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Effects of Alkylation of Phosphodiesters and of Bases on Infectivity and Stability of Tobacco Mosaic Virus RNA

(ethyl nitrosourea/ribophosphotriesters/RNA chain breakage/biological inactivation)

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ABSTRACT **Upon ethyl nitrosourea treatment of RNA of tobacco mosaic virus, up to four phosphodiester groups may be alkylated per molecule without chain breakage, as shown by sucrose gradient centrifugation. This indicates that ribophosphotriesters are quite stable. However, when this alkylation reaction is of longer duration and 6 to 10 triesters are formed, then an average of 1 to 2 breaks occurs and little or no intact RNA can be isolated. Methyl nitrosourea is less effective in forming triesters (about 25% of total alkyl groups compared to about 65% for ethyl nitrosourea), and a greater number of alkyl groups can, therefore, be introduced before breaks occur. Diethyl sulfate and dimethyl sulfate, which alkylate almost only the bases of nucleic acids, do not cause significant degradation of RNA of tobacco mosaic virus, even when as many as 70 alkyl groups are bound.**

All types of alkylation cause similar losses in viral infectivity at low levels of alkylation. Thus, an average of two chemical events leads to one lethal event, regardless of the nature of the alkylating reaction, which, for example, is with dimethyl sulfate about 65% on the N-7 guanine while with ethyl nitrosourea it is about 65% on phosphodiesters. It is thus concluded that all alkyl groups, whether on the base or on the phosphate, have the same potential to cause inactivation and that inactivation of RNA can result from phosphotriester formation *per se*.

Alkylation of nucleic acids by ethyl nitrosourea (EtNU) in neutral aqueous solution has been shown to occur primarily on the O^6 of guanine and on the oxygen of phosphodiesters (1, 2). About 65% of the total alkylation of RNA *in vitro* and of HeLa cell DNA *in vitro* and *in vivo* is in the form of phosphotriesters, while 8–12% is in the form of O^6-ethylguanine (1, 2). EtNU is a very potent carcinogen (3, 4), and the mechanism of its biological effect has been studied by Goth and Rajewsky (5–7), Lijinsky *et al.* (8), and Swann and Magee (9). All agree that there is no correlation between tumor formation in rats and the amount of 7-ethylguanine found in the RNA or DNA of various tissues. Goth and Rajewsky (6, 7) propose that ethylation of the O^6 of guanine is the significant biological event, but they do not consider phosphate alkylation, although their data can now be interpreted as indicating about 70% phosphotriester formation (1, 2).

In the course of a general study of the alkylation of nucleic acids, this laboratory has now focused on the chemical and biological effects of phosphate alkylation. In this paper we present data on the stability of ribophosphotriesters in tobacco mosaic virus (TMV) RNA and the effects of triester formation on the infectivity of intact (30S) TMV RNA.

It has long been believed that, in contrast to deoxyribophosphotriesters, ribophosphotriesters were extremely unstable, and as soon as a triester was formed, chain scission occurred (10–13). This was based primarily on indirect evidence, since no ribophosphotriester had been isolated or synthesized while deoxyribophosphotriesters had been synthesized and found to be stable under a variety of conditions (14–16). Two exceptions to this generalization are that cyclic-AMP alkyl phosphotriesters have been synthesized and found to be stable in dilute solution at room temperature for days (17), and that Szer and Shugar found that uridine 5′-phosphate dimethyl ester was resistant to hydrolysis (18).

The first isolation of ribophosphotriesters from an alkylated RNA was reported by Singer and Fraenkel-Conrat (1), who chromatographically separated material from enzyme digests of EtNU-treated TMV RNA which corresponded to dinucleoside ethyl phosphate and in magnitude represented about a third of the total phosphate diester alkylation. In the present study we present evidence that TMV RNA remains intact at neutrality when up to four phosphotriesters are present and that such RNA can be subjected to repeated centrifugation in a sucrose gradient at 20° without degradation. The relative alkali instability of TMV RNA containing phosphotriesters, as compared to unmodified or base-modified RNA, was demonstrated after heating at or above pH 8.

