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The Site of Alkylation of Nucleic Acids by Mitomycin*

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ABSTRACT: Alkylation of synthetic polyribonucleotides with [^{3}H]mitomycin or [^{14}C]porfiromycin is shown to proceed at least four times as easily on guanine as on the other common bases. Alkylation of s-RNA with porfiromycin is demonstrated to yield both monoguanyl- and diguanyl-porfiromycin, the latter probably arising from an interstrand linkage. The alkylated guanine moieties are labilized from the s-RNA on storage, leaving depurinated strands in these areas.

Evidence has been increasing to show that mitomycin and chemically related antibiotics may act in large measure by combining with DNA. Mitomycin C is known to prevent DNA synthesis in bacteria (Shiba *et al.*, 1959) and to cause lysogenic induction (Korn and Weissbach, 1962).

The work of Iyer and Szybalski (1964) gave the first insight into the possible mode of action. When DNA was treated with reduced mitomycin C, its physical properties were changed in a manner suggesting the formation of cross-linkages. This process, which occurs *in vivo* or *in vitro*, may represent direct alkylation of the DNA. The extent of such "cross-linking" increased roughly with the G-C content of the DNA.

Weissbach and Lisio (1965) have provided a more direct demonstration that chemically reduced mitomycin C and porfiromycin can attach to isolated DNA, s-RNA, and ribosomal RNA. Incubation of the nucleic acids with hydrosulfite-reduced [^{3}H]mitomycin C or [^{14}C]porfiromycin led to the binding of one antibiotic molecule for approximately 500 nucleotides in each case.

In view of this relatively high degree of alkylation of the nucleic acids, the site of attachment of the antibiotic to the polynucleotide became of interest. This paper describes the isolation of fragments containing both guanine and antibiotic, either mitomycin or porfiromycin, from alkylated s-RNA.

Materials and Methods

Mitomycin C was obtained from Kyowa Hakko Kogyo Co., Tokyo. Porfiromycin was obtained from the Lederle Laboratories Div., American Cyanamid Co. Tritiated mitomycin C and [1a-^{14}C]porfiromycin were prepared by the method of Weissbach and Lisio (1965). Yeast s-RNA was obtained from General Biochemicals, and was freed from any residual traces of nucleases by passing the solutions three times through a column of Amberlite IRC-50 resin equilibrated with 0.2 M phosphate, *p*H 5.55. Sephadex G-25 was obtained from Pharmacia, Uppsala. Synthetic homopolymers and poly-GU were a gift from Dr. Leon Heppel. Venom phosphodiesterase was prepared according to the method of Koerner and Sinsheimer (1957). Pentose was determined by a micromodification of the method of Dische (1951).

Experimental

Reduction of the Antibiotics. It has been shown (Iyer and Szybalski, 1963) that mitomycin or porfiromycin requires a preliminary reduction in order to serve as alkylating agents. In the present work, this was accom-

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plished with potassium borohydride and the process was followed spectrophotometrically. Serial spectra taken during the course of reduction of mitomycin (Figure 1) indicate that there is a progressive increase in the absorption at 305 mμ accompanied by a decrease in the absorption at 360 mμ. The transient peak at 250 mμ is seen in concentrated mitomycin solutions (>1 × 10^{-4} M) but does not appear when dilute solutions (1 × 10^{-5} M) are similarly reduced. It may represent a transitory quinhydrone-type complex.

In practice, reduction was carried out in a spectrophotometer cell with a 1 mm light path, and the absorption at 250 or 360 mμ followed with time. When the absorption decreased to a constant level, the excess borohydride was discharged with a small amount of 1 M acetate buffer, *p*H 5, and the substrate to be alkylated was added to the cell immediately.

Reduction of mitomycin has been postulated to involve loss of the 9a-methoxy group and the carbamyl residue (Iyer and Szybalski, 1964). The use of tritiated mitomycin makes it necessary to determine the correction in specific activity to compensate for the loss of these tritiated fragments. Since mitomycin is adsorbable on Norit, the increase in nonadsorbable counts after reduction of the antibiotic was used to establish this correction. Table I shows that only 2.7% of the radioactivity of [^{3}H]mitomycin was not adsorbed on Norit. When this preparation of mitomycin was reduced with KBH_4 and treated with Norit under the same conditions, 13.6% of the radioactivity remained in the supernatant, and represented the lost tritiated fragments. When this same amount of reduced mitomycin (0.06 μmole) was allowed to react with 8 μmoles (4 mg) s-RNA for 2.5 hours before adsorption, the nonadsorbable radioactivity remained at 12%. This indicates there is no further release of labeled fragments from the reduced antibiotic during the alkylation reaction.

