6.0, and then elute the nucleoside triphosphate with 0.4 M sodium acetate, pH 6.0. To the solution of purified nucleotide, we add a volume of 1 M mercuric acetate (in 0.01 M sodium acetate, pH 6.0) that yields a 5:1 molar ratio of mercuric acetate to nucleotide, incubate the mixture at 50 °C for 90 min, dilute the sodium acetate to 0.01 M with distilled water, and load the sample onto a fresh column of DE-52. After washing the column exhaustively with 0.01 M sodium acetate, pH 6.0, to remove unreacted mercuric ions, we elute the mercurated nucleotide with 0.5 M NaCl and precipitate it by adding three volumes of ethanol. When stored at −20 °C as an ethanol precipitate, HgCTP is stable for at least 1 year. As evidenced by binding to sulfhydryl-Agarose (Agthiol, P-L Biochemicals), at least 98% of the nucleotide in each of our preparations was mercurated.

Mercurated and Nonmercurated RNA. We synthesize RNA complementary to SV40 DNA (cRNA) as described by Lindstrom & Dulbecco (1972). For nonmercurated RNA, our reaction mixtures consist of 0.4 M Tris-HCl, pH 7.9, 0.15 M KCl, 0.01 M $MgCl_2$, 0.5 mM ATP, 1 mM GTP, 1 mM CTP, 0.1 mM dithiothreitol, 0.04 mM [5,6-^{3}H]UTP (28 Ci/mmol) or 0.04 mM [α-^{32}P]UTP (50 Ci/mmol), 30 μg/mL SV40 DNA I, and 30 units/mL *E. coli* RNA polymerase (Miles Biochemicals). We prepare mercurated cRNA (Hg-cRNA) in the same way except that we substitute HgCTP (1.6 mM) for CTP and 2-mercaptoethanol (12 mM) for dithiothreitol. After incubating the reaction mixtures at 37 °C for 2.5 h, we chromatograph them on Sephadex G-50-80 columns equilibrated with 0.1 M sodium acetate, pH 6.0, 20 mM EDTA, and 0.05% $NaDodSO_4$, pool the fractions of the excluded volume that contain RNA, and store the RNA at −20 °C as an ethanol precipitate.

Sulfhydrylcellulose Chromatography of RNA. For analytical chromatography, we routinely use a column of 1.0-mL bed volume in a 3.0-mL plastic syringe. After activating the sulfhydrylcellulose with 5 mL of 0.5 M 2-mercaptoethanol and 0.1 M NaCl, we wash it with 20 mL of 0.1 M NaCl. We generally load a sample in 0.2 mL of 0.1 M NaCl, mix it with the cellulose, and after 5 min collect the flow-through and reload it onto the column. To remove unbound RNA, we wash the column with 0.5 M NaCl, 20 mM EDTA, and 0.05 M Tris-HCl, pH 7.2. Finally, we elute the bound RNA with 0.5 M NaI, 20 mM EDTA, 0.5 M cysteine, and 0.1 M Tris-HCl, pH 7.2. At any step, the column can be allowed to run dry for brief periods. We collect 0.15-mL fractions at each step and monitor the presence of RNA by measuring either the absorbance at 260 nm or the radioactivity.

Under Results and Discussion, we describe other suitable buffers for the wash and elution steps.

Results and Discussion

Preparation of Sulfhydrylcellulose. To prepare sulfhydrylcellulose, we react *N*-acetylhomocysteine thiolactone (AHT) with aminoethylcellulose. To optimize the extent of addition of AHT to amino groups on the aminoethylcellulose,

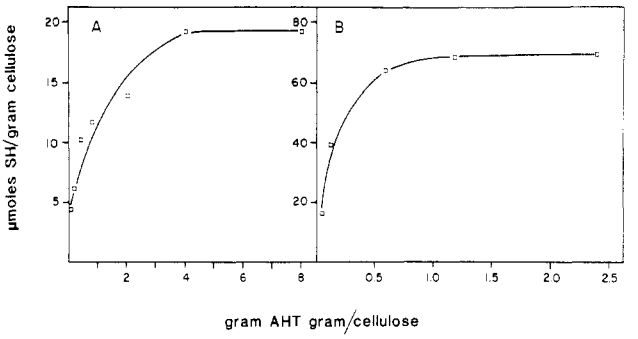

FIGURE 1: Effect of concentrations of aminoethylcellulose and *N*-acetylhomocysteine thiolactone on the capacity of the sulfhydryl-cellulose product.

Table I: Effect of Reaction Medium on the Addition of AHT to Aminoethylcellulose

reaction medium	pH	μmol of SH/g of cellulose
0.5 M $NaHCO_3$	9.7	80
1 M $NaHCO_3$	9.7	80
0.1 M borate buffer	9.7	74
0.5 M Tris–glycine buffer	9.7	72
0.2 M borate buffer	9.0	50
0.5 M Tris-HCl buffer	7.0	45
0.1 M sodium acetate buffer	4.0	12

we investigated the dependence of the reaction on the concentrations of both aminoethylcellulose and AHT, the reaction time, and the composition of the reaction medium.