MATERIALS AND METHODS

[^{14}C]Ethyl nitrosourea (4.1 mCi/mmol) was obtained from Farbwerke Hoechst AG, Germany; [^{3}H]methyl nitrosourea (17.5 mCi/mmol) from New England Nuclear; [^{14}C]diethylsulfate (4.09 mCi/mmol) from ICN; and [^{14}C]dimethyl sulfate (8.3 mCi/mmol) from Schwarz/Mann. The actual specific activity of each reagent was determined from the specific activity of purified labeled alkyl derivatives with known absorption coefficients (7-alkylguanine, 1-alkyladenine). They are 4.1 mCi/mmol, 6.5 mCi/mmol, 2 mCi/mmol, and 4.4 mCi/mmol, respectively.

TMV RNA was prepared by the phenol method used in this laboratory. Prior to precipitation of the RNA from the aqueous phase with alcohol, an equal weight of bentonite ($^1/_{20}$ of virus weight) was added and remained present until it was removed in the final centrifugation (19, 20). Since the

Abbreviations: EtNU, ethyl nitrosourea (*N*-ethyl-*N*-nitrosourea); MeNU, methyl nitrosourea (*N*-methyl-*N*-nitrosourea); Et_2SO_4, diethyl sulfate; Me_2SO_4, dimethyl sulfate; LE, lethal event; TMV, tobacco mosaic virus.

RNA during its isolation from the virus, as well as from reaction mixtures, was frequently in distilled water containing traces of EDTA, and thus fully hyperchromed, it may be assumed that most secondary structure was lost and any "hidden breaks" exposed.

Treatment of TMV RNA with Ethyl Nitrosourea, Methyl Nitrosourea, Diethyl Sulfate, and Dimethyl Sulfate. TMV RNA (1 mg) in 1 ml of 0.1 M EDTA pH 7.0 was treated with 10 mg of EtNU or 1 mg of methyl nitrosourea (MeNU) at 22°, or with 10 μl of diethyl sulfate (Et_2SO_4) or 1 μl of dimethyl sulfate (Me_2SO_4) at 37° in order to achieve the desired degree of alkylation. Each reagent was first dissolved in 0.1 ml of 95% ethanol. Aliquots (0.15 ml each) were taken from each reaction mixture immediately after reagent was added and various times thereafter (15–120 min) and immediately precipitated with 0.4 ml of cold 95% ethanol containing 0.1 M sodium acetate pH 7. After 2 hr at −20°, the treated TMV RNA was pelleted by centrifuging at 10,000 rpm for 30 min. Each pellet was washed with ice-cold 70% ethanol containing 0.1 M sodium acetate pH 7, and redissolved in 0.1 ml of H_2O.

When radioactive reagents were used under the same conditions as above, the alkylated TMV RNA was freed from excess reagent by 4–5 ethanol precipitations. The final pellet was redissolved in 0.1 ml of H_2O. The recovery of RNA after alkylation was >90%. The radioactivity was determined in 5 ml of Bray's scintillation fluid with a Beckman liquid scintillation counter.

Sucrose Gradient Fractionation. Alkylated TMV RNA (50–150 μg) in 0.3 ml of 0.01 M Tris·HCl buffer pH 7.4 containing 0.1 M sodium chloride and 1 mM EDTA was made 0.25% in sodium dodecyl sulfate. The whole sample was layered on a 10–25% linear sucrose gradient made up in the same Tris buffer containing 0.1% dodecyl sulfate, and centrifuged in the SW 50.1 rotor at 49,000 rpm and 20° for 2 hr in a Beckman L2-65B ultracentrifuge.

When a preparative experiment was carried out, fractionation was performed with the SW 41 rotor. Up to 1 mg of TMV RNA was layered on a 10–30% linear sucrose gradient and centrifuged at 40,000 rpm and 20° for 5 hr.

