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Electron Microscopic Study of the Base Sequence in Nucleic Acids. VI. Preparation of Ribonucleic Acid with Marked Guanosine Monophosphate Nucleotides*

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ABSTRACT: It has been previously shown that the compound 2-diazo-*p*-benzenedisulfonic acid can be treated with deoxyribonucleic acid (DNA) at pH 9 to form an addition product specifically with the guanosine monophosphate (GMP) nucleotides. These can then be stained with uranyl acetate for identification of the GMP nucleotide sites by electron microscopy. Further investigaton of the chemical reaction, reported here, has shown that the reaction at pH 9 also causes an acid-labile attachment of the diazonium marker to adenosine monophosphate (AMP) and cytidine monophosphate (CMP) nucleotides. This attachment is reversed by the acid precipitation which had been used for purifying the marked DNA. A much milder acid treatment, involving incubation at pH 4, has been developed to reverse this attachment with less damage to the nucleic acid and the marked GMP. The selectivity of the reaction with ribonucleic acid (RNA) was found to be slightly less than with DNA. When 60% of the GMP's have been marked, about 10% of each of the other nucleotides is also marked. The RNA molecules are somewhat fragmented during the reaction as found by sedimentation analysis and by measuring molecular length by electron microscopy. Using sucrose gradient sedimentation to fractionate the molecules according to size it has been possible to purify small amounts of marked MS-2 RNA molecules which are more than one-half their native length. The preparation of specimens for electron microscopic analysis is described.

A method has been proposed (Beer and Moudrianakis, 1962) for determining the base sequence of nucleic acids by examining single strands at high resolution in the electron microscope. The method requires the development of reagents, called "markers," which attach selectively to certain bases in the nucleic acid, and which are, or can be made, visible in the electron microscope. For example, one such marker that has been described is the compound 2-diazo-*p*-benzenedisulfonic acid (Moudrianakis and Beer, 1965a,b). It reacts with the nucleotides, and under certain conditions primarily with GMP,[1] to form addition products. Nucleic acid which has been treated with this reagent can be extended on thin carbon film and stained with uranyl acetate, so that each of the sulfonic acid residues binds complexes of uranyl ions, thus making the markers visible in the electron microscope. High-resolution electron microscopy has demonstrated that individual markers can be identified (Moudrianakis and Beer, 1965b; P. Bartl, H. Erickson, and M. Beer, to be published).

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[1] Abbreviations used: GMP, guanosine monophosphate; AMP, adenosine monophosphate; CMP, cytidine monophosphate; UMP, uridine monophosphate; TCA, trichloroacetic acid.

The chemical specificity of this marker was first studied by examining its reaction with the mononucleotides, where it was found that at pH 9.0 it reacted with GMP 60 times faster than with any other nucleotide (Moudrianakis and Beer, 1965a). To confirm the selectivity for the polynucleotide, DNA was treated with an excess of diazonium at pH 9.0 and the nucleic acid was purified from the reaction mixture by repeated precipitation with acid, redissolving the precipitate in buffer (Moudrianakis and Beer, 1965b). The reacted DNA had a spectral absorption peak at 386 mμ, which was characteristic of the diazonium–GMP addition product. Extensive reaction could bring the ratio of OD_{386}:OD_{260} to 0.23, which, on the basis of known extinction coefficients, indicated that 80% of the GMP's were marked. Base analysis of this marked DNA, determined by enzymic hydrolysis to nucleotides and electrophoresis at pH 3.5, confirmed the result, showing that 80% of the GMP's were marked while less than 5% of any of the other nucleotides had been changed.

Kössel (1965) has studied the reaction of the related compound, diazosulfanilic acid, with the nucleotides and has shown that an addition product was formed with AMP and CMP as well as with GMP. The GMP product was more stable at low pH so that some selectivity for GMP addition could be obtained after mild acid hydrolysis. These results suggested that the greater acid stability of the GMP addition product during the acid precipitation might be essential in determining the high selectivity for GMP marking by 2-diazobenzenedisulfonic acid.

In this paper we show that acid-labile addition products are indeed formed with AMP and CMP nucleotides and that selectivity for GMP marking is obtained only after acid hydrolysis. Conditions for the acid hydrolysis, milder than acid precipitation, have been determined and have been combined with gentle techniques for purifying the nucleic acid from the diazonium reaction mixture. The application of the marker has been extended to RNA for which the selectivity appears to be somewhat less than for DNA. About 10–15% of the non-GMP nucleotides is marked when 60–70% of the GMP's has been marked. The amount of breakage of the RNA molecules during the reaction has been investigated, and methods are described for preparing samples of high molecular weight, GMP-marked RNA suitable for electron microscopy.

