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Effect of Ultraviolet Light on Mengovirus: Formation of Uracil Dimers, Instability and Degradation of Capsid, and Covalent Linkage of Protein to Viral RNA

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UV irradiation of purified mengovirus resulted in a very rapid inactivation of the infectivity of the virions (D_{37} [37% survival dose] = 700 ergs/mm^2) which correlated in time with the formation of uracil dimers in the viral RNA. During the first 2 min of irradiation, an average of 1.7 uracil dimers were formed per PFU of virus inactivated. Hemagglutination activity of the virions began to decrease only after a lag period of about 5 min and at a much lower rate (D_{37} = 84,000 ergs/mm^2). This decrease coincided in time with the appearance of altered proteins in the capsid and a structural change in the capsid. Although 10- to 20-min irradiated virions appeared intact in the electron microscope and sedimented at 150*S* in sucrose density gradients, the RNA of the virions became accessible to RNase and extractable by low concentrations of sodium dodecyl sulfate, and the virions broke down upon equilibrium centrifugation in CsCl gradients. During longer periods of irradiation (30 to 60 min), a progressively greater proportion of the virions were converted to 14*S* protein particles and 80*S* ribonucleoprotein particles composed of intact viral RNA and about 30% of the capsid proteins, alpha, beta, and gamma. Empty capsids were not detectable at any time during 60 min of irradiation, by which time disruption of the virions was complete. Irradiation of complete virions also resulted in an increased sedimentation rate of the viral RNA and in the covalent linkage to the viral RNA of about 1% of the total capsid protein in the form of heterogeneous low-molecular-weight polypeptides. The two observations seem to be causally related, since irradiation of isolated viral RNA did not result in an increase in sedimentation rate of the RNA, even though uracil dimer formation in viral RNA occurred at about the same rate and to the same extent whether intact virions or viral RNA were irradiated.

The structural arrangement of the viral RNA and the structural proteins in picornaviruses is still not entirely understood (6, 19, 27). One approach to investigating the structure and composition of these viruses has been the use of controlled degradation of the virions as a result of UV irradiation (11, 12, 26), heating (7, 9, 12, 26), or incubation under alkaline conditions (2, 3, 6, 7) followed by the analysis of the degradation products. When poliovirus is exposed to any of these treatments, the virions are rapidly converted to empty capsids, with degradation of the viral RNA and a change in the antigenic specificity of the capsid. The antigenic change is probably triggered by the release of the viral RNA (9, 11) and related to the release of the smallest of the structural proteins of poliovirus, VP4 (3).

Our studies show that UV irradiation affects mengovirus, another picornavirus, in quite a different manner, especially in that no empty capsids are formed. Upon degradation of the virus, the viral RNA is released intact, associated with about 30% of the capsid protein in the form of 80*S* ribonucleoprotein (RNP) particles. Dissociation of the virions, however, is preceded by a progressively increasing instability of the virus capsid which correlates with the loss of its hemagglutination (HA) activity and ultrastructural changes in the capsid proteins. Also, some of the proteins or parts thereof become covalently linked to the viral RNA. Some of the results have been presented in a preliminary report (R. L. Miller et al., Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 225, 1973).

MATERIALS AND METHODS

Cells and cell culture. Novikoff rat hepatoma cells (subline N1S1-67) and mouse L cells (subline L-67G) (31) were propagated in suspension culture in Swim medium 67 as described previously (24).

Preparation of radioactively labeled mengovi-

rus. N1S1-67 cells were infected with 10 to 30 PFU/cell of mengovirus as described previously (21, 24). For the preparation of virus with labeled RNA, the infected cells were suspended in basal medium 42 (BM42; 24), and the suspension was supplemented with 2 μg of actinomycin D/ml at 0.5 h after infection and with 0.5 μM [5-^{3}H]uridine (2 mCi/μmol) at 1 h. For the preparation of virus with labeled protein, infected cells were suspended in leucine-free BM42 or amino acid-free BM42, and the suspensions were supplemented with 0.067 μM [^{3}H]leucine (60 mCi/μmol) or 1 μCi/ml of ^{3}H-reconstituted protein hydrolysate, respectively, 1 h after infection. For the preparation of double-labeled virus, the infected cells were suspended in amino acid-free BM42 and supplemented with actinomycin D and [^{3}H]uridine as already described and with 1 μCi/ml of ^{14}C-reconstituted protein hydrolysate. All radioactive precursors were purchased from Schwarz/Mann.

The virus was harvested at 7 h after infection and purified as previously described (15), except that the gradient-purified virus was freed of sucrose by dialysis rather than by Diaflo-membrane filtration. Virus suspensions collected from sucrose density gradients were dialyzed overnight at 4 C against 2 liters of a solution composed of 10 mM Tris-chloride (pH 8.2) and 2 mM EDTA and then concentrated by pervaporation at room temperature. The final suspensions contained between 5×10^9 and 10^{10} PFU/ml and were stored at −20 C.