Gradients were eluted from the bottom of the tube through a Gilson continuously recording spectrophotometer set to measure the absorbance at 260 nm, and collected in fractions.

[^{14}C]- or [^{3}H]alkylated TMV RNA was isolated from the ascending fractions of the elution profile, then freed from the sucrose by coprecipitation in ethanol with 150 μg of unlabeled TMV RNA. About 1/4 of the sample was heated in 1 M HCl at 100° for 1 hr, and the released alcohol was distilled in order to determine the number of labile oxygen-bound alkyl groups (1, 2). The released alcohol originates from O^6-alkylguanine and ribophosphotriesters. The relative amounts of O^6-alkylguanine and phosphotriesters formed for each reagent acting on TMV RNA is given in a previous paper (1).

The remainder of the sample again underwent gradient centrifugation with the SW 50.1 rotor as described above, and 0.38-ml fractions were collected. The absorbance at 260 nm and radioactivity of each fraction were measured.

Infectivity Assay of Alkylated TMV RNA. The alkylated TMV RNA and various fractions were assayed for remaining infectivity at levels of 0.05–50 μg/ml in the presence of bentonite according to Singer and Fraenkel-Conrat (20). At

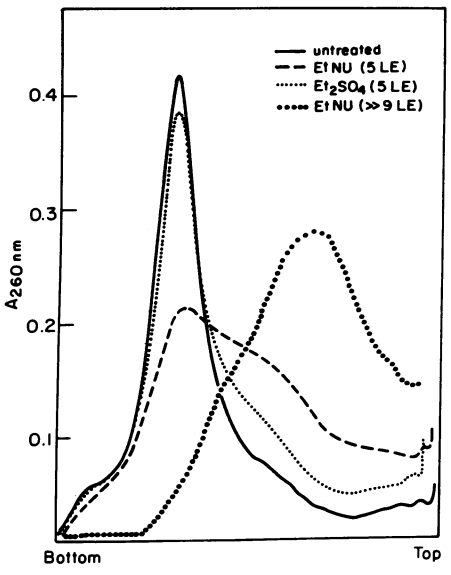

FIG. 1. Sucrose gradient sedimentation profiles of TMV RNA treated with EtNU and Et_2SO_4. The experimental method and determination of lethal events (LE) is given in *Materials and Methods*. The individual profiles have been superimposed to facilitate comparisons in this and all subsequent figures.

times, the RNA samples were also reconstituted with TMV protein prior to assay (21). The number of lethal events was calculated from the first order equation, $N/N_0 = e^{-x}$ (N_0 = initial infectivity, N = infectivity after alkylation, x = average number of lethal events).

RESULTS

Rate of Inactivation (Lethal Events) of TMV RNA Reacted with EtNU, MeNU, Et_2SO_4, and Me_2SO_4. Earlier work from this laboratory indicated that methylating agents were much more inactivating to TMV RNA than ethylating agents (1, 22) and that this was certainly due in part to the lesser rate of chemical modification by the ethylating agents used (alkyl sulfates, alkyl sulfonates, and nitroso alkyl derivatives) (23, 24). This was verified in the present work, which showed that 10-fold more reagent had to be used to make EtNU as effective as MeNU in inactivating TMV RNA at 22°.

A semi-logarithmic plot of the loss of infectivity against time at 22° (not shown) indicates that the reaction rates are first order and, therefore, the concomitant decomposition of reagent is not sufficient to noticeably affect the inactivation kinetics. The half-life of Me_2SO_4 at room temperature in H_2O at pH 7 has been determined to be 4 1/2 hr (25), and of MeNU, 1.2 hr (26). Under somewhat different conditions and at 37°, EtNU is reported to have a half-life of 15 min (27), and Et_2SO_4 a half-life of 22 min (28).