Materials and Methods

Growth of Virus and Purification of RNA. Bacteriophage MS-2 and its host, *Escherichia coli* c3000, were obtained from Dr. D. Nathans, Johns Hopkins Hospital, Baltimore, Md. Bacteria were grown to 4×10^8/ml as described by Strauss and Sinsheimer (1963) and infected at a multiplicity of 10. Lysis was seldom complete but a titer of $1–3 \times 10^{12}$ pfu/ml could usually be obtained after treating the culture with EDTA and $CHCl_3$ and incubating with 0.1 mg/ml of egg white lysozyme for several hours at room temperature (Shimura *et al.*, 1965).

The phage in about 20 l. of lysate was precipitated by the addition of 320 g/l. of $(NH_4)_2SO_4$ at 4° and collected, along with bacterial debris, by centrifugation for 15 min at 9000 rpm. The precipitate was resuspended in 200 ml of buffer (0.1 M NaCl–0.05 M Tris–0.01 M EDTA, pH 7.6) and 0.1 mg/ml of egg white lysozyme was added. The suspension was shaken at room temperature overnight and then centrifuged for 10 min at 15,000 rpm to remove bacterial debris. The pellet was reextracted once with 100 ml of buffer and the supernatants were combined. Magnesium acetate was added to the viscous suspension to a concentration of 0.02 M, and 5 μg/ml of DNase (beef pancreas, Worthington) was added. After 2 hr at room temperature the phage was collected by centrifugation at 27,000 rpm for 3 hr. The pellet was resuspended in 7 ml of buffer and centrifuged for 10 min at 10,000 rpm. The pellet was reextracted with 4 ml of buffer and the supernatants were combined and centrifuged once more for 10 min at 10,000 rpm. The final supernatant was weighed (11.63 g) and 0.550 g of CsCl was added per g. The solution was divided into three tubes and spun in the SW 39 rotor for 40 hr at 35,000 rpm at 5°. The phage was found in a pale brown band, about 0.25 in. thick, two-thirds of the way down the tube, and was clearly separated from the small pellet on the bottom and the protein band on top. Drops were collected from a hole in the bottom of the tube, and the fractions containing the phage were pooled and dialyzed into 0.2 M sodium acetate–0.001 M EDTA (pH 6.0) at 4°. The phage suspension was diluted to 8.5 ml and was stored with $CHCl_3$ at 4°.

The spectrum of a $^1/_{1000}$ dilution of this suspension in 0.1 M sodium phosphate (pH 6.8) showed an absorption maximum at 260 mμ and a minimum at 240 mμ, with ratios OD_{260}:OD_{240} = 1.55 and OD_{260}:OD_{280} = 1.77, in agreement with previously published values (Strauss and Sinsheimer, 1963; Fiers *et al.*, 1965). Assuming an absorbancy coefficient OD_{260} = 8.03 for a 1-mg/ml solution (Strauss and Sinsheimer, 1963), the total yield of phage was 670 mg, corresponding to 1.1×10^{17} phage. A titer of the phage stock showed that the plaque-forming efficiency was 9%.

RNA was extracted from portions of the phage solution as described by Fiers *et al.* (1965) except that it was done at room temperature. After precipitation with ethanol the RNA was stored in the ethanol mixture at −15° and was dissolved in water before use.

Radioactively labeled (^{32}P) MS-2 was prepared and purified as described by Fiers *et al.* (1965). In one case the phage was purified from a lysate prepared and generously donated by Dr. D. Nathans. Yeast RNA (1 mg/ml) was added to the radioactive phage suspension before extraction of the RNA.

Diazonium Preparation. 2-Amino-*p*-benzenedisulfonic acid was diazotized and washed as described previously (Moudrianakis and Beer, 1965a). The diazonium precipitate was mixed as a slurry in cold 0.05 N HCl

at a concentration of 0.8 M as determined by the β-naphthol test and was stored frozen at −70°. The diazonium activity decayed very slowly in this frozen state, dropping to one-half to one-third of the original value after 1 year.

Reaction Procedure. The nucleic acid to be marked was dissolved in 1 ml of water or buffer, usually 0.1 M $NaHCO_3$, and adjusted to pH 9. The diazonium slurry (1 ml), at 0°, was brought to pH 8–9 by the addition of about 0.03 ml of 50% NaOH and quickly added to the nucleic acid solution. A Radiometer TT-lc pH meter–titrator with combination glass electrode GK 2021C was used to monitor the pH and to add automatically the titrating solution (saturated K_2CO_3 which had been adjusted to pH 11.5 with HCl) as needed to keep the pH at 9.0 during the reaction. Titration was rapid at first but slowed down as the diazonium decayed, in 15–30 min (Moudrianakis and Beer, 1965a). After 30–45 min another milliliter of diazonium was added in the same way. The reaction mixture was kept at about 25°, and excessive exposure to light was avoided. After the desired number of diazonium additions, the mixture was kept at room temperature for 2 hr to allow complete decay of diazonium activity.

