Mercurated Dextran Column Chromatography for Fractionating Mononucleotides (borate/thin layer chromatography/pH gradients)

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ABSTRACT Mercurated dextran (polysaccharide) was found to fractionate mononucleotides according to their affinity for organomercurial Hg^+ . The nucleotides are released from the chromatographic column in the sequence CMP, AMP, GMP, and TMP when borate buffer of increasing pH is used as an eluent.

In this communication, we report the fractionation of mononucleotides on mercurated dextran*. At neutral or alkaline pH, the first site of reaction of nucleosides and methylmercuric ion is the N(3)-H bond of uridine and/or thymidine and the second site is the N(1)-H bond of guanosine (1-3). At higher organomercurial concentrations, and at acidic pH, there are additional reactions at other nitrogen atoms of guanosine, adenosine, and cytidine. By contrast, the oxygen atoms of the bases (and of the sugar moieties), and of the phosphate groups, do not appear to interact with methylmercuric hydroxide at concentrations up to 0.1 M.† Under suitable experimental conditions (e.g., pH > 8), the base residues will be complexed by the methylmercury group in the order $\text{T(U)} > \text{G} > \text{A} > \text{C}$ (1, 2).

Based on these findings, we felt that mercurated dextran should be a ligand-exchange resin containing monofunctional mercury as the sole reactive group, with binding characteristics similar to those exhibited by the methylmercury group. In particular, since the complexing of a base residue by $\text{CH}_3\text{-Hg}^+$ can be reversed by anions such as CN^- , Cl^- , Br^- , OH^- , that interact strongly with the organomercurial (2), the use of suitable salt- and pH gradients should allow the fractionation of constituents of nucleic acids based on differences in their chemical composition.

Attempts to prepare mercurated resins have been described in the literature (4-8). These resins were used exclusively in separating HS- from nonthiolated proteins. In the present investigation, we have followed the recommendations of Shainoff (8) in preparing mercurated dextran. In our hands, mercurated dextran proved to be a chromatographic material superior to the ash-free filter pulp originally used by Shainoff. The results of our investigation demonstrate clearly that mercurated dextran exhibits a considerable degree of "base-specificity" which, in view of the obvious interest in the availability of highly specific chromatographic techniques,

might prove particularly helpful in the chemical analysis of genetic material.

MATERIALS AND METHODS

Reagents

Mononucleotides were purchased from Miles Laboratories. Highly crosslinked dextran, Sephadex G-25 (fine grade), was purchased from Pharmacia Fine Chemicals, Inc. 1-allyloxy-2,3-epoxypropane was obtained from Matheson, Coleman, and Bell. Cellulose for thin-layer chromatography (Sigmacell, Type 19) was a product of Sigma Chemical Co. All other chemicals were of reagent grade. Distilled and subsequently doubly-deionized water was used throughout.

Mercurated Sephadex

Sephadex G-25 was first etherified with 1-allyloxy-2,3-epoxypropane and then treated with mercuric acetate (8). Careful attention should be given to the washing procedure of the etherified dextran before mercuration: under the alkaline conditions of etherification, 1-allyloxy-2,3-epoxypropane, which in itself does not absorb substantially at wavelengths above 230 nm, yields small quantities of an ultraviolet-absorbing material (polymerization?) with an absorption spectrum strikingly similar to that of the nucleotides. The artifact can be removed from the resin by exhaustive washing with borate buffer of pH 9. If not removed completely, this artifact will travel with the solvent front and will be eluted first. This, on the other hand, might be advantageously used in determining certain parameters of the chromatographic bed, e.g., hold-up volume. The mercurated dextran was finally stored in water in brown bottles at 4°C. It is a white material that appears to be insensitive to light, at least over a period of 2-3 months. As a rule, the mercury content of the resin was found to range from 0.2 to 0.3 mmol/g of dry weight. This is close to the lower end of the concentration range reported by Shainoff (0.2-0.7 mmol/g) (8).

