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*BASE SEQUENCE DETERMINATION IN NUCLEIC ACIDS WITH THE ELECTRON MICROSCOPE, III. CHEMISTRY AND MICROSCOPY OF GUANINE-LABELED DNA**

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Some time ago we proposed an electron microscopic procedure for determining the order of nucleotides in nucleic acids.[1] The method required nucleotide selective reagents which could be made visible in the electron microscope. Subsequently it was shown[2] that the coupling of diazotized 2-amino-*p*-benzenedisulphonic acid with the 5′-nucleotide monophosphates is highly selective; at pH 9.2 the rate of the coupling reaction with guanylic acid was about 60 times higher than with any other nucleotide. Furthermore it was suggested that the reaction with the other nucleotides might involve the 3′-OH of the sugar, in which case this reaction should be hindered in the polynucleotide chain improving the observed selectivity.

For the reaction to be useful in labeling for sequence determination, it is important to know (1) whether the selectivity found with the nucleotides is retained in the polynucleotide chain; (2) if every guanine residue on the nucleic acid molecule can

be labeled; (3) the extent of degradation of the polynucleotide chain induced by the reaction treatments; and (4) the visibility of the marking group. The following bears on these questions.

Materials and Methods.—Diazonium compound was prepared as described in the previous paper. Polyadenylic (poly A), polyuridylic (poly U), and polycytidylic (poly C) were obtained from Miles Chemical Co., Elkart, Indiana. Salmon sperm DNA was obtained from Calbiochem, Inc. Yeast RNA was obtained from Schwarz BioResearch, Inc., New York. Purified snake venom phosphodiesterase was purchased from Worthington Biochemical Corp., New Jersey. Buffers were prepared as described in the previous paper.[2]

Colorimetric assay of the reactions of the polynucleotides: To 2.0 ml of a solution 1.00×10^{-3} *M* in polynucleotide-phosphate of each homopolymer or to DNA or RNA were added 1.0 ml of 0.06 *M* carbonate buffer at pH 9.0 and 1.0 ml of 1.24×10^{-3} *M* diazonium salt.

The reactions were carried out at room temperature (22°C) under reduced illumination. They were assayed for remaining diazonium activity in the same way as with the nucleotides,[2] i.e., by the β-naphthol method. The DNA used in this reaction was denatured by heating to 100°C for 10 min and then was quickly cooled.

Systematic labeling of the DNA molecule: A typical reaction mixture was as follows: heat-denatured DNA at a concentration of 0.2 mg/ml was prepared in 0.18 *M* carbonate buffer pH 9.0. To 50.0 ml of this, 10.0 ml of 2.6×10^{-1} *M* diazonium salt was added after adjustment to pH 9.0. The reaction was carried out at room temperature (22°C). The pH of the reaction was kept constant at 9.0. At discrete time intervals separate reaction mixtures were stopped by lowering the pH to 7.0 by adding HCl and cooling in ice. The DNA was precipitated by acid in cold (4°C) and then centrifuged. The sediment was redissolved in the pH 9.0 bicarbonate buffer and precipitated several times to get rid of coprecipitating diazonium compound and its side reaction products. The precipitations were continued until a constant ratio between the visible and the ultraviolet absorption of the material was obtained.

Aliquots of the labeled DNA after purification were hydrolyzed with purified snake venom phosphodiesterase. The precipitated DNA was taken into solution with 0.1 *M* ammonium carbonate containing 0.02 *M* Ca^{++} as $CaCl_2$. No precipitation occurred during the preparation of the buffer.

To the resulting mixture dry snake venom phosphodiesterase was added and incubation carried out at 45°C. When all the phosphate appeared as mononucleotide-phosphate, the enzyme was precipitated with ethanol and removed by centrifugation. The hydrolysate was applied on Whatman 3 MM chromatographic paper and electrophoresis was carried in 0.04 *M* sodium acetate buffer at pH 3.5, 21 v/cm for 1.5 hr.