Plaque and hemagglutination assays for mengovirus. The plaque assay was conducted as follows. L-67G cells were collected by centrifugation from suspension cultures in the late exponential phase of growth between 10^6 to 1.5×10^6 cells/ml. The cells were suspended to about 5×10^5 cells/ml in minimal Eagle medium (MEM) supplemented with 5% (vol/vol) calf serum, and 5-ml amounts of this suspension were seeded into plastic petri dishes (60 mm diameter; Falcon Plastics). After 24 h of incubation at 37 C in an atmosphere of 5% CO_2 in air, the culture fluid was aspirated off and the monolayers were inoculated with 0.2 ml of 10-fold dilutions of mengovirus per plate (three plates per dilution). Virus dilutions were prepared in a solution composed of 136 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 4.2 mM Na_2HPO_4, 0.8 mM KH_2PO_4, and 0.02 mg of phenol red/ml. After 30 min of incubation at 37 C, 5 ml of MEM supplemented with 5% (vol/vol) calf serum and 0.5% (wt/vol) agarose (Seakem, Marine Colloids) were added. The plates were incubated at 37 C for 24 h and then overlayed with 3 ml of the same MEM-agarose solution, but supplemented with 0.01% (wt/vol) of neutral red. Plaques were counted after 1 to 2 h of incubation at 37 C. HA activity was determined as previously described (24).

Isolation of viral RNA. RNA was extracted by treatment of the virus with sodium dodecyl sulfate as described in the appropriate experiments. Viral RNA was also isolated by extraction with phenol and sodium dodecyl sulfate as described previously (25) or by the phenol-chloroform method of Perry et al. (17).

Irradiation of mengovirus or its RNA with UV light. Suspensions of purified mengovirus or viral RNA were diluted 1:10 in 10 mM Tris-chloride (pH 7.4). One milliliter of the dilution was placed in 60-mm plastic petri dishes and irradiated at 4 C with a General Electric 15-W germicidal lamp at a distance of 8 cm and an intensity of about 7,000 ergs per min per mm^2.

Isolation of uracil dimers. Samples (1 ml) of irradiated suspensions or purified [^{3}H]uridine-labeled mengovirus or viral RNA were mixed with 2 mg of bovine serum albumin and 1 ml of 1 N perchloric acid at 0 C. The resulting precipitate was collected by centrifugation at $1,000 \times g$ for 5 min and hydrolyzed in 88% formic acid at 175 C for 30 min as described for DNA (29, 30). The RNA was completely degraded to its constituent bases by this treatment. The hydrolysates were analyzed by ascending chromatography on Whatman no. 1 paper. The paper was developed with a solvent composed of 70 ml of isopropanol, 10 ml of concentrated NH_4OH, and 20 ml of 0.1 M H_3BO_3 at room temperature for 18 h. The dried chromatograms were cut into 1-cm segments at right angles to the direction of migration. The segments were agitated with 1 ml of water, and the paper and eluate together were analyzed for radioactivity.

Velocity sedimentation in sucrose density gradients. Linear sucrose density gradients in either buffer 6 or buffer 13 were prepared with a gradient former (ISCO model 570). Buffer 6 consisted of 10 mM EDTA, 10 mM Tris-chloride (pH 7.4), 50 mM NaCl, and 0.5% (wt/vol) sodium dodecyl sulfate. Buffer 13 consisted of 100 mM Tris-chloride (pH 8.2) and 20 mM EDTA. Conditions of centrifugation are described in the appropriate experiments. Except where indicated otherwise, 1-ml fractions were collected by means of a density gradient fractionator (ISCO model 183) which was attached to a continuously recording UV light analyzer (ISCO model UA-2) and a fraction collector (ISCO model 272). Gradient fractions were analyzed for radioactivity in acid-insoluble material as previously described (21, 24). Sedimentation rates of RNA were estimated by the method of Martin and Ames (14) by using hot phenol-extracted 29*S* rRNA from N1S1-67 cells as a standard (22).

Isolation of viral proteins and separation by gel electrophoresis. Suspensions of protein-labeled virus were supplemented with 10% (vol/vol) glacial acetic acid, 1% (wt/vol) sodium dodecyl sulfate, and 1% (vol/vol) mercaptoethanol and incubated at 37 C for 1 h. The samples were then dialyzed overnight against a solution composed of 10 mM sodium phosphate (pH 7.4), 0.1% sodium dodecyl sulfate, and 0.1% mercaptoethanol and then concentrated by pervaporation to about 0.2 ml. The samples were supplemented with 0.02 ml of glycerol and 5 μliters of a 0.1% (wt/vol) solution of bromphenol blue. The proteins were separated by electrophoresis in 10% (wt/vol) polyacrylamide gels containing 0.2% *N,N'*-methylene-bis-acrylamide as described by Maizel (13). Electrophoresis was in 20-cm tubes at about 100 V for 8 h. The gels were sliced into 2-mm slices. The slices were allowed to swell in 1 ml of water for 1 h at room temperature, and the slices and eluates together were analyzed for radioactivity.

