DETERMINATION OF BASE SEQUENCE IN NUCLEIC ACIDS WITH THE ELECTRON MICROSCOPE: VISIBILITY OF A MARKER

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Communicated by Bentley Glass, January 3, 1962

At the present time, the determination of the nucleotide sequence of various nucleic acids is engaging the attentions of a number of laboratories. Two general approaches are being employed. In one, an attempt is made to reconstruct the complete sequence following isolation and analysis of polynucleotide fragments. The other method involves the stepwise removal and identification of terminal bases. The difficulties in both methods are considerable.

In principle, the electron microscope offers a powerful approach to this problem providing that marker groups can be found which are detectable and which will bind selectively to the nucleotides. Such discriminating markers would reflect the occurrence of a particular base at a particular site. If the position of a base is to be indicated with precision, the dimensions of the attached marker group must not be large compared with the separation of the sites. In the present study, we discuss the visibility of a possible marker.

In electron micrographs, dense areas correspond to regions in the specimen which are rich in heavy atoms. In a previous investigation, this density difference, or contrast, was measured for single DNA molecules to which known numbers of uranyl ions had been attached. From the magnitude of the observed contrast, it was inferred that a single heavy atom attached to a base pair could in principle give a detectable level of contrast. However, the structure of all specimen-supporting films currently available is such that in high resolution electron micrographs there always exists an irregular background pattern, the contrast of which is comparable to that resulting from a single heavy atom. This makes the localization of single heavy atoms difficult. Therefore, we have chosen a marker by which several heavy atoms could be attached to a site on the macromolecule. This was done in the hope of obtaining a marker which is distinguishable from the background "noise." The marker used was the diazonium salt of 8-amino-1,3,6-naphthalenetricarboxylic acid. It was coupled to DNA, and single strands were stained in uranyl salt solutions and examined in the electron microscope. The results presented below indicate that three to six uranyl ions are coupled to each marker group during the staining procedure and that such marker groups give a detectable level of visibility.

Methods.—1. Determination of the number of uranyl ions attached to one sulfonic acid group: The stoichiometry of attachment of uranyl ions to sulfonic acid groups was inferred from results with the ion exchange resin Dowex 50W which carries benzene sulfonic acid groups. Uranyl acetate or nitrate solutions were prepared in varying concentrations from 10^{-2} to 10^{-3} M with pH ranging from 2.0 to 5.0 adjusted with sodium hydroxide or nitric acid. Dowex 50W resin, which had been allowed to swell for three hours in deionized distilled water, was added in the proportions 0, 1, 2, 4, 5, 10, 20 ml of wet resin per liter of uranyl salt solution. The solutions were shaken for 1/2 hour and then the absorption spectra of the supernatant was determined between 380 and 530 nm. The amount of uranyl ion adsorbed to the resin was calculated from the decrease in absorption of the solution. The number of equivalents of acid per ml of resin was given.
by the manufacturer. Using this the ratio of uranium atoms to benzene sulfonic acid groups could be determined.

2. Preparation of marked DNA: 8-Amino-1,3,6-naphthalenetrisulfonic acid was obtained from Matheson Coleman and Bell, Norwood, Ohio, and was purified by recrystallizing once from water. 5 ml of 0.16 M of its solution was diazotized by the indirect method, added to 5 ml of 1 mg/ml of DNA in the presence of 0.5 M NaCl and shaken for 3 hr at 3° C. Under these conditions the coupling was not completed; it was carried further in some experiments by reacting for another 3 hr with fresh diazonium salt. Finally the DNA was separated from excess dye by precipitating five times with two volumes of ethanol and was redissolved in sodium hydroxide solution at pH 11.5. After coupling with the diazonium salt, the purified DNA solution had a yellow color and a visible absorption maximum at about 415 mμ.

