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Reconstitution of rods from tobacco mosaic virus protein and RNA modified with bulky carcinogens

(benzo[*a*]pyrene diol epoxide/acetylaminofluorene/virus assembly)

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ABSTRACT **Tobacco mosaic virus (TMV) RNA was treated with radioactive *N*-acetoxy-2-acetylaminofluorene (*N*-acetoxy-AAF) and (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (B*a*P diol epoxide) to obtain 3–25 adducts per molecule. Modified full length 30S RNAs and unmodified RNA were reconstituted for various time periods with TMV protein. The particulate products were separated by ultracentrifugation, and the amounts of virus-like material were quantitated by UV spectrophotometry. The length distribution and general appearance of the virus-like rods were studied by electron microscopy. Neither type of carcinogen prevented typical rod formation, but the rate of formation and the maximal yield of reconstituted particles diminished with increasing modification by both agents. The rod length distribution also showed progressively lesser numbers of full-length virus rods. The particulate material contained approximately the same number of adducts as the modified RNA. Thus, it appears that these carcinogen modifications of guanine residues at the N-2 or C-8 atoms did not prevent orderly protein assembly on the RNA but instead slowed up this process and frequently stopped it, possibly at sites where adducts happen to be clustered.**

It was previously reported from our laboratories that reconstitution of tobacco mosaic virus (TMV) RNA, modified by 2–11 groups of *N*-acetoxy-2-acetylaminofluorene (*N*-acetoxy-AAF) or (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (B*a*P diol epoxide) with TMV protein decreases the yield of sedimentable material, though not proportionately to the extent of adduct formation (1). To test the hypothesis that assembly initiation might be the critical event, we now have extended these studies to investigate the kinetics of reconstitution both with TMV A-protein and with TMV protein preparations rich in the disk aggregates needed for rod initiation (2). Electron micrographs of the reconstituted virus particles were studied both in terms of length distribution and appearance of the rods. The finding that assembly of sedimentable material proceeded more slowly and less completely, and that this material consisted of rods of widely varying lengths, mostly shorter than 300 nm, suggests that adduct formation does not specifically interfere with initiation. The failure to detect any gross deformation in the resultant rods is not surprising if the radial location of the RNA and the loose protein conformation in that area (3) are taken into account.

MATERIALS AND METHODS

[^{3}H]B*a*P diol epoxide (specific activity, 565 mCi/mmol; 1 Ci = 3.7 × 10^{10} becquerels) and *N*-[^{14}C]acetoxy-AAF (specific activity, 49.6 mCi/mmol) were supplied by the National Cancer Institute, Division of Cancer Cause and Prevention, Bethesda, MD. TMV, TMV RNA, and TMV A-protein were prepared by the usual procedures (4–6). The viral protein was extracted with 67% acetic acid and precipitated by thorough dialysis against H_2O at 0°C. Disk-enriched protein preparations were obtained by dialyzing the protein suspensions against 0.05 M phosphate (pH 7.0) at 20°C (2). TMV RNA was modified in 10 mM Tris (pH 7.4) by addition of various amounts of carcinogens (1). Incubation was carried out in the dark at 37°C for 30 min for the B*a*P diol epoxide-modified samples and for 3 hr for the AAF-modified samples. The RNAs were extensively extracted with water-saturated ethyl acetate and precipitated with alcohol.

Modified and control RNAs were subjected to sucrose gradient centrifugation to separate undegraded 30S RNA from fragments. Sucrose gradient purification of the RNA was with 7.5–35% sucrose in 5 mM Tris, pH 7.8/50 mM NaCl/2.5 μM EDTA/0.05% diethylpyrocarbonate; 11-ml samples were spun in 9-cm tubes in a SW 41 rotor at 36,000 rpm for 15–16 hr at 4°C. Most of the modified samples showed doubtful or no evidence for degradation, but occasionally, and seemingly randomly, extensive degradation was observed. Usually only the ≈30S material was used for reconstitution.

Reconstitution was at 30°C with 50 μg of RNA and 1.8 mg of protein per ml of 0.1 M sodium pyrophosphate (pH 7.1) (5); aliquots containing about 30–40 μg of RNA were removed after 0.33–24 hr. These were diluted 1:20 in H_2O at 0°C and were subjected to three cycles of differential centrifugation (10 min at 8,000 rpm, followed by 2 hr at 40,000 rpm). The sediments obtained at high speed were taken up in 1 ml of 0.1 M NaCl.

After these repeated sedimentations, the UV absorption spectra of the samples were plotted. These were usually typical for TMV, with a maximum at 260 nm and a gently descending wiggly slope from 260 to 270 nm. Thus, any non-protein-covered RNA was not sedimented, apparently having been degraded. Based on authentic TMV, A_{260} = 0.27 was taken as the value equivalent to 0.1 mg of virus per ml. These samples were often tested for infectivity. The level of modification of the reconstitutable RNA, as compared to that of the original preparation of modified RNA, was determined by radioactivity measurement.