These reaction conditions were altered slightly during the experiments on molecular breakage. Best results were obtained by adding a volume of diazonium slurry equal to the total volume of the reaction mixture at 10- or 15-min intervals, thus keeping the concentration of active diazonium very high for as short a time as possible. As a precaution against the possible action of RNase, about 1 mg of bentonite was usually added to the reaction mixture. In an attempt to reduce the formation of bubbles during the reaction, a possible cause of breakage, a solution of concentrated potassium metaborate (boric acid plus 1 equiv of potassium hydroxide) was generally used instead of the potassium carbonate for titrating the reaction. Bubbling was reduced but there was no obvious reduction of breakage.

Purification. The nucleic acid was separated from low molecular weight reaction by-products by Sephadex chromatography. A column of Sephadex G-75 (1.8 × 40 cm) was equilibrated with 0.05 M $NaHCO_3$ at 4°. (The Sephadex chromatography has also been done at room temperature but reaction of the diazonium with the column material, staining it yellow, was more extensive, and occasionally led to contamination of the nucleic acid by the diazonium–Sephadex products.) The reaction mixture, usually 10–30 ml, was cooled to 0°, loaded on the column, and eluted with 0.05 M $NaHCO_3$. The yellow-colored, diazonium-marked nucleic acid was eluted ahead of the yellow-to-dark red diazonium decay products. Fractions containing the nucleic acid were pooled and the nucleic acid was precipitated by adding two volumes of ethanol–sodium acetate (90% ethanol containing 2% sodium acetate) and cooling to −15° for several hours, usually overnight. The precipitate was collected by centrifugation and dissolved in a small volume of water or buffer.

Incubation at pH 4. For incubation at pH 4, the ethanol precipitate was dissolved in 0.1 M sodium acetate (pH 4.0). After the desired incubation, routinely 1 hr at 37°, the nucleic acid was precipitated by adding two volumes of ethanol–sodium acetate and cooling to 0° for about 1 hr. The precipitate was collected by centrifugation and redissolved in 0.05 M $NaHCO_3$.

Nucleotide Analysis. A small sample of marked RNA, usually 0.1 ml containing 10^5 cpm of ^{32}P labeled MS-2 RNA and 1 mg of yeast RNA, was hydrolyzed to nucleotides by incubation in alkali. KOH (0.33 volume of 1 N) was added and the mixture was kept at 37° overnight. The hydrolysate was cooled in ice and a predetermined amount of 1 N $HClO_4$, sufficient to lower the pH to 10–11, was added. The crystals of $KClO_4$ were allowed to settle out. The stability of the marked nucleotides in the alkaline hydrolysis was examined by comparing the spectrum of a sample of marked RNA before and after alkaline hydrolysis. The OD_{260} showed the expected hyperchromic shift but the high-wavelength absorption band showed less than 5% change.

Whatman 3MM paper was prepared for electrophoresis by washing overnight by descending chromatography with water. The neutralized hydrolysate (10–30 μl) was applied to the dry paper in a small spot and dried. The paper was moistened with electrophoresis buffer, 0.05 M NH_4HCO_3 adjusted to pH 8.0 with NH_4OH, and run for 1 hr at 30 v/cm. Two elongated spots separated. The faster spot, designated X, which was colored yellow, was present only in the diazonium-marked samples and contained the marked nucleotides. The slower spot, which could be seen only by ultraviolet absorption, contained the unreacted nucleotides. The spots were cut out and the nucleotide spot was heated in an oven at 80° for 30 min to remove NH_4HCO_3. The nucleotides were transferred quantitatively to the origin of another electrophoresis paper by descending chromatography with water (Aronoff, 1958). This paper was moistened with the second electrophoresis buffer, 0.075 M citric acid adjusted to pH 3.5 with NaOH, and run for 2 hr at 30 v/cm. The four nucleotide spots were found by viewing the paper under ultraviolet light and cut out. The amount of ^{32}P in each of these spots and in the X spot was determined by counting it in a liquid scintillation counter.