The reaction between AHT and aminoethylcellulose depends on both the concentration of aminoethylcellulose and the ratio of AHT to the cellulose. In two experiments, we kept the concentration of aminoethylcellulose constant at either 5% or 8% (w/v), used a 3-h reaction time, and varied the ratio (w/w) of AHT to aminoethylcellulose. We measured the extent of the reaction by incubating the product, sulfhydrylcellulose, with Ellman's reagent, as described under Experimental Procedures. In both experiments the moles of SH per gram increased with increasing ratio of AHT to cellulose. In these experiments the maximum number of micromoles of SH per gram of cellulose was 18 with the 5% suspension of aminoethylcellulose (Figure 1A) whereas with the 8% suspension the reaction plateaued at 70 μmol of SH/g (Figure 1B).

We found the reaction of AHT with aminoethylcellulose to be quite rapid. We mixed an 8% (w/v) suspension of aminoethylcellulose with AHT at a ratio of AHT to cellulose of 1.2:1 and removed aliquots at various time intervals for analysis by the Ellman assay. After 1 h of incubation, the reaction had plateaued at 70 μmol of SH/g, and there was no further increase during an additional 24 h of incubation.

We also investigated the dependence of the reaction on the composition of the reaction medium. In this experiment, we used an 8% suspension of aminoethylcellulose, a ratio of AHT to aminoethylcellulose of 1.2:1, and a 4-h incubation time. The reaction appears to depend on pH and not on ionic strength (Table I). The best reaction media that we used were 0.5 M $NaHCO_3$, pH 9.7, and 1 M $NaHCO_3$, pH 9.7.

We have obtained sulfhydrylcellulose that carries up to 90 μmol of SH/g. This value corresponds to the addition of AHT to approximately 30% of the available amino groups on the aminoethylcellulose (0.29 mequiv/g, according the manufacturer). With more highly substituted aminoethylcellulose as starting material, it should be possible to obtain sulfhydryl-cellulose with even higher SH activity.

Stability of Sulfhydrylcellulose. When stored at 4 °C in 0.1 M NaCl, sulfhydrylcellulose suffers time-dependent loss of active thiol groups as monitored by Ellman's assay. For example, the sulfhydryl concentration falls to 50% of the original value after 1 week of storage. This loss is almost entirely a result of oxidation of the SH groups because treatment with 0.5 M 2-mercaptoethanol largely restores the sulfhydryl activity. Even after 1 month of storage, the sulfhydryl activity can be restored to about 90% of the original value.

Sulfhydrylcellulose is stable to treatment with 0.1 N NaOH: neither the SH activity nor the ability of the cellulose to bind mercurated RNA is affected. Therefore, columns of sulfhydrylcellulose can be pretreated with alkali to remove nucleases that may have been introduced during the preparation of the sulfhydrylcellulose.

Though we have found that sulfhydrylcellulose can be reused for experiments that involve the separation of mercurated RNA from nonmercurated nucleic acids, we generally do not recycle it because it is so inexpensive, easy to prepare, and stable.

Separation of Mercurated RNA from Nonmercurated RNA on Sulfhydrylcellulose. So that it may be a useful tool for separating mercurated RNA from nonmercurated nucleic acids, sulfhydrylcellulose must satisfy three requirements. It must bind mercurated RNA. Under the same experimental conditions, it must *not* bind nonmercurated RNA. Furthermore, it must bind mercurated RNA reversibly so that the RNA can be recovered. In developing sulfhydrylcellulose chromatography, we varied experimental parameters in order to find conditions that would satisfy these requirements.

Our first consideration was to determine conditions under which nonmercurated RNA can be completely removed from a column of sulfhydrylcellulose before an elution buffer containing a reducing agent is applied. We loaded samples of radiolabeled nonmercurated RNA in 0.1 M NaCl onto columns and found the most effective means for overcoming nonspecific interactions to be simply raising the salt concentration to 0.5 M NaCl and including 20 mM EDTA and 0.05 M Tris-HCl, pH 7.2, in the wash buffer. In addition, pretreatment of the column with 2-mercaptoethanol not only activates the SH groups on sulfhydrylcellulose but also reduces nonspecific binding of nonmercurated RNA. Using a column pretreated with 2-mercaptoethanol and the high-salt buffer, we routinely are able to remove at least 97% of the nonmercurated RNA in two column bed volumes.