3. Examination of marked DNA: The DNA in the pH 11.5 solution was diluted with 1% formaldehyde to a concentration of 40 μg/ml and stored for 1.5 hr at 22° C. 0.5 ml of this solution was placed in the depression of a standard staining dish and was picked up on carbon-coated grids by a procedure essentially similar to that used for double-stranded DNA. The marked DNA was stained in 10−3 M uranyl acetate solution at pH 5 by streaking the grids over the stain solution. Dry polystyrene spheres were scattered over the grids, and these were shadowed with platinum at an angle of 8 to 1. As previously, micrographs were obtained of the molecules where they passed through the shadows cast by the polystyrene spheres. Here, no platinum was deposited by the shadowing procedure, and the molecules were visible entirely as a result of their own density and that of the attached markers. Electron micrographs were obtained on a Siemens Elmiskop I at electron optical magnifications of 160,000 times with 50μ objective apertures and the astigmatism corrected to better than 0.05μ. To minimize errors in focus always a series of micrographs was taken with a separation in the focal setting of 0.1μ. After the exposures of DNA, test plates were obtained to determine the increase in astigmatism. It was generally found that after ten micrographs the astigmatism was still less than 0.1μ. The contrast of the micrographs was enhanced by enlarging fourfold on Kodak Lantern Slide contrast plates, projecting these again on plates without enlarging further, and finally printing from these second intermediate negatives.

Results and Discussion.—Distribution of uranium atoms in uranyl-stained marker: Uranyl acetate and nitrate solutions absorb in the spectral region between 400 and 525 mμ. When Dowex 50W resin was added to uranyl salt solutions the visible absorption decreased. This decrease was a measure of the quantity of uranyl ion adsorbed to the resin. The number of equivalents of sulfonic acid in one ml of wet resin is given by the manufacturer. Knowing the number equivalents of resin added and the number of moles of uranyl ion adsorbed, the number of uranium atoms per sulfonic acid group was calculated. The results from fifteen determinations are summarized in Table 1.

<table>
<thead>
<tr>
<th>Salt</th>
<th>pH</th>
<th>Concentration (in moles)</th>
<th>Atoms of uranium</th>
<th>Atoms of sulfur</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uranyl Acetate</td>
<td>5.0</td>
<td>10⁻²</td>
<td>1 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Uranyl Acetate</td>
<td>3.5</td>
<td>10⁻³</td>
<td>1 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Uranyl Nitrate</td>
<td>2.0</td>
<td>0.1</td>
<td>0.5 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

It is apparent from the table that at low pH one half atom of uranium is taken up per equivalent of resin whereas at pH 3.5 and 5.0 at solution concentrations of 10⁻³M the uptake is approximately twice this. Cation exchange resins are known to take up cation in amounts which will very nearly just neutralize the charges on the resin. The results at pH 2 agree with this fact and the accepted view that under such acid conditions uranyl salts exist as the doubly charged uranyl ions, UO₂⁺⁺. At the higher pH values the absorption spectrum of uranyl salts changes,
and it is believed the cation undergoes hydrolysis. Species of the type $\text{UO}_2(\text{OH})^+ = A$ as well as $(\text{UO}_2)[(\text{OH})_n(\text{UO}_2)]^m = B$ have been proposed. Ahrlund has argued persuasively that the products are the latter and his view agrees with that of Hearne and White who could account for their experimental data in terms of a system richer in $B$ than in $A$.

Our results with the ion exchange resin indicate that at the higher pH values one uranium atom is taken up for every sulfonic acid group. These results are consistent with the presence of either $A$ or $B$ with $n = 1$ but are not consistent with high concentrations of $B$ with $n = 2$ or more.

The anionic groups in the marker are essentially the same as those of Dowex 50W resin and it will be assumed that the results found for the resin are applicable in the marker.

In Figure 1, the marking group has been drawn to scale. The stroke represents 10 Å. The dimensions of the rings are based on the crystallographic data for naphthalene while those for the sulfonic acid group are derived from the dimensions of the benzenesulfonic acid salts. By analogy with those structures, the naphthalene ring systems and the three sulfur atoms are coplanar. The three oxygen atoms have threefold symmetry about an axis along the C-S bond. Clearly, two sulfonic acid groups on the same naphthalene ring system are so far apart that it is unlikely that a single ion of type $B$ with $n = 1$ could attach to both. Thus, if the dominant cation is $B$ with $n = 1$ then one such cation would be attracted to each acid group of the marker, with charge neutrality probably insured by additional OH$^-$ ions. Conceivably these OH$^-$ ions could act as bridges between the cations on adjacent sulfonic acid groups.
If the dominant cation is A, then on staining probably one such cation would become attached to each sulfonic acid group. In either case the uranyl ions would form a triangle with sides 10 to 12 Angstroms in length.