The spots of the unreacted nucleotides and of a new yellow band were cut out, eluted in the pH 9.0 bicarbonate buffer, and their solutions were measured in the spectrophotometer. At the same time nucleotide-phosphate was determined in each solution, as described earlier.[2]

Electron microscopy of labeled DNA: Purified labeled DNA was stretched on carbon-coated grids, as described before.[3, 4] The grids were shadowed perpendicular to the streaking direction with evaporated platinum metal at an angle of 8:1. The shadowed grids were streaked over the surface of a 10^{-2} *M* uranyl acetate solution, pH 4.6, made in quartz-distilled water. They were examined in a Phillips EM200 electron microscope operating at 60 kv with double condenser illumination, a 50-μ diameter molybdenum objective aperture, and a cold finger cooled to −152°C by liquid nitrogen.

Through focal series were taken at a spacing of either 0.05 μ or 0.025 μ per step, and astigmatism was corrected to better than 0.05 μ. Instrumental magnification was always 100,000 ×.

To minimize image movements due to specimen drift exposure time was kept down to two seconds. Enhancement of the contrast of the electron micrograph was carried out as described elsewhere.[5]

Results and Discussion.—Colorimetric assay of the polynucleotide reactions: The aim of the present experiment was to test if the rate-dependent selectivity of the diazonium reaction with the mononucleotides held also for the polynucleotides by studying the coupling with poly A, poly C, poly U, RNA, and denatured DNA.

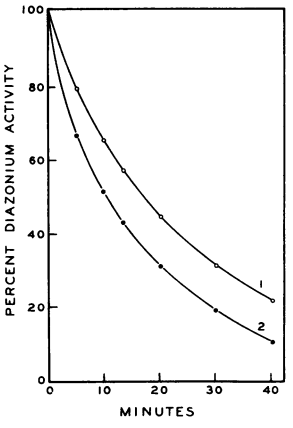

FIG. 1.—Loss of diazonium activity with time, at pH 9.0 as a function of the particular polynucleotide present. Line *1* represents reaction mixtures containing poly U, poly C, poly A, or no polynucleotide at all (control). Line *2* represents reaction mixtures containing RNA or denatured DNA.

The concentration of the stock solutions of polynucleotides used was 10^{-3} *M* polynucleotide-phosphate.

The results of these experiments (Fig. 1) indicate that although the loss of diazonium activity in the presence of poly A, poly U, and poly C, is no more rapid than in the control reaction mixture containing no polynucleotide, when RNA or denatured DNA is used, the disappearance of diazonium activity proceeds at a higher rate than in the control. This difference in rate was attributed to diazonium uptake by the guanine residues of the RNA or DNA. A possible objection to the straight comparison between homopolymers and RNA and DNA is the fact that their secondary structure is known to be different,[6] and the effect of this to the diazonium coupling reaction cannot be predicted.

By using the same method of calculating rates as with the mononucleotides,[2] the value obtained for the initial rate of the reaction of RNA or denatured RNA with the diazonium salt at pH 9.0 was 3.8 $L.M.^{-1}$ sec^{-1} as compared to 4.1 $L.M.^{-1}$ for the deoxyguanylic acid. This is considered a surprisingly good agreement in the rate constants and it can be concluded that at least at the beginning of labeling of the guanine residues on the chain no extra steric complication exists.

Under the same conditions no reaction by poly A, poly U, or poly C, is detectable. It should be noted that a reaction with the diazonium salt of any other nucleotide with a rate lower than 5 per cent of that for guanylic would not be detectable.

Labeling of the DNA molecule: DNA isolated and purified after it has been reacted with diazonium salt was colored. In addition to the 260-mμ absorption maximum it had a second maximum at 386 mμ at pH 9.0. This broad peak shifted to 345 mμ when the solution was acidified to pH 2.0 (Fig. 2).

The ratio of the absorption at 386 mμ to that at 260 mμ increased with prolonged treatment of the DNA with the diazonium compound. This ratio was taken to indicate the extent of the coupling reaction.