Relationship Between Lethal Events (LE), Number of Bound Alkyl Groups, and Integrity of TMV RNA. In a group of experiments using unlabeled EtNU to alkylate TMV RNA at pH 7, it was found that when about three lethal events (loss of infectivity, 95%) had occurred, the sedimentation pattern of TMV RNA was virtually unchanged, which indicates that no chain breaks had occurred. As the number of lethal events increased, progressive degradation occurred, and after about six lethal events, little or no 30S RNA could be detected in sucrose gradients. An intermediate stage of degradation (5 LE)

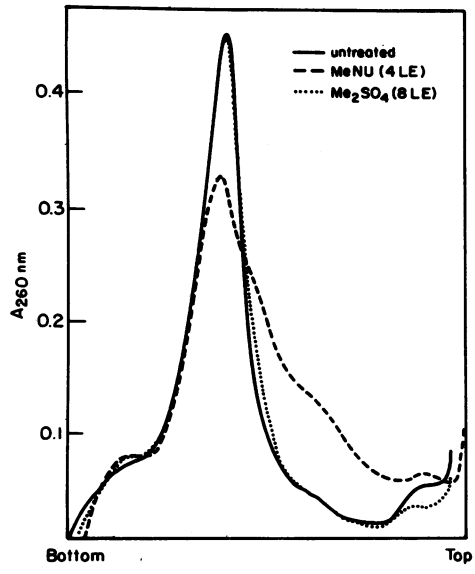

FIG. 2. Sucrose gradient sedimentation profiles of TMV RNA treated with MeNU and Me_2SO_4. The experimental method and determination of lethal events (LE) is given in *Materials and Methods*.

and complete degradation (≫9 LE) are illustrated in Fig. 1, with the comparable sucrose gradient profile for the untreated RNA and Et_2SO_4-treated RNA, with five lethal events. Methylating agents were found to act similarly, in that MeNU-treated RNA showed beginning degradation with four lethal events, while a more extensively Me_2SO_4-treated RNA (8 LE) was indistinguishable from untreated RNA (Fig. 2).

Radioactive reagents were used to determine the number of alkyl groups bound to RNA, and these data were correlated

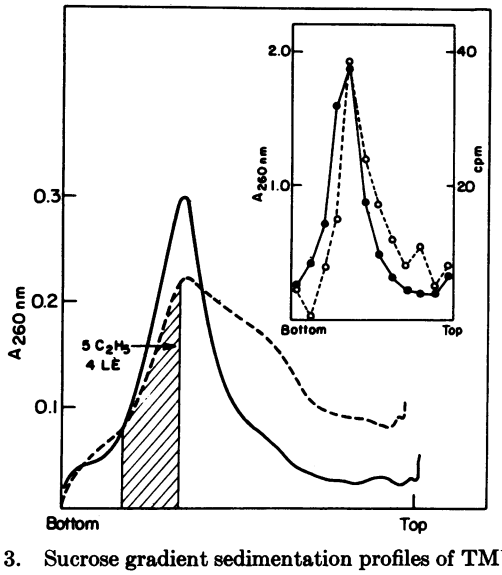

FIG. 3. Sucrose gradient sedimentation profiles of TMV RNA before (solid curve) and after (broken curve) treatment with [^{14}C]EtNU. The shaded area in the large drawing indicates the portion of [^{14}C]ethylated RNA corresponding to undegraded RNA. The number of ethyl groups and lethal events in this fraction are shown in the figure. The inset figure shows the sucrose gradient sedimentation profile of the shaded "30S" fraction after precipitation with untreated TMV RNA. ●, Absorbance of untreated TMV RNA; ○, radioactivity of [^{14}C]-ethylated TMV RNA.