Column chromatography

A swollen slurry of washed mercurated dextran was packed into a chromatographic column (inner diameter, 9 mm; length, 700 mm; Lab-Crest, Fisher and Porter Co.) by pouring it down a rod through a funnel. The upper part of the slurry in the tube was stirred gently during filling so that air bubbles could rise to the surface. After the column was packed, a No. 4 Whatman filter of appropriate size was placed on top of the organomercurial to prevent disturbance of the resin during sample application or elution. The column was equilibrated with 0.1 M borate, pH 9.2. Then, a 2-ml sample was applied to

* Dextran is a polysaccharide, built up from glucose residues by α -1,6 glucosidic linkages. The chains possess a certain degree of branching and crosslinking via other than 1,6 glucosidic linkages.

† As judged from the sedimentation behavior of single-stranded T7 DNA in dilute salt media (Gruenwedel, D. W., and D. S. Lu, unpublished results).

the drained surface of the bed and, after restart of the solvent flow, the sample was allowed to pass into the bed. Finally, the top of the column was washed twice, each time with 2 ml of 0.1 M borate buffer. Each sample consisted of one or several mononucleotides dissolved in 0.1 M borate, pH 9.2, at concentrations of 1.6 mM. The column was then eluted, first with 0.1 M borate, pH 9.2, and finally with a gradient from pH 9.2 to 13. A polystaltic pump (Buchler Instruments, Inc.) was used to transport the eluent through the column. The pH gradient was generated in a rectangular variable gradient mixer (9) (Varigrad, Buchler Instruments, Inc.) with two neighboring chambers each filled, respectively, with equal volumes of 0.1 M borate, pH 9.2, and 0.1 M borate, pH 13. The flow rate through the column was 75 ml/hr per cm². Eluted nucleotides were monitored by ultraviolet-absorbance measurements at 260 nm (dual-beam UV monitor, Buchler Instruments, Inc.) in a quartz flow cell of 0.1-ml capacity and 3-mm optical path length. Elution profiles were recorded with a galvanometric recorder (Texas Instruments, Inc.). Fractions were collected at 2.5-min intervals; each fraction contained about 2 ml (Fractometre 200, Buchler Instruments, Inc.). Gradient elution was usually started after passage of 84 ml of 0.1 M borate buffer, pH 9.2. This volume sufficed to elute in sequence, for instance, 5'CMP and 3'AMP, whereas 5'GMP and 5'TMP could not be eluted at this pH.

After use, the column can be regenerated by passing 10% acetic acid through the bed and washing exhaustively with water until the eluate is neutral. It is easier, however, to regenerate the organomercurial in batches.

Characterization of eluted mononucleotides

Eluted nucleotides were identified (a) spectrophotometrically, (b) with the help of thin-layer chromatography (TLC), and (c) by their elution volumes, i.e., in the mixture and as single samples.

Spectroscopy. The fraction representing the center part of an eluted peak was used for analysis. Its pH was determined and its UV spectrum recorded from 340 to 200 nm (Beckman DB Spectrophotometer). 0.1 M borate buffer, adjusted with 2 N NaOH to the same pH as that of the sample, served as an absorbance blank. The spectrum was then compared with the spectra of authentic samples obtained by dissolving the various nucleotides in 0.1 M borate solutions of appropriate pH.

TLC. All fractions comprising about 70% of a recorded peak area (centered around the mean) were combined and evaporated to a small volume (about 1 ml) under reduced pressure. Each peak was then spotted (about 50 μ l) on a TLC plate, together with authentic nucleotide samples, and the spots were dried in a stream of warm air. Excess salt (Na borate) was removed by inserting the plate in a flat dish for about 10 min with anhydrous reagent-grade methanol (10). Subsequently, the plate was dried. Ascending chromatography was in a solvent of *n*-butanol-acetone-acetic acid-water 7:5:5:4. The nucleotides were traced under UV light.

RESULTS AND DISCUSSION

Fig. 1 shows the fractionation pattern obtained when a mixture of the nucleotides 3'AMP, 5'TMP, 5'GMP, and 5'CMP is passed through mercurated Sephadex with 0.1 M Na borate, of increasing pH, as the eluent. Peak 1 is 5'CMP, whereas

peak 2 contains 3'AMP. Peaks 3 and 4 are 5'GMP and 5'TMP, respectively. Recovery of the nucleotides from the column was quantitative, with the exception of 5'TMP. Only 70–80% of the original amount was recovered. We have no explanation for its loss.