Having determined the extinction coefficient at 386 and 260 mμ of the product obtained from coupling the diazonium salt to guanylic acid, and knowing the coefficients of the other nucleotides at 260 mμ, the ratio of 386/260 mμ absorption for a DNA of known G-C content can be calculated for different extents of guanine labeling. For this we have to make the assumption that the relative absorption of the nucleotides is the same for the free nucleotides as for the fully denatured polymer. The fact that the 386/260 mμ ratio of the labeled DNA preparation is the same before and after complete enzymatic hydrolysis seems to support the assumption.

For most complete labeling of DNA it was found necessary to repeat the reaction-purification cycle three or four times until no further increase of the 386/260 mμ ratio was observed. This DNA was considered saturated with diazonium salt.

DNA's with different 386/260 mμ ratios were hydrolyzed with snake venom

phosphodiesterase, electrophoresis of the hydrolysate was carried out at pH 3.5, the resulting spots were eluted, and the solution was measured in the spectrophotometer. It was found that the increase in the 386/260 mμ ratio was paralleled by a decrease in free guanylic recovered from the hydrolysate, and this in turn by an increase of a fast-moving yellow-colored band having the same electrophoretic and spectroscopic properties as the product obtained by reacting deoxyguanylic acid with the diazonium salt.

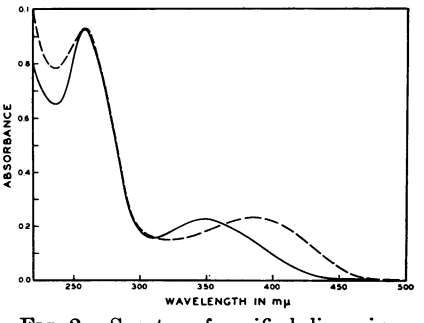

FIG. 2.—Spectra of purified diazonium-labeled DNA, in acid and alkali. ——, in pH 2.0; — —, in pH 9.0.

A series of such analyses were as follows: 200.0 mμmoles unlabeled DNA phosphate and an equal amount from diazonium-labeled DNA were applied separately on

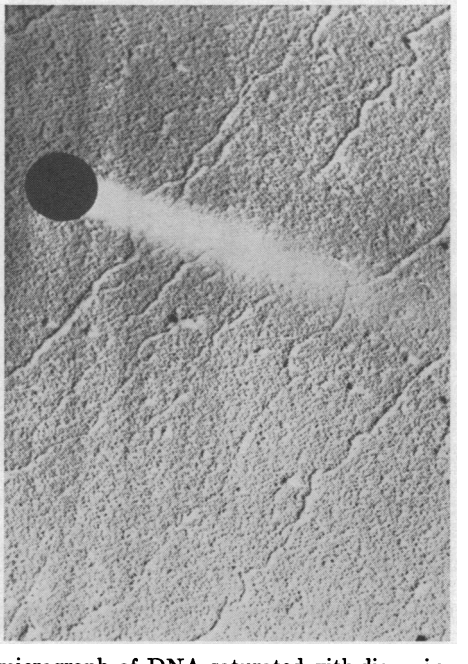

FIG. 3.—Electron micrograph of DNA saturated with diazonium salt. Carbon-coated grids were streaked over the DNA solution, polystyrene spheres 0.188 μ in diameter were sprayed on them, and then shadowed with platinum at an angle 8:1. Very long parallel strands are visible crossing the field. Magnification 90,000 ×.

paper for electrophoresis. Three different samples of labeled DNA with increasing 386/260 mμ ratio were used. The amount (in mμmoles) of recovered nucleotides from each run were as follows:

386/260 mμ	dGMP	dCMP	dAMP	dTMP	Product
Control	37.2	39.3	57.3	56.5	0.0
0.08	25.5	39.1	58.1	57.2	11.5
0.15	14.3	38.7	56.4	55.3	22.3
0.23	7.6	39.9	58.2	56.8	30.0