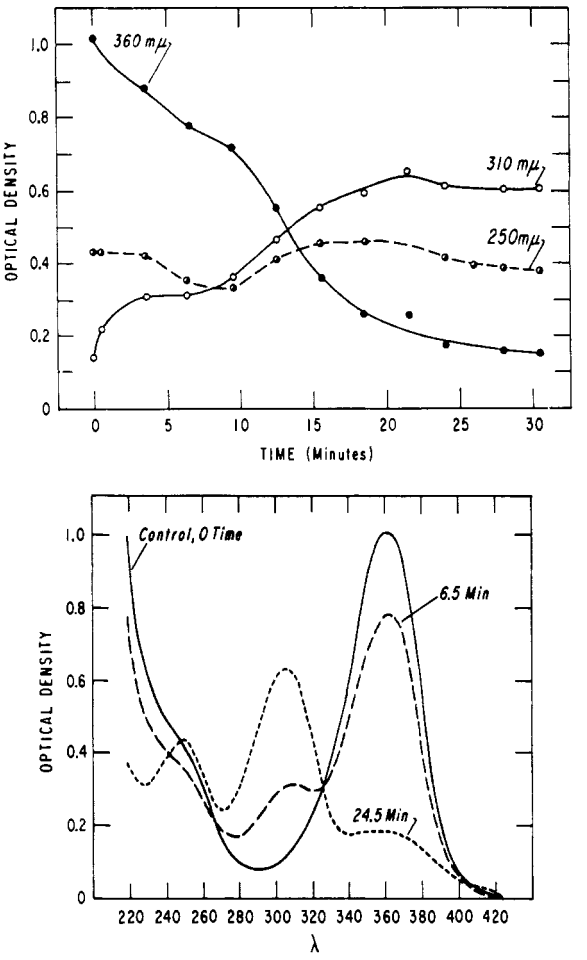

FIGURE 1: Spectral changes during reduction of mitomycin C. The spectrum of a mixture of 0.3 ml mitomycin C (50 μg) and 2 μl freshly prepared 1 M KBH_4 was followed using a cell with a 1-mm light path. (A) Changes in optical density with time at three wavelengths. (B) Mitomycin C spectrum at three stages of reduction.

TABLE I: Correction for Tritium Loss on Reduction of Mitomycin.[a]

	Reduced	Cpm Nonadsorbable on Norit (%)
[^{3}H]Mitomycin	0	2.7
	+	13.6
s-RNA alkylated with [^{3}H]mitomycin	+	12

[a] Tritiated mitomycin (20 μg, 30,000 cpm) in 0.04 ml H_2O and 0.15 ml 0.1 M acetate buffer, *p*H 4.5, was adsorbed on 0.05 ml 10% (w/v) Norit in water. After being stirred in ice for 20 minutes, the suspension was filtered on a Millipore filter and washed with 0.5 ml cold buffer, and an aliquot of the combined supernatant and wash fluids was examined for radioactivity in Bray's solution. For reduction of the mitomycin, 2 μl of 0.1 M KBH_4 was added to the water solution and allowed to react in a spectrophotometer cell until the D_{250} reached a minimum, and the reaction was stopped by the addition of *p*H 4.5 acetate buffer to a final concentration of 0.08 M. Norit adsorption was then carried out as above.

Alkylation of Homopolymers. The extent of alkylation of synthetic polyribonucleotides was studied using ^{14}C-labeled porfiromycin (Table II). The amount of alkylation in poly-GU was used to calculate the labeling of guanine. After subtracting the value for alkylation of the uridine moieties calculated from the known value for poly-U, the guanosine portions were estimated to contain 1.28 μmoles porfiromycin/100 μmoles nucleotide. This value is at least four times higher than those for poly-A, poly-C, or poly-I, and over

TABLE II: Alkylation of Synthetic Polyribonucleotides.[a]

Polymer	G	I	A	C	U	GU[b]
μMole porfiromycin to 100 μmoles base	1.0	0.32	0.33	0.34	0.20	0.74[c] (1.28)
Preference for G over other bases[c]		4.0	3.9	3.8	6.4	