The elution of the nucleotides at alkaline pH in the order 5'CMP, 3'AMP, 5'GMP, and 5'TMP agrees very well with the known affinity of the nitrogen atoms of the purines and pyrimidines for the methylmercury group; at pH values above 8, T and U are complexed most strongly by CH₃HgOH followed, in sequence, by G, A, and C (1, 2). Since 5'CMP, 3'AMP, 5'GMP, and 5'TMP are eluted from nonmercurated dextran only as one unresolved peak, immediately after the solvent front, we conclude that it is indeed the organomercurial group in the dextran matrix that is responsible for the observed fractionation pattern.

To a first approximation, elution can be assumed to proceed according to

where *R* represents the etherified dextran and N[−] stands for a base residue, e.g., guanine at pH values above 9. Obviously, any other ligand that exhibits a high affinity for the organomercurial can replace OH[−] in Eq. [1]. A more detailed analysis of the fractionation pattern is presented in Fig. 2. We have plotted *Q*, the calculated degree of binding of a nucleotide to the column, as a function of pH.

$$Q = \Sigma[N_b]/(\Sigma[N_f] + \Sigma[N_b]) \quad [2]$$

$\Sigma[N_b]$ is the concentration of a nucleotide bound to the column, summed over all possible binding sites, and $\Sigma[N_f]$ is the concentration of a nucleotide not bound to the column, summed over all known protonated and unprotonated species. In calculating *Q*, we assumed that the binding of the nucleotides by the monofunctional mercury groups in the dextran is identical with the complexing of nucleosides by CH₃HgOH (1, 2). We further assumed that at pH values greater than 7, and in the absence of other complexing ligands, mercurated dextran exists in aqueous solutions predominantly as the species R-Hg-OH (2). Thus, Eqs. [3] and [4] are the

FIG. 1. Column chromatography of mononucleotides at room temperature on a 0.9 $\times$ 70 cm column of mercurated dextran (mercurated Sephadex G-25). Left ordinate: absorbance at 260 nm. Right ordinate: pH (--- pH of eluent). Peak 1: 5'CMP; peak 2: 3'AMP; peak 3: 5'GMP; peak 4: 5'TMP. Eluent: 0.1 M Na-borate of increasing pH. Flow rate: 75 ml/hr per cm². Each fraction contains 2 ml.

two principal binding reactions that occur at pH values >7:

To the extent that NH represents the N(1)-H site of guanosine and the N(3)-H site of uridine or thymidine, reaction [3] is pH dependent due to the ionization of $\text{NH} \rightleftharpoons \text{N}^- + \text{H}^+$. If NH designates the primary amino groups of A, C, or G, reaction [3] is independent of pH unless we expect the amino groups to dissociate according to $\text{NH}_2 \rightleftharpoons \text{NH}^- + \text{H}^+$ at pH values greater than 12. Eq. [4] describes complexing reactions involving, for instance, the unprotonated, basic N(1) site of A, the N(3) site of C, or the N(7) site of G. Obviously, these equilibria are pH dependent.

We have calculated Q according to Eq. [2], using the complex-formation constants and proton-association constants given by Simpson (1). The latter constants, by the way, are almost identical with those published for nucleotides (11). As is customary in ion-exchange chromatography, we have expressed the mercury content of the column in terms of *concentration of fixed binding sites* in the resin, e.g., $[\text{RHgOH}]_{\text{free}}$ = molarity of fixed monofunctional mercury, M' , in milliequivalents per cm^3 of swollen resin (12). In order to calculate $[\text{RHgOH}]_{\text{free}}$, one needs the solvent content of the mercurated swollen resin and its density. We have determined both parameters. The solvent content amounts to 73 % by weight (determined as absorbed water), which is almost identical with the value given for untreated Sephadex G25, and the density is 1.02 g/ml. This yields $[\text{RHgOH}]_{\text{free}} = 0.05 M'$, based on a mercury content of the dextran of 0.2 mmol/g of dry weight and on the formula weight of OH as the molecular weight of the reference group bound to the mercury (12). We have used this $[\text{RHgOH}]_{\text{free}}$ value to calculate Q .