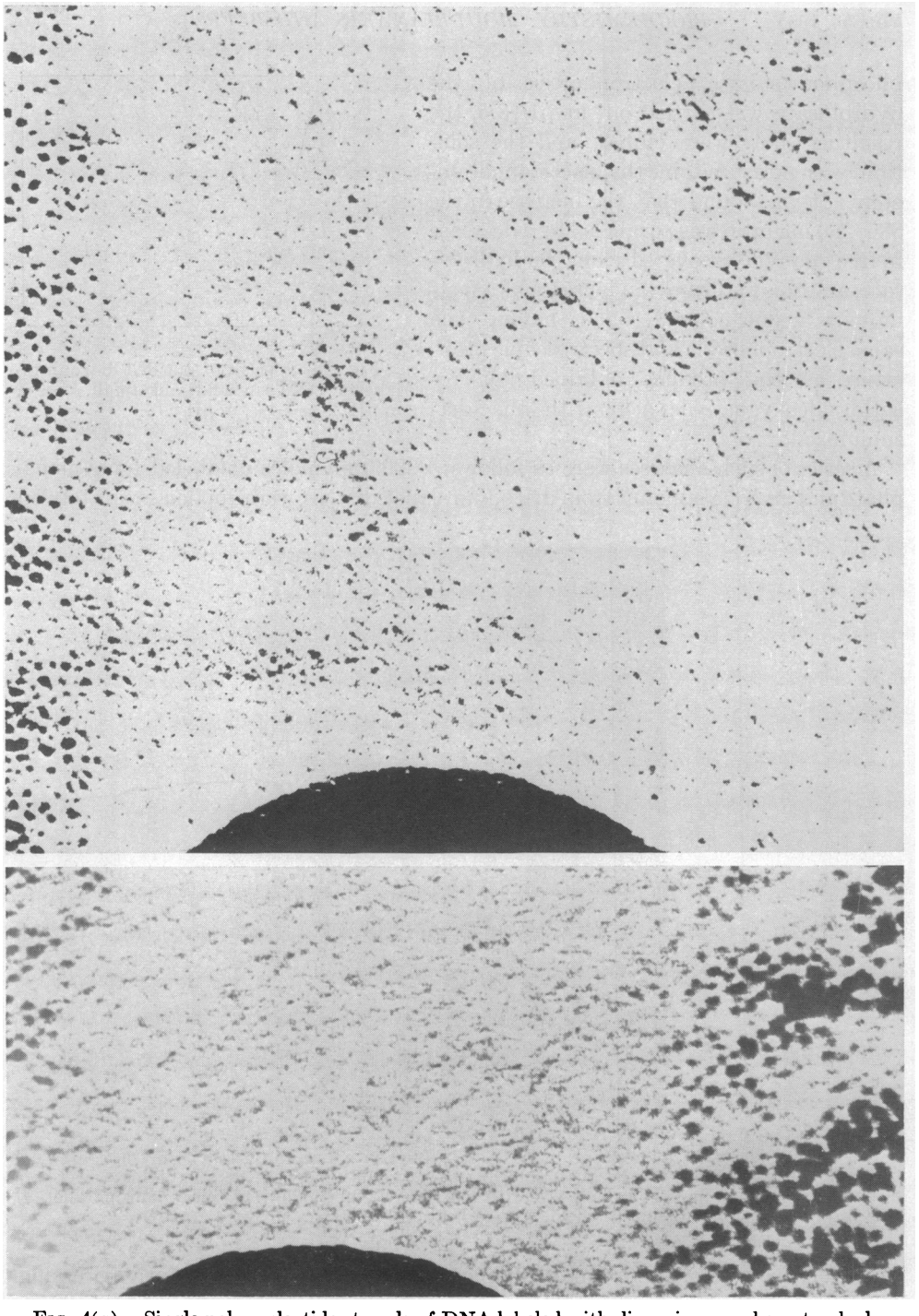

FIG. 4(*a*).—Single polynucleotide strands of DNA labeled with diazonium marker, streaked on carbon-coated grids, sprayed with polystyrene spheres, shadowed with platinum at an angle of 8:1, and stained with uranyl acetate. Several dots are visible along the direction of the strands entering from the left of the picture and joining in the middle of the field behind the polystyrene sphere (bottom). Background noise is introduced by the carbon supporting film. Magnification 850,000 ×.