Recovery of applied ^{32}P was 98–99%, with 1.0–1.5% accounted for by incomplete transfer of the nucleotide spot to the second electrophoresis paper. Duplicate electrophoretic runs gave values for the ^{32}P recovered in each individual spot which were normally within 3% of each other. For each analysis the fraction of the total applied ^{32}P recovered in each of the spots, CMP, AMP, GMP, UMP, and X, was determined and two or more duplicate runs were averaged. Since the X spot contained all the marked nucleotides and was not resolved into individual components, the amount of each nucleotide which was marked could not be determined directly. It was calculated indirectly by subtracting the mole fraction of each nucleotide from the mole fraction found for

unmarked control RNA, thus determining the amount of that nucleotide which was in the X spot.

With the samples prepared for molecular weight determination and electron microscopy, which were not radioactively labeled, the level of GMP marking was estimated from the spectrum by determining the ratio of OD_{380}:OD_{260} at pH 9. Using the known extinction coefficients (Moudrianakis and Beer, 1965a) a ratio of 0.16 corresponds to about 50% of the GMP's marked. This estimate has been confirmed experimentally on one sample of ^{32}P-labeled RNA and on DNA (Moudrianakis and Beer, 1965b). (To apply this estimate directly to the marked RNA, instead of to the nucleotides from alkaline hydrolysis, it is necessary to correct for the hypochromic shift in OD_{260}.)

Determination of Sedimentation Coefficient. For sedimentation runs the RNA was dissolved in 0.2 M NaCl–0.01 M Tris (pH 7.6) at a concentration of about 40 μg/ml and was spun in the Spinco Model E ultracentrifuge at 44,770 rpm at 5°, using ultraviolet optics to observe the boundary. The photographs were traced on a Joyce-Loeble densitometer and the sedimentation coefficient was determined for the midpoint of the boundary. This was taken to be the point of 50% maximum absorption for the diffuse boundaries obtained with the heterogeneous samples of fragmented RNA. The value of $s_{20,w}$ was calculated from this.

Measurement of Molecular Length. For preparation of electron microscope specimens the method of Highton and Beer (1963) was modified to avoid treating the marked RNA with formaldehyde, which was found to reduce the level of marking significantly (P. Bartl, unpublished experiments). Instead the RNA was denatured by heating to 70° for 1 min in 0.02 M sodium phosphate–0.001 M EDTA (pH 6.8). Thin hydrophobic carbon films, on copper grids which had been coated with polybutene, were streaked once across the surface of the hot solution, about 0.05 ml in a depression of a glass-staining dish. A concentration of RNA of 10-μg/ml was found to give the best distribution of individual extended strands.

Fractionation on Sephadex G-200. A column of Sephadex G-200 (2.6-cm diameter and 30 cm long) was equilibrated with 0.01 M $NaHCO_3$ at 4°. A 2.0-ml sample of RNA was loaded and eluted in the same $NaHCO_3$. Fractions of 3 ml were collected and the OD_{260} of each was measured. The first four fractions showing significant OD_{260} were assumed to include all completely excluded (high molecular weight) molecules and were combined for further analysis and purification.

Sucrose Gradient Sedimentation. Sucrose solutions were made in 0.2 M NaCl–0.01 M Tris (pH 7.6), treated with acid-washed Norit by mixing at room temperature for several hours, and then filtered through a 0.45-μ Millipore filter. Gradients of 5–20% sucrose were prepared for either the SW 39 or the SW 25 rotor and a 0.2- or 1.0-ml sample, containing up to 10 mg/ml of RNA, was loaded on top. Before loading, the RNA sample was concentrated by precipitating with ethanol and collected by centrifugation. The ethanol was thoroughly drained off the precipitate and the RNA was dissolved by adding the desired volume of water. The gradients were centrifuged at 10°, the SW 39 for 6 hr at 35,000 rpm, the SW 25 for 17 hr at 20,000 rpm. Fractions were collected by counting drops from a hole in the bottom of the tube and the spectrum of each tube was recorded. For preparation of electron microscope specimens from these fractions the RNA was collected by precipitation with ethanol and was dissolved in 0.02 M sodium phosphate–0.001 M EDTA.