TABLE 1. *Sucrose gradient fractionation of alkylated TMV RNA**

Reagent	Extent of degradation	Characteristics of 30S fraction: No. of alkyl groups	Approximate no. of: Lethal events	Approximate no. of: Alkyl groups/ lethal event
EtNU	Barely detectable	6	3	2
	Noticeable degradation†	5	4	1.2
	Noticeable degradation	10	4.5	2
	Little or no 30S left	12	5.5	2
	No 30S left	15	9	1.5
MeNU	None‡	3	1	3
	Beginning degradation‡	6	4	1.5
	Little 30S left	10	5	2
	Little or no 30S left	18	7	2.5
Et_2SO_4	None	5	2.5	2
	Barely detectable§	6	4	1.5
	Barely detectable	10	5	2
	Barely detectable	12	6	2
Me_2SO_4	None	20	6.5	3
	None¶	24	8	3

* See *Materials and Methods* for reaction conditions, determination of number of alkyl groups and lethal events, and method of sucrose gradient fractionation. The 30S fraction is defined as that which coincides with the ascending portion of untreated TMV RNA. The shaded areas in Figs. 3–5 illustrate this fraction.

† Illustrated in Fig. 3.
‡ Illustrated in Fig. 5A.
§ Illustrated in Fig. 4.
¶ Illustrated in Fig. 5B.

with the number of lethal events as determined from infectivity assays. Table 1 presents data for a number of representative experiments. The entire alkylated RNA without sucrose gradient fractionation generally had slightly more alkyl groups bound than the RNA on the gradient. Since this was particularly the case for [^{14}C]Me_2SO_4, which does not cause any RNA degradation, it is probably due to variably small amounts of nonspecific binding of methyl sulfate, not easily removed by repeated alcohol precipitation, but evidently dissociated in the dodecyl sulfate-containing sucrose used in the gradients.

The ratio of alkyl groups to lethal events, determined on RNA alkylated by all four reagents and purified by sucrose gradient fractionation, ranges from one to three, the ratio being highest for Me_2SO_4 (Table 1). For example, the EtNU-treated RNA in Fig. 3 has been degraded so that less than 50% of the RNA is 30 S. However, the high-molecular-weight fraction (shaded area) has five ethyl groups bound and the infectivity loss indicates four lethal events. When this RNA fraction is precipitated with alcohol, redissolved in water, and again fractionated on a sucrose gradient with intact TMV RNA as an internal absorbance marker, the radioactivity coincides with the 30S marker (insert, Fig. 3), indicating that the remaining, undegraded alkylated molecules are stable during the various procedures. Analysis of the refractionated RNA shows that it has retained its original alkyl groups and

TABLE 2. *Effect of alkaline pH on infectivity of EtNU- and Et_2SO_4-treated TMV RNA**

Reagent	Infectivity before treatment: % of control	Infectivity before treatment: Lethal events	Infectivity after treatment (37°) % of untreated: pH 8, 0.15 M Tris·HCl, 60 min	pH 8, 0.15 M Tris·HCl, 90 min	pH 9, 0.02 M borate, 30 min	pH 9, 0.02 M borate, 90 min
None	100	0		73		74
	100	0	85		105	
EtNU	27	1–2		14		10
	2.9	3–4	26		45	
Et_2SO_4	14	2		72		77
	0.8	4–5	85		100	

* TMV RNA, alkylated and fractionated by sucrose gradient as described in *Materials and Methods*, was assayed for infectivity before and after heating in buffered solution containing 300 μg of bentonite per ml. A total of 29 to 36 half-leaves was used for each assay sample, and the reproducibility was ±15%. Infectivity before heating is expressed in % of untreated RNA, while infectivity after heating is expressed as % of the unheated sample. Thus, EtNU-treated RNA (2.9% infectivity = 3–4 LE) further loses infectivity at pH 8, 60 min, equivalent to 1 to 2 breaks and at pH 9, 30 min, equivalent to approximately 1 break.

about 85% of the radioactivity was O-alkylation (65–70% on phosphodiesters and the remainder on the O^6 of guanine). Fig. 4 illustrates the comparable experiment with Et_2SO_4-treated TMV RNA, which shows only minimal degradation. The shaded portion has six methyl groups/molecule and four lethal events. The pattern found on refractionation is shown in the insert to Fig. 4. About 20% of the radioactivity in the refractionated sample appears to be O-alkylation.