The technique of specimen preparation and staining with uranyl formate that was used for electron microscopy was as described by Williams (7).

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Abbreviations: TMV, tobacco mosaic virus; B*a*P diol epoxide, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; *N*-acetoxy-AAF, *N*-acetoxy-2-acetylaminofluorene.

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RESULTS

The yields upon reconstitution of TMV RNA modified by *N*-acetoxy-AAF and B*a*P diol epoxide, respectively, with TMV A-protein are shown in Fig. 1. The data from experiments using protein preparations rich in disk aggregates showed the same general pattern of decreasing nucleoprotein yields upon increasing adduct formation. The difference in the kinetics, indicating a relatively slower assembly for the modified RNAs, is more clearly shown by the tabular presentation of data for several experiments with the two types of modified RNA (Table 1). It is evident that adduct formation slowed up the assembly, besides decreasing the maximal amount of sedimentable material. This was so for modification by both reagents, even though their mode of action differs. The AAF residue, bound to the C-8 position of guanosine, is located between the neighboring bases and causes a displacement of guanosine in DNA (8). On the other hand, the bulky B*a*P diol epoxide, preferentially bound to N-2 of guanosine, is not intercalated in DNA but lies outside of the phosphodiester backbone (9).

Not shown are data on the residual infectivity of the lesser treated reconstituted RNAs, which were ≈0.6% and 0.2% of TMV for AAF (5 residues) and B*a*P diol epoxide (3.3 residues), respectively. The calculated infectivity for five lethal events is 0.7%. Thus, it appears, in confirmation of the earlier conclusion(s), that each adduct formation represents a lethal event.

The specific radioactivities (due to the adducts) in the reconstituted samples were, within the error of calculation, the same as those of the respective RNAs and differed in expected proportions with the levels of adduct formation.

Electron microscopy of the reconstitution products, with enlargements of 300-nm rods to up to 16 cm, revealed no difference in the appearance of the rods. Histograms of the distribution of rod lengths after reconstitution of modified and control RNAs are shown in Figs. 2 and 3. It is evident that adduct formation led to great predominance of rods shorter than the 300-nm class that predominated in the control. The more extensively AAF-modified RNA yielded on reconstitution almost no long rods, and, therefore, no histograms of these preparations are shown.

DISCUSSION

The first question posed in this study was whether TMV assembly initiation was particularly affected by the formation of bulky

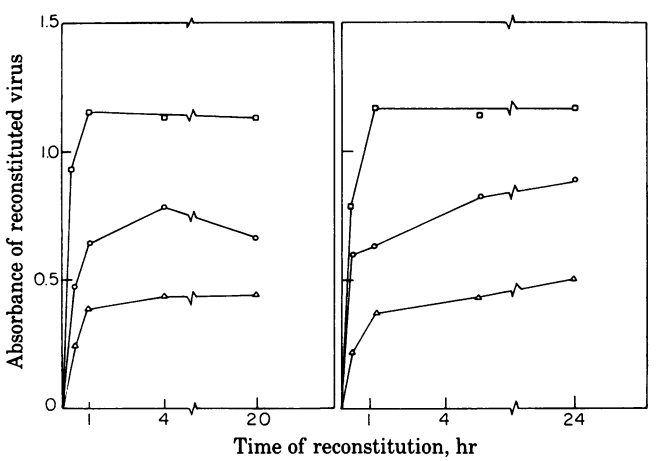

FIG. 1. Rate and extent of interaction of TMV protein and modified and control TMV RNA. The extent of modification is expressed in terms of the percentage of total nucleotides modified by B*a*P diol epoxide (*Left*) and *N*-acetoxy-AAF (*Right*). □, Control; ○, 0.08% (*Left*) and 0.11% (*Right*); △, 0.15% (*Left*) and 0.55% (*Right*).

Table 1. Comparative reconstitution rates

Modification		% of maximal reconstitution		
		A-protein		Disk-enriched protein
Agent	%	20 or 30 min*	1 hr	30 min
AAF	0.11	72, 91	71	73
	0.55	51, 53	49	45
None	—	72, 96	83	87
B*a*PDE	0.08	60		92
	0.15	54		69
None	—	83		99

AAF, *N*-acetoxy-AAF; B*a*PDE, B*a*P diol epoxide.
* See Fig. 1 for specific time.

adducts in that part of the RNA, about 5,400 and 800 nucleotides, respectively, from the 5′ and 3′ ends. This would only be expected to be the case if the many guanine residues in that part of the molecule were particularly available for modification. If assembly initiation were easily blocked by modification, one would predict that most of the rods that were formed would be derived from unmodified RNA molecules and would be 85–100% of standard length. The great preponderance of variable short rods appears to rule out this mechanism. It may be noted that upon intracellular reaction of *N*-acetoxy-AAF with simian virus 40, reaction does not occur as randomly as it appears to do with RNA *in vitro* (10). This may well be due to the site-specific loss of doublestrandedness of that DNA at the beginning of its replication.