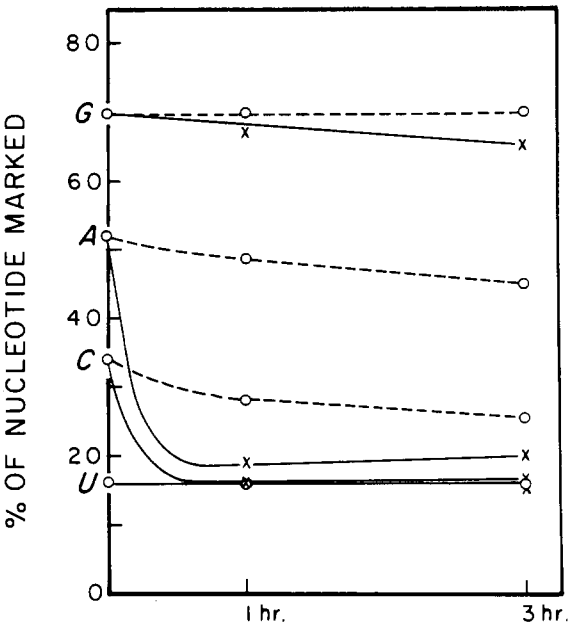

FIGURE 1: Diazonium-marked RNA (Table I, column b) was dissolved in 0.1 M sodium acetate (pH 4.0) and incubated at 0° (⊙- - -⊙) or at 37° (×——×). At 1 and 3 hr the RNA was precipitated with ethanol and hydrolyzed in alkali, and the nucleotide composition was determined.

Results

Spectroscopic Studies. RNA or denatured DNA which was marked extensively with the diazonium and purified by Sephadex chromatography had an absorption peak at 385 mμ considerably larger than that previously reported (Moudrianakis and Beer, 1965b). A ratio of OD_{385}:OD_{260} in excess of 0.45 could be obtained, which is much higher than the ratio of 0.30 expected for complete GMP marking. When poly A was similarly treated and purified it showed a large peak at 350 mμ with OD_{350}:OD_{260} in excess of 0.6, and OD_{380}:OD_{260} over 0.45. Poly C showed a smaller peak at 340 mμ and poly U showed a small increase in OD_{310} after the marking reaction. When the marked poly A was precipitated with 10% cold TCA, the peak at 350 mμ completely disappeared, and when the extensively marked DNA or RNA was precipitated with TCA there was a large decrease in the absorption

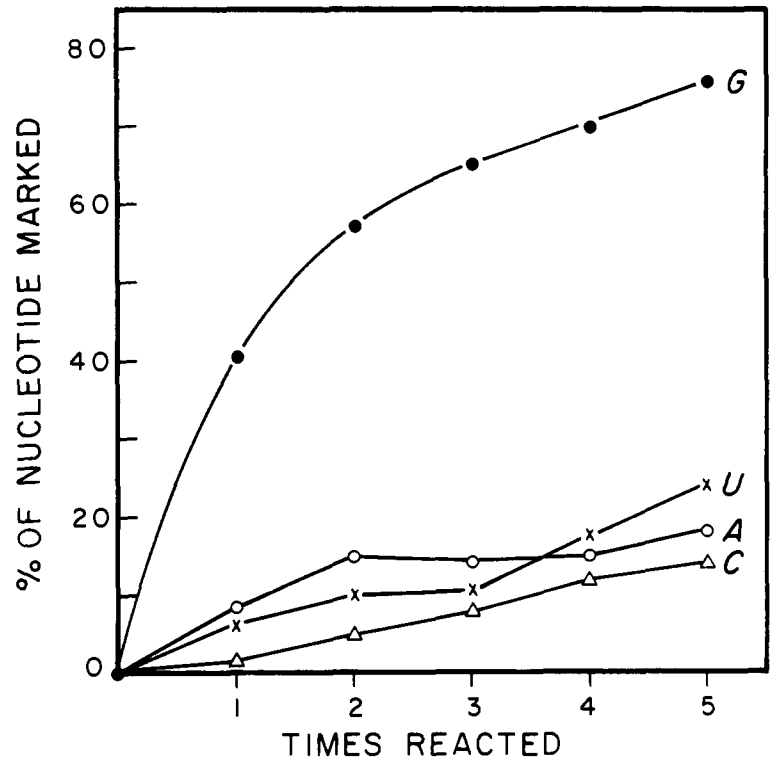

FIGURE 2: RNA was treated with the diazonium by five 1-ml additions of diazonium slurry and purified as described in Materials and Methods. The purified RNA was incubated at pH 4.0, 37° for 1 hr. A sample was removed for analysis and the reaction was repeated on the remainder of the RNA.

at 385 mμ. This same loss of absorption was found after incubating poly A or RNA at room temperature in 0.1 M sodium acetate buffer (pH 3.5) for 3 hr.

Nucleotide Analysis. The results of a nucleotide analysis of RNA which had been extensively marked with the diazonium and purified by Sephadex chromatography is given in Table I (column b). A similar analysis of unmarked control RNA is shown in column a. The mole fraction of each nucleotide is lower in the reacted RNA, with a total of 42% of the ^{32}P appearing as marked nucleotides (X). GMP and AMP have both been extensively marked, while CMP and UMP have been marked to a smaller extent. The specificity for GMP marking was greatly improved by incubating the marked nucleic acid at pH 4.0 at 37° as shown in column c. The time course of the release of marker at pH 4.0 is shown in Figure 1. Most of the marking of AMP and CMP is reversed rapidly at 37° and more slowly at 0°. The marked AMP and CMP seems to involve two types, one which is quickly hydrolyzed in the mild acid, and a second which is apparently resistant to acid hydrolysis. This second type, which amounts to 16–19% of the CMP, AMP, and UMP in this sample, is equally resistant to incubation at pH 2.5.