Representative data for the two methylating agents are shown in Fig. 5. In Fig. 5A, the MeNU-treated RNA containing six methyl groups is considerably less degraded than the comparable EtNU-treated RNA (5 ethyl groups) in Figs. 1 and 3. The O-alkylation of MeNU-treated RNA is about 25%. Me_2SO_4, which does not cause appreciable O-alkylation, does not cause degradation even when 70 methyl groups are introduced. Fig. 5B shows TMV RNA still intact when methylated with Me_2SO_4 to as high an extent as is possible while still retaining detectable infectivity (24 methyl groups and 8 LE).

Lability of TMV RNA Containing Alkylated Phosphodiester Bonds. Chain breakage, as a secondary consequence of phosphotriester formation, was demonstrated in various ways. During reaction with EtNU or MeNU some breakage takes place, since, with increasing amounts of alkylation, the amount of 30S RNA decreases (Fig. 5A), until with about 0.3% alkylation (18 alkyl groups), no 30S RNA is left (Fig. 1). The alkyl-sulfate-treated RNAs show little if any evidence of breakage even with 0.4% (Fig. 5B) or more alkylation.

Undegraded EtNU-treated TMV RNA containing 1 to 4 triesters (2–10 alkyl groups) loses infectivity when incubated at 37° in pH 8 or pH 9 buffers (Table 2), while Et_2SO_4-treated RNA (4–8 alkyl groups) is as stable as untreated RNA. After incubation, representative samples were centrifuged in sucrose gradients (see *Materials and Methods*) to determine the extent of chain breakage. Of the two ethylating reactions, only EtNU-treated RNA showed marked loss of 30S RNA, indicating triester breakage. Similarly, MeNU-treated RNA exhibited alkali lability resulting in chain scission, while no degradation was observed with Me_2SO_4-treated RNA containing 70 methyl groups.

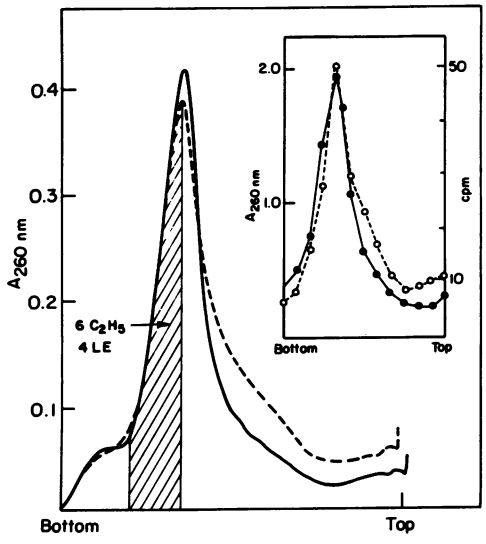

FIG. 4. Sucrose gradient sedimentation profiles of TMV RNA before (solid curve) and after (broken curve) treatment with [^{14}C]Et_2SO_4. See legend to Fig. 3 for details.

When reconstitution was used in our procedure for increasing the sensitivity of assaying TMV RNA, it was found that reconstituted alkyl-nitrosourea-treated RNA was less infective, relative to reconstituted untreated RNA, than the treated RNA itself, relative to untreated RNA. Since this was not the case for reconstituted alkyl-sulfate-treated RNA, it is likely that the reconstitution conditions (0.1 M pyrophosphate pH 7, 30°, 6 hr) were sufficient to cause some phosphotriester breakage. This can be illustrated by the following data. Six Et_2SO_4-treated RNA samples with an average remaining infectivity of 0.7%, when reconstituted had an average infectivity of 0.6%. The same type of experiments with EtNU-treated samples showed 3.3% infectivity as the RNA and 1.6% infectivity upon reconstitution, a loss of about 50%.