FIG. 5(*a*).—Photograph of a DNA preparation treated exactly as the one in Fig. 4 except that no diazonium marker was coupled to it. Strands entering in the shadow of the sphere become almost invisible. In some places they can be followed in lines of small dots (arrows) presumably due to uranium held by the phosphate groups of the molecule. This signal is almost the same order as the background noise. Notice the complete absence from this picture of the dark dots observed in the preparation of Fig. 4. Magnification 850,000 ×.

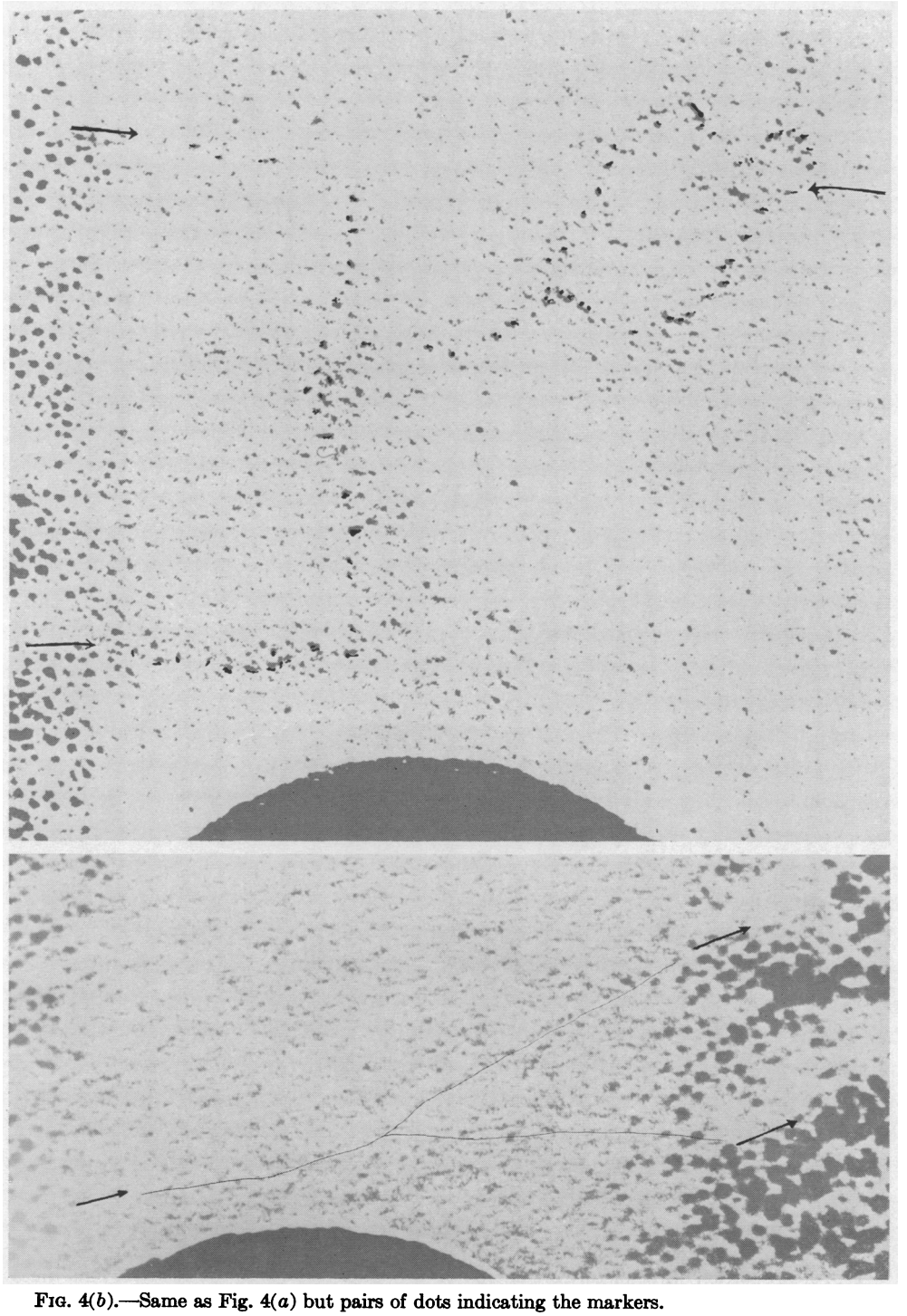

FIG. 4(*b*).—Same as Fig. 4(*a*) but pairs of dots indicating the markers.

FIG. 5(*b*)—Same as Fig. (5*a*) but arrows and drawn lines indicate faintly visible unmarked strand.