Figure 2 shows the results of successive reactions followed each time by mild acid hydrolysis. The rate of marking AMP, CMP, and UMP is about the same as that found previously, about 10–15% average for each nucleotide when GMP is 65–70% marked. It has not been determined whether the apparent differences in the rate of marking each nucleotide are significant.

In a study of the possible influence of temperature on the specificity of marking, a sample of RNA was treated with the diazonium at 0°, using much longer reaction times to obtain a fairly high level of GMP marking. The selectivity was essentially the same. When 62% of the GMP was marked, an average of 8–10% of each other base was marked.

Molecular Weight Determination. Before marking, the MS-2 RNA sediments in a sharp boundary with an $s_{20,w}$ of 29 in agreement with previously published observations (Strauss and Sinsheimer, 1963). In the electron microscope specimens prepared from this RNA many individual strands could be found. The longest of these was 1.75 μ, and after correcting for doubled regions at the ends (Highton and Beer, 1963) the most frequent length was 1.75 ± 0.1 μ. This is in good agreement with the average length of 3.5 μ found for TMV-RNA (Highton and Beer, 1963), which has a molecular weight twice that of MS-2 RNA. It is longer than the average length of 1.05 μ found for single strands of MS-2 RNA prepared by the Kleinschmidt technique (Granboulan and Franklin, 1966), implying a smaller degree of extension of the RNA molecules prepared by the Kleinschmidt technique.

Reaction of the RNA with the diazonium marker causes extensive breakage of the RNA molecules as

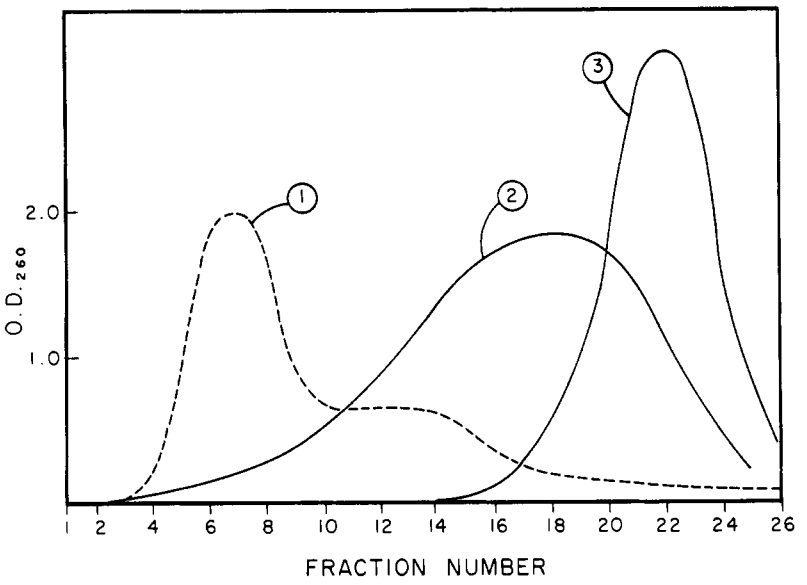

FIGURE 3: Zone sedimentation of GMP marked MS-2 RNA. Samples (1 ml) containing 1–2 mg of RNA were sedimented through sucrose gradients as described in Methods. The sample in each bucket and its average $s_{20,w}$ determined from boundary sedimentation were: (1) unmarked control RNA, $s_{20,w}$ = 29 S; (2) 30% GMP-marked RNA, $s_{20,w}$ = 17 S; (3) 35% GMP-marked RNA, $s_{20,w}$ = 7 S.

TABLE I: Nucleotide Analysis of RNA.