Marked polynucleotide chains: When the diazonium salt was shaken at high pH with DNA, the product obtained on repeated ethanol precipitation was colored with a weak absorption maximum near 415 μ and a strong one near 260 μ. The material was DNA with the diazonium salt coupled to some of the bases. Details of the chemistry of coupling of this reagent will be discussed in a forthcoming publi-
cation. At the high pH used, the hydrogen bonds of DNA are broken. After coupling, the pH was lowered to about 8 as the DNA solutions were diluted. Formaldehyde solutions were used for dilution to prevent hydrogen bond formation and to insure that the DNA persists as individual polynucleotide chains. After the marked DNA was transferred to grids covered with carbon films and shadowed, long thin fibrils could be observed in the electron microscope, as shown in Figure 2. Although some variation in thickness was observed, the thinnest strands had a diameter considerably less than that found for native DNA and are believed to be single polynucleotide chains. The presence of thicker strands suggests that

Fig. 3.—A portion of marked, stained single strand of DNA. A number of markers are visible along the strand. Magnification 8 × 10⁴.
some aggregation of the strands occurs. This problem of aggregation will be discussed hereafter in another publication.\(^9\) The thin strands, when observed after staining but in the absence of shadowing, consisted of a row of dots as shown in Figure 3. The dots were absent in micrographs of unmarked DNA and appeared more abundant in DNA if the coupling reaction was carried out for a long time. Consequently, we attribute the dots to the markers.

**The uranyl-stained markers:** The shapes of the dots seen at high magnification were either triangles of 10 or 12 Å along each side or strokes 10–12 Å long and about 5 Å wide. This is shown in Figure 4 A, which is an enlargement of a portion

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**Fig. 4 A.**—A portion of the micrograph given in Fig. 2 at higher magnification. Some of the markers are clearly triangular while others are a stroke. Magnification 3 × 10^4.
Fig. 4 B.—Size of markers as predicted from Fig. 1. The separation of the dots represents the separation of the uranium atoms. The markers are assumed to lie either parallel or perpendicular to the plane of the specimen and are oriented to give best coincidence with the markers observed in Fig. 4 A.

of Figure 3. It is believed that the triangles are the images of the markers, with the three uranyl ions attached to the sulfonic acid groups. The strokes could arise from markers which lie in a plane perpendicular to the supporting film. This view is strengthened by the excellent agreement in size of the observed triangles and strokes, with that predicted from Figure 1. The sets of three dots shown in Figure 4 B correspond to the spacing of the three sulfonic acid groups with only the orientation varied to give best agreement with the triangles and strokes of Figure 4 A.

Theories of contrast in the electron microscope can be used to evaluate the rela-
tive contribution of the different atoms of the markers. According to Haine, the three uranium atoms account for about two thirds of the so-called amplitude contrast of the whole marker. Six uranium atoms would account for virtually all the amplitude contrast. Since the focal setting is chosen to give minimum background pattern, the phase contrast and that due to inelastic scattering cancel each other. Therefore, to this approximation, the triangles and strokes observed are largely due to the three or six uranium atoms.

The visibility of the one or two uranium atoms at the corners of the triangles is consistent with expectations given in an earlier study of stained DNA molecules, where it was found that as few as one uranyl ion for every two phosphate groups gave detectable contrast. Also, these results emphasize in a more direct way the limitations due to the background pattern. Indeed, it is doubtful that a single heavy atom could be reliably recognized in the background pattern, even though groups of three heavy atoms in a known configuration are readily recognized.

Summary.—8-Amino-1,3,6-naphthalenetrisulfonic acid was diazotized and coupled to DNA. Single polynucleotide chains of the marked DNA were transferred to specimen grids for electron microscopy, stained in uranyl acetate solutions, and examined. Along the DNA strands, triangular marks were observed. These are attributed to the three to six uranium atoms attached to the three sulfonic acid groups.

The competent photographic work of Ursula Ursprung is gratefully acknowledged.

* This work was supported by grants from the National Science Foundation (G-12323) and the National Institutes of Health (RG 8068). One of us (E. N. M.) is a holder of a NATO predoctoral fellowship and a fellowship from the Greek Atomic Energy Commission.

9 Moudrianaakis, E. N., and M. Beer, to be published.
11 Highton, P. J., and M. Beer, to be published.