Another requirement for sulfhydrylcellulose is that it bind mercurated RNA under the same conditions in which it fails to bind nonmercurated RNA. We found that ^{3}H-labeled Hg-cRNA readily binds to sulfhydrylcellulose when loaded in 0.1–0.3 M NaCl and that it remains bound under the wash conditions listed above.

To test the versatility of sulfhydrylcellulose, we also tested its binding capacity for mercurated RNA under a variety of other wash conditions that might be desirable for disrupting either aggregates of RNA (Konkel & Ingram, 1977; Yang et al., 1980) or protein–RNA complexes. Mercurated RNA remains bound to the column when detergents ($NaDodSO_4$, Sarkosyl, NP40), formamide, or dimethyl sulfoxide are included in the wash buffer. One of our reasons for developing sulfhydrylcellulose chromatography was to have a medium capable of binding large complexes that contain mercurated RNA. Preliminary results in our laboratory indicate that sulfhydrylcellulose indeed binds transcriptional complexes comprised of *E. coli* RNA polymerase, SV40 DNA, and nascent mercurated RNA (F. Haynes, unpublished data).

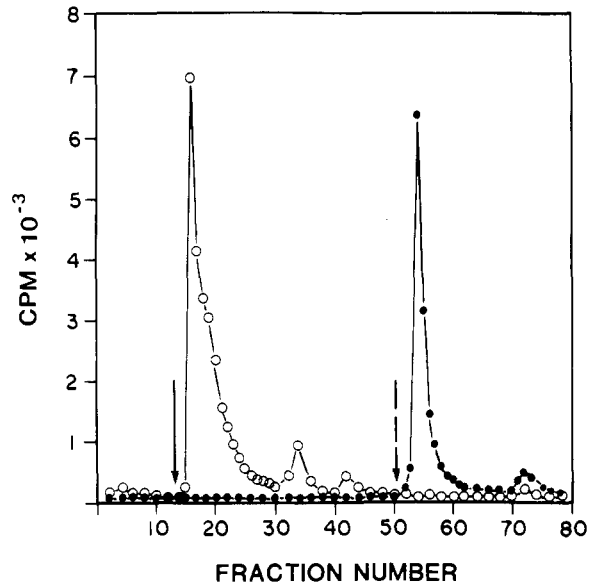

FIGURE 2: Separation of mercurated RNA from nonmercurated RNA by chromatography on sulfhydrylcellulose. A mixture of ^{3}H-labeled cRNA and ^{32}P-labeled Hg-cRNA was chromatographed as described in the text. (○) ^{3}H-Labeled, nonmercurated RNA; (●) ^{32}P-labeled, mercurated RNA.

Finally, mercurated RNA must bind reversibly to sulfhydrylcellulose. The addition of 0.5 M 2-mercaptoethanol to the wash buffer is sufficient to elute ^{3}H-labeled Hg-cRNA from sulfhydrylcellulose. However, sharper profiles result when cysteine is used in place of 2-mercaptoethanol and sodium iodide in place of sodium chloride. Therefore, for analytical columns, we routinely use the following elution buffer: 0.5 M NaI, 20 mM EDTA, 0.5 M cysteine, and 0.1 M Tris-HCl, pH 7.2. For preparative columns, we use 0.5 M 2-mercaptoethanol (in spite of its more offensive odor!) because we have encountered several problems with cysteine. Solutions of 0.5 M cysteine must be prepared shortly before use because of rapid oxidation to cystine, which has a low solubility, and, for the same reason, cysteine is difficult to remove from eluted samples. Moreover, mercurated RNA eluted with cysteine fails to rebind to sulfhydrylcellulose, thus precluding two cycles of chromatography. In contrast, RNA eluted with 2-mercaptoethanol not only can be rapidly separated from the reducing agent by ethanol precipitation but also will rebind to sulfhydrylcellulose once free 2-mercaptoethanol has been removed. We have not determined whether cysteine causes more rapid demercuration of RNA than 2-mercaptoethanol or whether cysteine as a counterligand of the mercury residues simply cannot be replaced by the SH groups on a second column.

Figure 2 illustrates a typical separation of mercurated RNA from nonmercurated RNA on sulfhydrylcellulose. We pretreated the sulfhydrylcellulose with 0.5 M 2-mercaptoethanol, washed the column with 20 bed volumes of 0.1 M NaCl, and loaded a mixture of ^{32}P-labeled Hg-cRNA (20 000 cpm) and ^{3}H-labeled cRNA (32 000 cpm) in 0.1 M NaCl. A peak of ^{3}H radioactivity, corresponding to nonmercurated RNA, is removed by wash buffer (the solid arrow indicates the addition of this buffer), and a peak of ^{32}P radioactivity, corresponding to mercurated RNA, is eluted after the addition of reducing agent (dashed arrow indicates the addition of "elution buffer"). No nonmercurated RNA elutes with the mercurated RNA. Less than 5% of the input radioactivity remained bound to the sulfhydrylcellulose.