	Control RNA (a)		Diazonium-Marked RNA, before pH 4 (b)		Diazonium-Marked RNA, after pH 4 (c)	
	Nucleotide Composition (mole fraction)	% of Nucleotide Marked	Nucleotide Composition (mole fraction)	% of Nucleotide Marked	Nucleotide Composition (mole fraction)	% of Nucleotide Marked
CMP	0.252	0	0.166	34	0.209	17
AMP	0.231	0	0.110	52	0.186	19
GMP	0.268	0	0.080	70	0.089	67
UMP	0.250	0	0.210	16	0.210	16
X	0.004		0.419		0.285	

indicated by sedimentation analysis. The extent of the breakage is somewhat variable and the exact cause has not been determined. Control experiments have shown that it occurs only when there is active diazonium in the reaction mixture and is probably therefore an unavoidable aspect of the marking reaction. Best results were obtained, in terms of the least breakage for a certain level of marking, by using fresh diazonium with a high activity (0.8 M) and combining a high concentration of diazonium with short reaction times. In this manner it has been possible to routinely produce samples of 30% GMP-marked RNA having an average sedimentation coefficient $s_{20,w}$ = 17 S. Further marking always results in more extensive breakage, reducing the average $s_{20,w}$ to 7 or 5. One sample, which was used for further analysis and preparation of electron microscope specimens, was prepared which had 50–60% of the GMP's marked and had an average $s_{20,w}$ = 7.2 S. It was impossible to identify reliably any single strands with a measurable length in microscope specimens prepared from either the 17S or the 7S samples of marked RNA. The shadowed specimens show mostly a very rough texture, presumably due to the deposit of many very short strands.

Since the sedimentation measurements indicated that the marked RNA samples were very heterogeneous with regard to molecular length we felt that it would be possible to purify small amounts of marked RNA which were longer than the average broken molecules by using preparative techniques which fractionate the molecules according to molecular size. One method which proved effective was fractionation on a column

of Sephadex G-200. Since this gel has a nominal exclusion limit of 200,000 mol wt, the highest molecular weight fractions from this column should give strands which are at least one-fifth of the native length. This expectation was confirmed by chromatographing either 17S or 7S marked RNA. Electron microscope specimens prepared from the highest molecular weight (excluded) fractions showed strands of length 0.3 μ and longer.

The most useful fractionation technique has been sedimentation in sucrose gradients. Figure 3 shows the results of sedimenting about 1 mg each of unmarked control RNA and samples of 30% GMP-marked RNA which had an average $s_{20,w}$ of 17 and 7 S. The 17S RNA shows clearly the heterogeneous size distribution, most of it sedimenting much more slowly than the control RNA. A small fraction, about 5% (fractions 5–8), does sediment as fast as the major peak of the 29S control RNA. In microscope specimens prepared from fractions 5 and 6 a high frequency of apparently unbroken strands (1.5–1.8 μ) were found. Fraction 7 gave shorter strands (0.5–1.0 μ). Fractions 10 and higher gave no strands which could be reliably identified and measured, indicating that they are probably less than 0.2 μ.

The 7S RNA sediments even more slowly and generally has no significant fraction of high molecular weight RNA (Figure 3). We have been able to obtain some long strands from 7S samples by combining the Sephadex chromatography with the sucrose gradient sedimentation and starting with a much larger amount of RNA. A total of 15 mg of RNA, which was 50% GMP marked and had an average sedimentation coefficient of 7.2, was first chromatographed on the Sephadex G-200 column to eliminate the bulk of low molecular weight RNA. The fractions corresponding to the highest molecular weight material, about 3 mg in all, were pooled and then fractionated by sucrose gradient sedimentation. A very small fraction of this sample, about 0.05 mg of the total, was now found to sediment with the 29S control RNA, and microscope specimens prepared from this showed a high frequency of strands longer than 1.0 μ.

It was important to determine that the small fraction of unbroken molecules isolated by these methods had the same extent and specificity of marking. Because of the small amounts recovered, a complete base analysis was not attempted. The spectrum, including the ratio of OD_{385}:OD_{260}, was, however, determined for each fraction of the sucrose gradient sedimentation and each was identical with the spectrum of the sample before fractionation.

Discussion

The results of the nucleotide analysis show that a simple reaction of RNA with the diazonium marker at pH 9 results in formation of acid-labile addition products with the adenylic and cytidylic residues. The spectral results indicate that the same is true for DNA. These products are probably diazoamino couplings, as proposed by Kössel (1965) for the reaction products with diazosulfanilic acid. The greater acid stability of the guanylic addition product is consistent with the previous proposal that it is an azo coupling at the C-8 of the purine ring (Moudrianakis and Beer, 1965a). Except for the characteristics of acid and alkali stability and high electrophoretic mobility, the nature of the UMP, CMP, and AMP addition products is not known. Reaction of this diazonium compound with the ribose of the nucleotides has been observed previously (Moudrianakis and Beer, 1965a) and may be involved here.

The rate of nonspecific marking sets a limit to the useful extent of GMP marking. After 60% of the GMP nucleotides have been marked, the rate of GMP marking decreases (Figure 2), so that further reaction results in decreased specificity for GMP marking.