[a] [^{14}C]Porfiromycin (86 mμmoles, 3.9×10^9 cpm/mμmole) was reduced with 2 μl freshly prepared 0.1 M KBH_4. After 4 minutes at 24°, the excess borohydride was discharged with 40 μl 0.1 M acetate, *p*H 6, and the mixture was diluted to 0.1 ml. To tubes containing 0.1 mg of each polymer in 0.1 ml H_2O were added 10 μl of the reduced porfiromycin. After 2 hours at 24° the mixtures were separated on Sephadex columns, and the ultraviolet absorption and radioactivity of the peak tubes containing the polymer were read. The greatest amount of porfiromycin bound in any reaction mixture represented less than 2% of the total amount present. [b] Base ratio approximately 1:1 G/U. [c] Correcting for the known binding of porfiromycin to the uridylic residues, the alkylating agent is present to the extent of 1.28 per 100 guanine residues in the GU copolymer. This value is used in calculating the ratios in line 2.

six times higher than the value for alkylation of poly-U.

The observation that guanylic nucleotides are alkylated to a greater extent in poly-GU than in poly-G may have several explanations. The very stable secondary structure of poly-G (Fresco and Massoulié, 1963) may sterically prevent the antibiotic from approaching vulnerable sites. Even more likely, however, is the possibility that the N-7 position is the site of attack on the guanine residue, as it is for other alkylating agents (Brookes and Lawley, 1963; Jones and Robins, 1963). This nitrogen is tied up in the hydrogen bonding scheme in G-G interactions (Gellert *et al.*, 1962), and hence is doubly hindered from reacting with mitomycin. In poly-GU, on the other hand, a large number of the guanylic residues are unfavorably situated for G-G interactions, and are open to attack.

The role of G-G bondings in the availability of the bases for alkylation was further studied using a preparation of GpGpG which had aggregated to a high degree (Ishikura, 1962; Ralph *et al.*, 1962; Lipsett, 1964). After exposure to reduced mitomycin at 3° for 16 hours, the solution was separated on a Sephadex G-25 column equilibrated with 0.05 M NaCl–0.002 M Tris buffer, *p*H 7.5. In this system, aggregates of GpGpG are excluded and emerge from the column before the monomeric form. The aggregate fraction from the column showed no evidence for the presence of mitomycin. Thus the spectrum of this material showed no peak at 310 mμ either before or after heat dissociation of the complex. Paper chromatography in solvent 1 of the dissociated complex revealed only a single spot, with the expected R_F for GpGpG, and a normal spectrum. On the other hand, the monomeric GpGpG fraction, which was not excluded on the Sephadex column, was alkylated by the antibiotic. The GpGpG was separated from free mitomycin by paper chromatography in solvent 1. The spectrum of the eluted GpGpG band had a shoulder at 310 mμ, indicating the presence of mitomycin.

Alkylation of Mononucleotides. No spectral evidence could be obtained for alkylation of 5′-AMP, 2′(3′)-UMP, or 2′(3′)-CMP when the nucleotide-mitomycin reaction mixture was separated on paper in system 1. Guanine, guanosine, and 5′-GMP were all observed to undergo alkylation, as shown by the appearance of two new ultraviolet-absorbing spots on paper chromatography in solvent 1. The two new substances contained mitomycin, for their spectra revealed a shoulder at 310 mμ; their R_F values were 0.8 and zero. In all three cases the component moving in system 1 with an R_F of 0.8 was present in much greater amount than the compound at the origin. It was thought that these two spots might represent guanyl- and diguanyl-mitomycin, respectively, and the following experiment was devised to test this point.

[8-^{14}C]Guanine sulfate was dissolved in water and adjusted to *p*H 5 with KOH, and the specific activity was determined to be 6.38×10^6 cpm/μmole in Bray's solution (Bray, 1960). Tritiated mitomycin, 0.5 μmole, specific activity 350,000 cpm/μmole, was diluted in 0.75 ml water and reduced with 2 μl freshly prepared 0.1 M KBH_4. After 5 minutes, the excess borohydride was removed by acidification with 50 μl 1 M acetate buffer, *p*H 4.5. Two μmoles of the [^{14}C]guanine in 0.4 ml water was added to the reduced mitomycin and the mixture was allowed to stand at room temperature for 19 hours. The mixture was streaked on a single sheet of Whatman 3MM paper and chromatographed in system 2. In this solvent there were two spots not ascribable to guanine or mitomycin, one at the origin and the other at R_F = 0.26. (Guanine has an R_F of 0.35.)