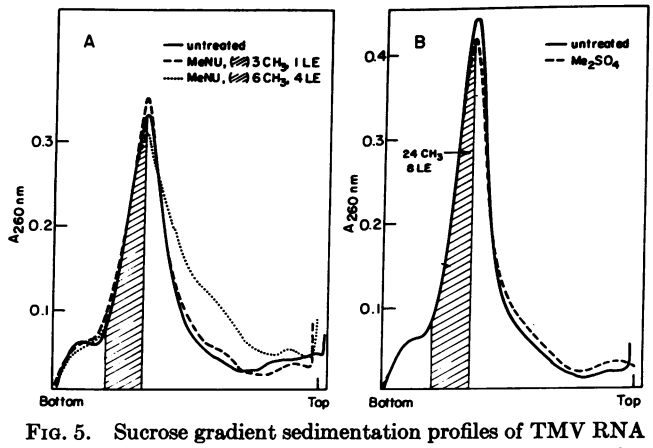

FIG. 5. Sucrose gradient sedimentation profiles of TMV RNA before and after treatment with (A) [^{3}H]MeNU and (B) [^{14}C]-Me_2SO_4. The shaded areas indicate the fractions used to determine the number of bound methyl groups and the number of lethal events shown in the figures. See *Materials and Methods* for experimental details.

DISCUSSION

The biological effect of ribophosphotriester formation has been assumed to be inactivation as a result of chain scission (11–13). We find, however, that as many as four phosphotriesters can be present in undegraded TMV RNA. Although such triesters can be shown to be significantly alkali-labile, triester-containing TMV RNA can be subjected to repeated sucrose gradient centrifugation and alcohol precipitation at neutrality, without breakage. "Hidden breaks" due to secondary structure, which would not be likely to survive our methods of handling the RNA, also were not detected when RNA treated with 1% HCHO in 90% formamide was subjected to gradient centrifugation (data not shown).

Intact EtNU-treated TMV RNA, isolated from sucrose gradients and by analysis having 6 to 15 alkyl groups, loses infectivity corresponding to one lethal event per two alkyl groups (Table 1). Since two out of three of the alkyl groups are found on analysis to be on phosphates, and less than 0.4 are on the O^6 or N-7 of guanine, it is concluded that the formation of a triester can be lethal. The same must be true for 7-methylguanine since alkyl-sulfate-treated TMV RNA (5–24 ethyl or methyl groups), in which this alkylation accounts for two out of three chemical events and no other alkylation exceeds 0.3, is equally efficiently inactivated, that is, one lethal event occurs per 2 to 3 alkylations. This conclusion differs somewhat from an earlier one where we reported that while *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment of TMV RNA caused one lethal event per 1 to 2 bound alkyl groups, dimethyl sulfate treatment indicated about 10 methyl groups per lethal event (29). We now find that methyl sulfate remaining after hydrolysis of dimethyl sulfate tends to be nonspecifically bound and completely removed only upon sucrose gradient fractionation, a procedure not used in the earlier work. It may be noted that intact TMV RNA with 70 methyl groups has been obtained by such procedures, illustrating the lack of chain breaks as a secondary effect of RNA base methylation, as contrasted to DNA in which depurination of alkyl purines may lead to breaks.

The lability of ribophosphotriesters can be roughly calculated from the approximate molecular weight of a degraded TMV RNA containing 10 triesters, which shows a broad peak of about 18S (average, 700,000 daltons) on a sucrose gradient (Fig. 1). This would indicate that an average of two breaks occurs during the time that 10 triesters are formed. Assuming that the kinetics of alkylation and chain breakage are constant, this is in agreement with our observation that EtNU-alkylated TMV RNA with 4 triesters was only partially degraded (Fig. 1) and the 30S component remained undegraded through additional separations. We observe little difference in the number of alkyl groups of 30S and degraded RNA which is in line with expectation, since the act of chain breakage at the triester, probably after intermediate 2′:3′ cyclization (11), would produce a stable 3′-alkyl phosphate terminus.

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