As is seen in Fig. 2, Q decreases with increasing pH. In the pH range 9–11.5, Q is of the order $Q(\text{U(T)}) > Q(\text{G}) > Q(\text{A}) > Q(\text{C})$, i.e., the sequence of observed elution. On the other hand, below pH 8.5, C should be bound more tightly by the resin than A. Since our calculation considers only pH effects, $Q(\text{G})$, $Q(\text{C})$, and $Q(\text{A})$ do not become zero, due to the pH-independent binding of the amino groups of the bases. This, perhaps, explains our finding that washing of the column with distilled water (pH 7) and with water made slightly alkaline by adding NaOH (pH 9–10) does not remove any of the nu-

FIG. 2. Plot of Q , the degree of binding of a nucleotide to mercurated dextran, as a function of pH. The letters with the curves refer to the respective base residues. For details, see text.

cleotides. Yet, 0.1 M borate (pH 9.2) does elute CMP and AMP (Fig. 1). We would conclude that borate also complexes monofunctional mercury in the column, thereby reducing $[\text{RHgOH}]_{\text{free}}$ to a value smaller than $0.05 M'$. Indeed, it can be readily shown that a decrease in $[\text{RHgOH}]_{\text{free}}$, say, by a factor of 10, not only shifts the midpoint of the binding curves in Fig. 2 by about one pH unit to the left, but also renders $Q(\text{C})$, $Q(\text{G})$, and $Q(\text{A})$ essentially zero in the pH-independent region. Since release from the column can be expected as soon as $Q \rightarrow 0$, both the observed pH of elution for GMP (pH 10.7) and TMP (pH 11.7) and the observed sequence of elution of the four nucleotides are in good agreement with the numerical arguments presented above. Thus, it appears to us that the fractionation principle of the column can be fully understood in terms of the complexing behavior of the organomercurial. We elected to use borate as an eluent since its wide buffer range eliminates the need for frequent solvent changes. However, by replacing 0.1 M borate with 0.1 M Tris sulfate in the pH range 8–9 and using, for instance, 0.1 M NaHCO_3 instead of 0.1 M borate in the pH range 9–13, one can also elute the nucleotides in the sequence mentioned above. This finding rules out the possibility that the fractionation is brought about by anion exchange via borate-*cis* diol complexes (13). Additional evidence against a borate-induced separation on mercurated dextran was found by replacement of 5'CMP with 5'dCMP or 2'(3')CMP, and by substitution of 2'(3')GMP for 5'GMP; the elution pattern presented in Fig. 1 remains unchanged, although the deoxyribosyl analogue and the nucleosides phosphorylated in the 2'(3') positions do not complex with borate. We have not yet investigated the possibilities by which, for instance, a family of differently phosphorylated guanosines and deoxyguanosines can be separated from one another on mercurated Sephadex; perhaps the simultaneous application of a pH and salt gradient might prove useful. Preliminary results show that dinucleotides also can be fractionated according to base composition and that last, but not least, whereas native calf-thymus DNA passes through the column at the solvent front, heat- and/or alkali-denatured calf-thymus DNA is retained. The denatured DNA can be released from the column by applying, for instance, a NaCN gradient (Gruenwedel, D. W., and J. C. C. Fu, manuscript in preparation). This behavior is to be expected, since in native DNA the nitrogen atoms that possess a high affinity for monofunctional mercury are engaged in intrastrand hydrogen bonding and are thus unavailable for complexing (2, 3, 14). By contrast, single-stranded DNA has these binding sites available and can therefore react with the organomercurial. In view of the ability of methylmercuric hydroxide in solution to denature native DNA when its concentration exceeds a certain critical value (2, 3, 14), there exists the possibility that local denaturation might also occur at the solid-liquid interface of the matrix.

In conclusion, the demonstrated capability of mercurated dextran to separate base residues according to their affinity for monofunctional mercury should prove helpful in the chemical analysis of genetic material. In particular, we think of the fractionation of the complementary strands of DNA (3), the separation of tRNAs from one another, and, in general, fractionating nucleotide sequences.

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