The material at the origin was eluted with water, lyophilized, and examined for ^{14}C and ^{3}H in hyamine-toluene-PPO-POPOP. After application of the 13% correction factor for loss of tritium labilized by reduction of the mitomycin, the material was found to have a ratio of 1.19 mitomycin/1 guanine (Table III). This deviation from a 1:1 ratio is probably not significant considering the error involved in approximating

TABLE III: Properties of Guanine-Antibiotic Derivatives[a]

Guanine Source	Antibiotic	R_F of Derivative in System 1	R_F of Derivative in System 2	Molar Ratio Guanine/Antibiotic
(I) [8-^{14}C]Guanine	[^{3}H]Mitomycin	0.8	0	0.84
		0	0.26	Undetermined
(II) [^{3}H]Guanine-s-RNA	[^{14}C]Porfiromycin	0.8	0	0.89
		0	0.23	2.07

[a] For details, see text. The specific activities used for calculation of the molar ratios are: Expt I, [8-^{14}C]guanine, 6.38×10^6 cpm/μmole; [^{3}H]mitomycin, 3.5×10^5 cpm/μmole; Expt II, [^{3}H]guanine, 4.48×10^6 cpm/μmole; [^{14}C]-porfiromycin, 6.1×10^6 cpm/μmole.

the correction. This compound migrates with an R_F of 0.8 in system 1, and the neutral spectrum is shown in Figure 2.

The second new substance, $R_F = 0.26$, was partly contaminated with streaking from the faster-moving guanine. This second material is very difficult to elute from paper, even with 50% ethanol. The losses incurred by elution, rechromatography, and reelution were so high that examination of the substance was abandoned. Identification of this material as the diguanyl derivative was accomplished using s-RNA as described in the following paragraphs.

Alkylation of s-RNA. The maximum extent of alkylation of s-RNA observed here is approximately 1 mitomycin per 500 nucleotide units, which is of the same order of magnitude as has been reported for DNA and ribosomal RNA by Weissbach and Lisio (1965). Alkylation in these experiments was carried out by adding the s-RNA solution to KBH_4-reduced ^{14}C- or ^{3}H-labeled antibiotic in 0.1 M acetate, *p*H 6, and allowing the alkylation to proceed for the specified time. The alkylated polymer was separated from the unreacted mitomycin on a column of Sephadex G-25 (Figure 3).

Labeled alkylated s-RNA prepared in this manner showed no change in specific activity on repeated Sephadex fractionation, even when refractionated in the presence of unlabeled reduced antibiotic. However, a gradual loss of radioactivity to the medium was noted after storage of the s-RNA solutions at 3° for several days. While almost all of the s-RNA was still excluded from Sephadex G-25, an ever-increasing amount of radioactive label was retarded on the Sephadex. Likewise, while almost all of the ultraviolet-absorbing material remained precipitable with 2 volumes of cold ethanol at the end of several days, an appreciable amount of radioactivity appeared in the ethanol supernatant. These results were of great interest to us. They indicated that initially the antibiotic was firmly bound by s-RNA, and that subsequently certain alkylated groups at the binding sites were split off into the medium. We therefore investigated the nature of the material set free.

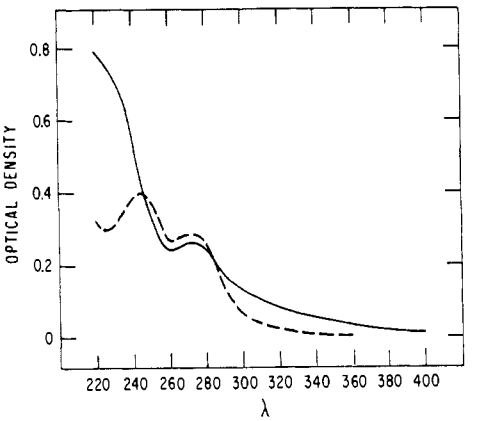

FIGURE 2: Spectrum of 1:1 guanine-mitomycin compound. – – – –, guanine spectrum, 0.01 M PO_4, *p*H 7; ———, compound containing 1.19 mitomycin C–1 guanine, 0.01 M PO_4, *p*H 7. See text for details.