Sulfhydrylcellulose readily adapts to a "batch" technique for separating mercurated RNA from nonmercurated RNA.

In a typical experiment, we added a mixture of ^{3}H-labeled cRNA and ^{32}P-labeled Hg-cRNA in 0.2 mL of 0.1 M NaCl to 0.5 mL of sulfhydrylcellulose (packed volume) in a 1.5-mL microfuge tube. We agitated the sample with a vortex mixer for 15 s, then centrifuged it for 30 s in a microfuge, removed the supernatant fluid, and analyzed it for radioactivity. To the pellet we added 1 mL of 0.1 M NaCl and repeated the process until the level of radioactivity in the 0.1 M NaCl wash was negligible. We then repeated the procedure with wash buffer and finally with elution buffer.

This method produced an elution profile identical with column chromatography profiles of an identical sample (see Figure 2), with the same volumes of wash and elution buffers needed to remove nonmercurated RNA and to elute mercurated RNA, respectively. This application should prove useful for large samples of RNA, since sizable amounts of sulfhydrylcellulose can be processed rapidly in a tabletop centrifuge.

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Anion-Dependent Sodium Ion Conductance of Platelet Plasma Membranes†

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ABSTRACT: External anions stimulate Na^+ efflux from platelet plasma membrane vesicles. Efflux is apparently electrogenic since K^+ diffusion potentials induced with valinomycin (interior positive) accelerate and potentials of the opposite polarity (interior negative) inhibit. In the presence of stimulatory anions, voltage-dependent Na^+ efflux is much faster than Na^+–Na^+ exchange in the absence of an induced membrane potential. Anions stimulate voltage-dependent efflux in the following order: $SCN^- > I^- > NO_3^- > Br^- \sim$ acetate $\sim Cl^- > F^- \sim SO_4^{2-} > HPO_4^{2-}$, gluconate, and isethionate. Thiocyanate, the most stimulatory anion, increases Na^+ efflux 20-fold in the presence of a membrane potential (interior positive). Stimulation of efflux by Cl^- is a saturable phenomenon with a $K_{0.5}$ of 41 mM and a maximal 2–3-fold stimulation over the basal level of efflux. Neither basal nor valinomycin-stimulated efflux was influenced by the presence of the platelet-aggregating agents thrombin, epinephrine, or ADP in the presence of fibrinogen.

Plasma membrane vesicles isolated from osmotically lysed platelets have proven extremely useful in the study of platelet transport phenomena (Rudnick, 1977; Nelson & Rudnick, 1979; Talvenheimo et al., 1979). These vesicles are functionally sealed as evidenced by their ability to maintain high internal concentrations of serotonin and lipophilic cations (Rudnick & Nelson, 1978; Nelson & Rudnick, 1979). Preliminary experiments measuring exposure of sialic acid residues suggest that greater than 90% of the vesicles have the same orientation as that of the intact platelet. The primary driving forces for serotonin accumulation in plasma membrane vesicles are experimentally imposed transmembrane gradients of Na^+ and K^+ (Nelson & Rudnick, 1979). Even though these gradients are not maintained by ongoing energy metabolism, the driving forces for serotonin transport in vesicles are apparently stable for at least 10 min. Thus, plasma membrane vesicles provide an experimental model system in which to study the ion permeability of platelet plasma membranes.

Horne & Simons (1978a) have reported that thrombin, which stimulates platelet aggregation, depolarizes the platelet plasma membrane and proposed that Na^+ influx is responsible (Horne & Simons, 1978b). In many cell types, hormones are believed to act, in part, by altering the plasma membrane potential, presumably through changes in ion permeability (Peterson, 1974; Zierler, 1972; Korchak & Weissman, 1978; Grollman et al., 1977). In the case of platelets, thrombin stimulation was inhibited by amiloride (Horne & Simons, 1978a,b), which blocks Na^+–H^+ exchange and Na^+ channels in such diverse tissues as sea urchin eggs (Johnson et al., 1976), mouse soleus muscle (Aickin & Thomas, 1977), and rabbit renal brush border vesicles (Kinsella & Aronson, 1980). Other pathways for Na^+ movements through membranes include the voltage-sensitive channel of nerve cells (Hodgkin & Huxley, 1952), the Na^+,K^+-ATPase (Glynn, 1957), and the Na^+–K^+ cotransporter found in a variety of cells (Geck et al., 1980). In this report, we describe a Na^+ transporter in the platelet plasma membrane with properties different from those of known Na^+ transport systems.

Materials and Methods

Materials

^{22}NaCl was obtained from New England Nuclear, bumetanide from Leo Pharmaceuticals (Denmark), and nigericin from Hoffmann-La Roche Inc. Porcine blood was obtained fresh at a local slaughterhouse. All other materials were

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