The finding that all rods, regardless of their length, show the typical appearance of TMV rods with no deformations, protru-

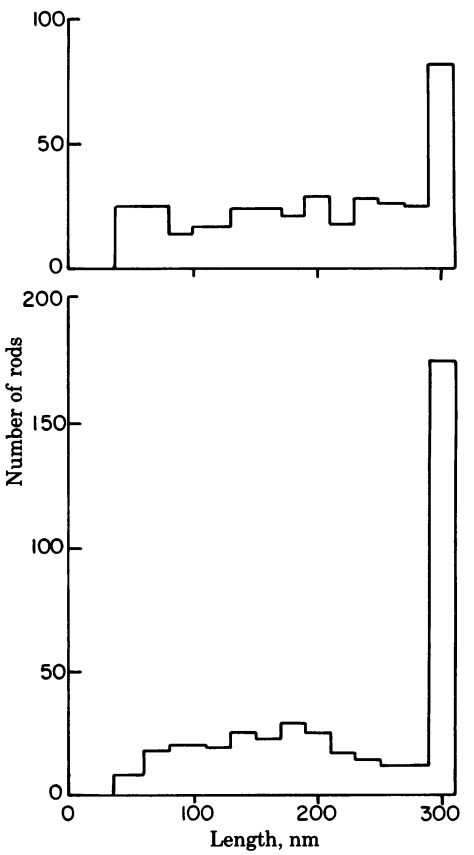

FIG. 2. Length distribution histogram of rod-like material reconstituted maximally after *N*-acetoxy-AAF treatment of the viral RNA. (*Upper*) A 0.11% adduct. (*Lower*) Control RNA.

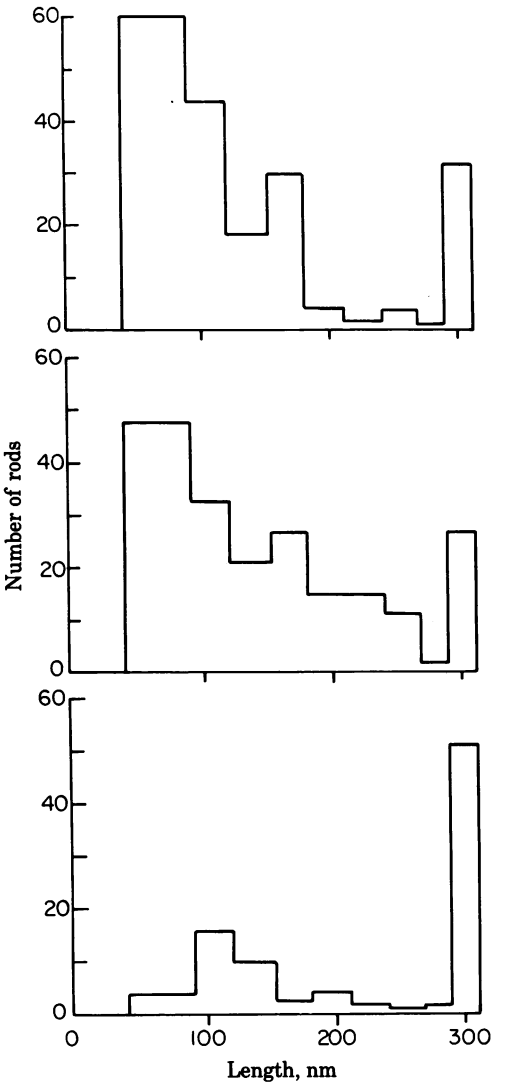

FIG. 3. Length distribution histogram of rod-like material reconstituted maximally after BaP diol epoxide treatment of the viral RNA. (*Top*) A 0.15% adduct. (*Middle*) A 0.08% adduct. (*Bottom*) Control RNA.

sions, or gaps might be interpreted as an indication that the presence of an adduct might stop further elongation. However, this cannot be the right interpretation because, in that case, the amount of adduct radioactivity in the particulate fraction should be much less than that of the total RNA, and this is not the case.

Thus, it appears that bulky substituents do not prevent protein aggregation but only diminish its rate and occasionally stop it, possibly when several adducts happen to be near one another. The fact that these large carcinogens can be accommodated in the virion is, *post facto*, not too surprising because conformational rigidity of the coat protein has been reported to exist only in the 5-nm shell exterior to the RNA-binding site. In contrast, the interior 2-nm cylindrical domain contains only 25 of the 158 amino acids of that protein and appears to lack extensive structural interactions (3).

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