Paper chromatography in solvent 1 of either the Sephadex-retarded fraction or the ethanol supernatant yielded materials behaving like the alkylated guanine compounds ($R_F = 0$ and 0.8). The ultraviolet spectra of the two substances showed the 310-mμ shoulder characteristic of the presence of the antibiotic, as well as the radioactivity associated with it.

A quantitative estimation of these two materials was made by subjecting a preparation of [^{3}H]mitomycin-treated yeast s-RNA, alkylated to the extent of 1 mitomycin per 1000 nucleotides, to degradation with snake venom phosphodiesterase. Subsequent chromatography in solvent 1 showed the same two radioactive spots, the first at $R_F = 0$, the second at $R_F = 0.8$. The ratio of counts present in these areas was 1:10.5, which indicates that more than 90% of the bound antibiotic is present as a monoalkyl substituent.

To identify these antibiotic-containing fragments, *Escherichia coli* K12λ was grown in a synthetic medium (Korn and Weissbach, 1962) containing 10 μmoles of uniformly tritiated guanosine, specific activity 481 mc/

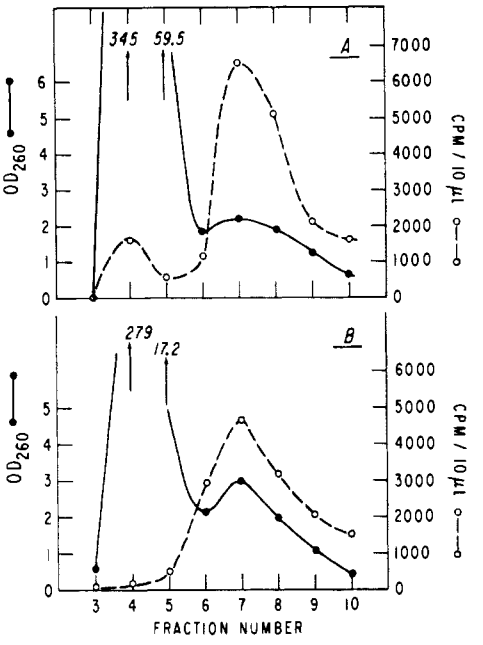

FIGURE 3: Sephadex separation of alkylated s-RNA. (A) Separation of yeast s-RNA alkylated with [^{14}C]-porfiromycin. [^{14}C]Porfiromycin, 30 µg containing 40,700 cpm, in 0.1 ml 0.1 N NaCl, was reduced with 2 µl fresh 0.1 M KBH_4. After 4 minutes, 0.4 ml yeast s-RNA (20 mg) in 0.1 M Na acetate, *p*H 6, was added. The mixture was put on a 0.8 × 26-cm Sephadex G-25 column equilibrated with 0.1 M NaCl–0.002 Tris, *p*H 7.5. Eight-drop (1.1-ml) fractions were collected. Samples of 10 µl were digested 20 minutes at 45° in 1 ml hyamine hydroxide and counted in 10 ml toluene scintillation fluid. (B) Control separations. Yeast s-RNA and borohydride-reduced [^{14}C]porfiromycin were chromatographed separately on similar Sephadex G-25 columns of the same dimensions. There is no peak of radioactivity in the s-RNA region.

mmole. The cells were harvested and washed four times with 30-ml portions of 0.02 M Tris buffer, *p*H 7.4, and the wet cells were stored frozen. s-RNA was prepared from 1.95 g of these cells by the method of Fleissner and Borek (1962). The resulting product contained 4550 cpm/mµmole nucleotide. Separation of the four nucleotides by alkaline hydrolysis and subsequent electrophoresis revealed the label to be divided in the following manner: (as cpm/mµmole nucleotide) GMP, 7740; AMP, 2280; CMP, 111; and UMP, 43.

The specific activity of the free guanine base in the isolated 2′(3′)-GMP was determined as follows: The nucleotide was degraded in turn to guanosine and free guanine by treatment with *E. coli* phosphomonoesterase and then with periodate and lysine, using the conditions described by Neu and Heppel (1964). Free guanine was separated from other reaction products by electrophoresis in 0.05 M sodium tetraborate, *p*H 9.1. The specific activity of the eluted guanine was found to be 4480 cpm/mµmole.[1] Determination of this value by hydrolysis of the GMP in 0.1 N HCl at 100° for 1 hour was not feasible, since the acid labilized the tritium and gave an apparent specific activity of only 492 cpm/mµmole, barely 6% that of the nucleotide.