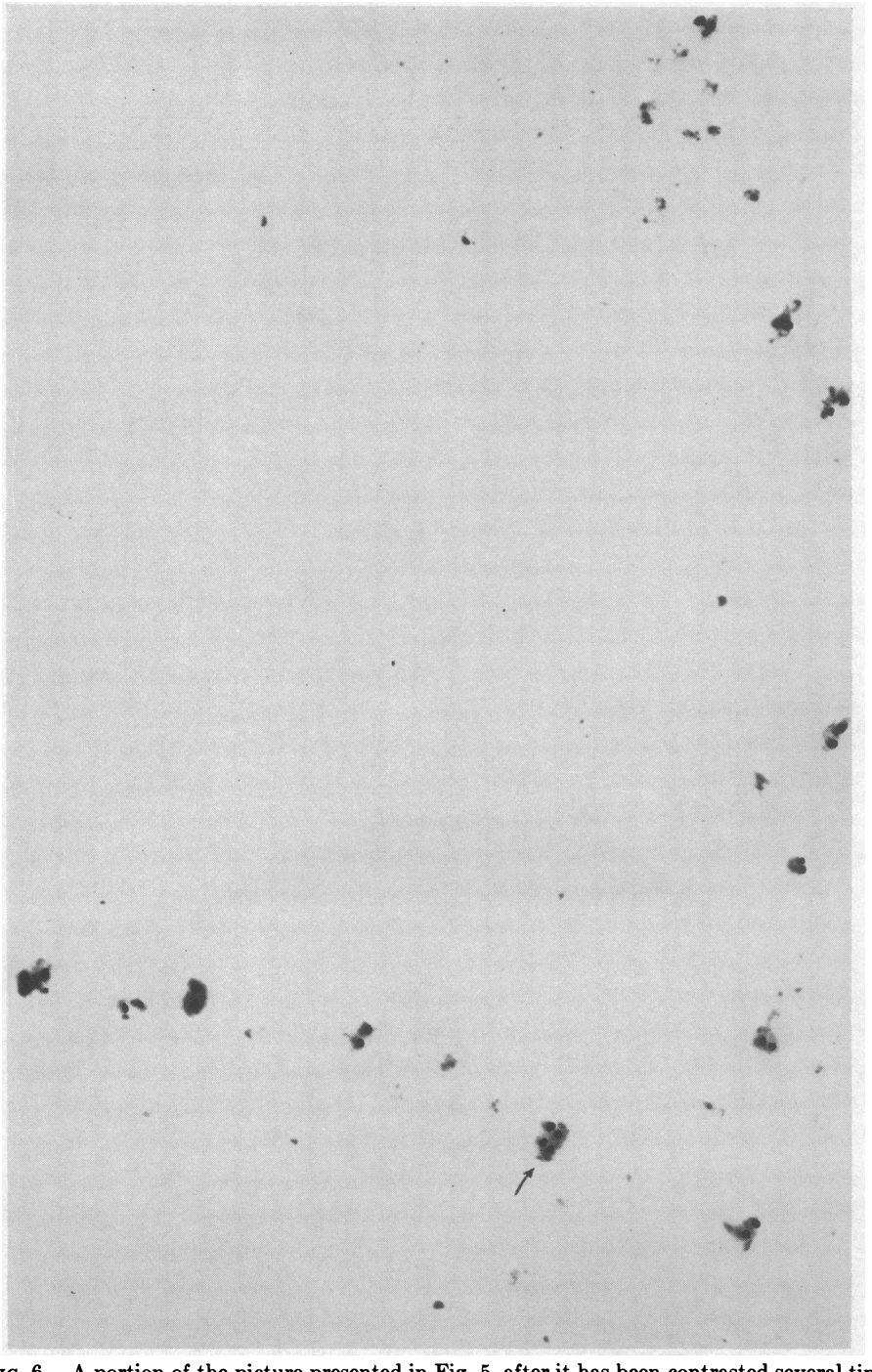

FIG. 6.—A portion of the picture presented in Fig. 5, after it has been contrasted several times and enlarged photographically to 4,000,000 ×. Marker dots are seen to split to a pair of smaller dots 10–12 Å apart. In some places (arrow) these pairs are found next to each other with a separation of 6–7 Å. Orientation of most of the pairs is unique, i.e., their long axis is perpendicular to the DNA strand. Magnification 4,000,000 ×.

Comparing dGMP recovered to product, it can be seen that they add up to the amount of dCMP recovered, that the decrease of free dGMP with progressively increasing 386/260 mμ parallels the increase in the amount of product recovered, and that about 80 per cent of the guanine residues have been labeled.

Electron microscopic examination of labeled DNA: Examining grids of labeled DNA in the electron microscope, strands are found with variable thickness and several micra in length. The thinnest ones are of the order of 10 Å thick and are believed to represent single polynucleotide chains (Fig. 3). When these thin strands run behind the polystyrene sphere, i.e., inside its shadow, they can be seen as a row of dots (Fig. 4). These were absent in unlabeled DNA (Fig. 5) and they are attributed to the marker.

At high magnification each dot is split into a pair of dots 10–12 Å apart. These are believed to represent the positions of the uranyl ions held by the two sulphonic acid groups para to each other on the benzene ring (Fig. 6).

By using crystallographic data from the study of the benzenesulphonic acid salts[7] the distance between the two sulphonic acid groups of the diazonium compound used is expected to be 10–12 Å.