The molecular breakage during the marking reaction at first presented another limitation to the extent of useful GMP marking. While it has been possible to study in the electron microscope samples of GMP-marked Ala-tRNA, which has a molecular weight of only 30,000 (P. Bartl, H. Erickson, and M. Beer, to be published), much longer molecules, at least 200,000 mol wt, about 0.4 μ long, are needed to obtain a good distribution of reliably identifiable single strands by the streaking technique and to make the microscopy and analysis convenient. It will obviously be preferable for the study of nucleotide sequence to work with the longest pieces available, preferably unbroken molecules. By the fractionation techniques described here we have been able to achieve these aims to a large extent.

For a sample with 50–60% of the GMP's marked, the chemical work indicates that an average of about 10% of each other nucleotide is marked. With this level of selectivity, calculations (A. M. Fiskin and M. Beer, to be published) have shown that 30–40 reliable observations of the presence or absence of a marker at each nucleotide site will be sufficient to determine the GMP nucleotide sites with about one mistake in 2000 nucleotides. This can be compared to the 3200 nucleotides in the complete MS-2 RNA molecule to indicate the precision of the sequence determination that could be obtained with these samples.

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The Biosynthesis of 1,6-Phenazinediol 5,10-Dioxide (Iodinin) by *Brevibacterium iodinum**

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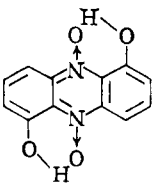
ABSTRACT: In experiments with resting cells of *Brevibacterium iodinum* the highest yield of iodinin occurred in the presence of some three-, four-, or five-carbon amino acids. Tricarboxylic acid cycle compounds, especially succinic acid, also gave high yields. However, the direct incorporation of labeled glutamic acid into iodinin was only 0.1%, and using growing cultures, the activity was diluted by the addition of compounds of the shikimic acid pathway. The average incorporation of labeled shikimic acid into iodinin was 3.7%. This activity was diluted by L-phenylalanine but not significantly by other amino acids or by fumaric acid. Anthranilic acid is not a direct precursor of iodinin. Labeled 1,6-phenazinediol and 1,6-phenazinediol 5-oxide were incorporated into iodinin in high efficiency (10 and 15%), confirming the idea that these two substances are the immediate biosynthetic precursors of iodinin.

The relatively good incorporation of shikimic acid into iodinin indicates that the pathway for the biosynthesis of iodinin is similar to that of the phenazines studied by others.

About 20 naturally occurring phenazines are known at present, all produced by microorganisms. The biosynthesis of four of them has been studied. Using labeled substrates to study the formation of pyocyanine (1-hydroxy-5-methylphenazinium betaine) by *Pseudomonas aeruginosa*, Blackwood and Neish (1957) showed the incorporation of glycerol and dihydroxyacetone; Frank and DeMoss (1959) of L-alanine, pyruvate, and glycerol; Ingram and Blackwood (1962) of glycerol, alanine, and leucine; Millican (1962) of shikimic acid; and MacDonald (1963a), of glycerol, shikimic, and quinic acids. Sheikh and MacDonald (1964) demonstrated that the 5-methyl group was derived from methionine. When unlabeled substances were used for the production of pyocyanine some amino acids and some tricarboxylic acid cycle compounds with ammonium ions as a source of nitrogen were found to be the most efficient (Grossowicz *et al.*, 1957; Valette *et al.*, 1964). Carter and Richards (1961) reported the incorporation of [carboxyl-^{14}C]anthranilic acid into chlororaphin (a 3:1 molecular compound of phenazine-1-carboxamide and its 5,10-dihydro derivative) produced by *Pseudomonas chlororaphis*. Levitch (1961) showed that DL-[3-^{14}C]tryptophan and/or [1,3-^{14}C]glycerol were precursors of phenazine-1-carboxylic acid in *P. aureofaciens*. Later, labeled shikimic acid was shown to be incorporated into phenazine-1-carboxylic acid (Levitch and Stadtman, 1964) and 2-hydroxyphenazine (Levitch and Rietz, 1966). Acetate was demonstrated to be a much less efficient phenazine precursor (Blackwood and Neish, 1957; Levitch, 1961; MacDonald, 1963b; Levitch and Stadtman, 1964).

Since no member of the important group of 1,6-dioxygenated phenazines had been studied, we decided to investigate the biosynthesis of iodinin. Produced by *Brevibacterium iodinum* (Clemo and McIlwain, 1938; Clemo and Daglish, 1950) this extremely insoluble pigment which, in bulk, looks like iodine was the first naturally occurring *N*-oxide known. It has antimicrobial (Gerber and Lechevalier, 1964; Oda *et al.*, 1966) and antitumor activity (Makino *et al.*, 1963; L. H. Pugh, unpublished data). In *B. iodinum*, *Microbispora*



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