This preparation of tritiated s-RNA was alkylated with [^{14}C]porfiromycin in the usual manner (see legend, Figure 3). After the alkylated s-RNA had been separated from the unreacted porfiromycin by passage over a Sephadex G-25 column, simultaneous counts of ^{3}H and ^{14}C showed that alkylation had proceeded to the extent of 1 porfiromycin for 528 nucleotide residues. This result is comparable to the limit values previously reported (Weissbach and Lisio, 1965).

The alkylated s-RNA solution was stored in 0.05 M NaCl–0.002 M Tris, *p*H 7.1, at 3° for 6 days in order to allow the release of alcohol-soluble fragments to take place. Two volumes of cold ethanol were added, and the solution was centrifuged after storage at 3° overnight. The supernatant solution was concentrated and chromatographed in system 2. Strip counting showed a heavy density of counts at the origin and a less active spot migrating with an R_F of 0.23 (Table III). The latter substance was eluted, rechromatographed, and examined as before for ^{3}H and ^{14}C. Using the known specific activities for porfiromycin and guanine, this material proved to contain 2.07 mµmoles guanine to 1 mµmole porfiromycin. Rechromatography of the origin material in system 1, where it had the expected R_F of 0.8, led to isolation of a substance containing approximately equimolar guanine and porfiromycin. Neither the monoguanyl nor the diguanyl derivative contained demonstrable amounts of ribose, as measured by the orcinol reaction; therefore the ^{14}C counts were a valid measure of guanine.

Discussion

The homopolymers are alkylated to a much greater extent than s-RNA. Even poly-U, the poorest-reacting one of those tested, gave a limit alkylation of 1 porfiromycin for 500 nucleotides, which is approximately the best level ever achieved with s-RNA. Poly-G was alkylated to a level of 1%, and the G in poly-GU approached 1.3%. It is indeed possible that the secondary structure of s-RNA is interfering, and that the N-7 position of guanine is not easily available for alkylation because of steric hindrance, even though it is unbonded.

Iyer and Szybalski (1964), on the basis of observations that mitomycin-treated DNA now undergoes a reversible rather than an irreversible heat denaturation, postulated that the antibiotic alkylates the DNA, and that this alkylation occurs in such manner as to "cross-link" the DNA strands. The susceptibility of DNA to such "cross-linking" increases with the G-C content

[1] Similar treatment of the isolated 2′(3′)-AMP gave a specific activity of 480 cpm/mµmole in the resulting adenine. This indicates that most of the radioactivity of the AMP (2280 cpm/mµmole) was contained in the ribose moiety.

of the DNA. This narrows the favored binding sites to G-G, C-C, or G-C. We have direct evidence that mitomycin does bind firmly to s-RNA, and that this reaction may yield either a monoalkylated guanine or a diguanyl-mitomycin linkage, since both substances were isolated. We have found no evidence for involvement of C-C or G-C binding sites.

The formation of mono- and diguanyl derivatives of mitomycin is analogous to the situation found with other alkylating agents (Brookes and Lawley, 1961). In their work, monoalkylating agents such as ethylene oxide reacted with guanosine and guanylic acid to give a monoalkylated derivative, while the bifunctional agents such as butadiene oxide or di-(2′-chloroethyl)-methylamine were also observed to yield some diguanyl compounds. On the basis of the data in the present paper, it is proposed that mitomycin and related compounds can alkylate nucleic acids either singly or bifunctionally. Also, in accord with the observations by Brookes and Lawley (1961), alkylation of the guanine residues labilizes the guanine-ribose bond and permits ejection of mono- and diguanyl derivatives of the alkylating agent.

The residual s-RNA shows no change in Sephadex behavior or alcohol precipitability but these methods would not distinguish intact s-RNA from fragments of one-fourth that size. It is very unlikely that the depurinated ribose-phosphate backbone remains intact for long. There is evidence from work on DNA (Weissbach and Lisio, 1965) that fragmentation of the DNA molecule may also occur after alkylation. A specific cleavage of a double-stranded molecule at the site of adjacent GC-CG pairs could be a very useful tool, and as homogeneous preparations of specific s-RNA's become available, mitomycin and its analogs may prove valuable in structure determination.

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