The minimum distance between two consecutive pairs of dots was found to be 6–7 Å as demonstrated in Figure 6. This would imply that at this particular site on the polynucleotide chain the marker is placed on two consecutive guanine residues.

The two dots constituting the marker are oriented in such a way that the line joining them is generally perpendicular to the axis of the polynucleotide strand. Such an orientation of the marker was predicted in the earlier paper[2] on chemical grounds.

Extent and randomness of labeling: Under the present conditions it was found that no more than 80 per cent of the guanine residues in the DNA could be labeled. The unlabeled 20 per cent of the guanines could represent the same sites in all the molecules in a population. If this is true—it seems unlikely—then there will always be a group of guanines that will remain undetected by this procedure.

Alternatively it may be that although only 80 per cent can be labeled, the unmarked residues are not the same ones in all the molecules of a population. In this case, several labeled molecules must be photographed to detect all the guanines.

Summary.—Diazotized 2-amino-*p*-benzenedisulphonic acid can selectively label the guanine residues in DNA. The reaction is mild and no excessive degradation of the polymer chain is observed. DNA so labeled and stained with uranyl acetate is visible in the electron microscope and the markers resolved in pairs of dots 10–12 Å apart. Pairs have been observed spaced 6–7 Å from each other, suggesting labeling of consecutive guanine residues.

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