Radioactivity determinations. Radioactivity was

measured by liquid scintillation counting. All samples were mixed with 15 ml of a modified Bray solution described previously (24).

Electron microscopy of mengovirus. Sucrose density gradient-purified mengovirus was allowed to adsorb to copper grids covered with a parlodion film while the virus was still in the sucrose solution. Sucrose was effectively removed by floating the grids on a series of droplets of 1% (wt/vol) phosphotungstic acid (pH 7.0). The stained preparations were allowed to dry and were examined with a Siemens Elmiskop electron microscope.

RESULTS

Effect of UV irradiation on infectivity and HA activity of mengovirus. Inactivation of infectivity followed single-hit kinetics (Fig. 1A), and 99% of the infectivity was lost within 1 min of irradiation. HA activity was lost much more slowly than infectivity, and the decrease in HA activity began only after a lag period of about 5 min (Fig. 1A). The doses resulting in 37% survival of infectivity and HA activity were about 700 and 84,000 ergs/mm^2, respectively.

Formation of uracil dimers in viral RNA upon irradiation of complete virions and isolated RNA. Samples of [^{3}H]uridine-labeled complete virus or of isolated viral RNA were irradiated and then hydrolyzed in formic acid, and the uracil dimers were separated from uracil chromatographically. Typical chromatograms of RNA hydrolysates of untreated mengovirus and 20-min irradiated virus are illustrated in Fig. 1C. The formation of uracil dimers upon irradiation was very rapid whether complete virus or isolated viral RNA was irradiated (Fig. 1B). In both cases, a maximum of 8 to 10% of the total uracil bases of the viral RNA had formed dimers within 10 min of irradiation. Only a small proportion of the total uracil dimers could have arisen by deamination of uracil-cytosine dimers, since [5-^{3}H]uridine is only very inefficiently incorporated into cytosine of mengovirus RNA during replication in N1S1-67 cells (23). About 80 to 85% of the total radioactivity in viral RNA was associated with UMP, and the remainder was associated with CMP.

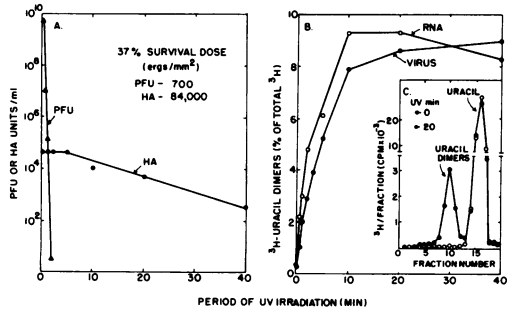

FIG. 1. *Effect of UV irradiation on (A) infectivity (▲) and HA activity (●) of mengovirus and (B and C) on formation of uracil dimers in viral RNA. (A) A suspension of purified mengovirus was irradiated, and samples of the suspension were analyzed for infectivity and HA activity as described in Materials and Methods. (B) Suspensions of [^{3}H]uridine-labeled mengovirus (●) or phenol-extracted viral RNA (○) were irradiated as in (A). At various times of irradiation, samples of virus or RNA were hydrolyzed and uracil dimers were separated by paper chromatography as described in Materials and Methods. (C) Chromatograms of hydrolysates of RNA from untreated (○) and 20-min-irradiated (●) virus. Migration is from left to right.*

Effect of irradiation on the integrity of mengovirus. Double-labeled virus ([^{3}H]RNA and [^{14}C]protein) was irradiated for various lengths of time and then analyzed by zone sedimentation in sucrose density gradients. Even after 20 min of irradiation, the virus particles still seemed intact since they sedimented at about the same velocity (150*S*; 28) as untreated virus (Fig. 2A and B). Degradation of virus only began to occur between 20 and 40 min of irradiation (Fig. 2C and D). It resulted in the release of 80*S* RNP particles which contained all of the viral RNA (^{3}H) and about 30% of the total viral protein (^{14}C). The remainder of the protein was released in the form of particles sedimenting at about 14*S*. Electron microscope examination of preparations of irradiated virus revealed no empty capsids at any stage during irradiation.

Early effects of irradiation on capsid proteins and stability of the capsid. Sedimentation analysis had shown that the virions remained essentially intact for at least 20 min of irradiation (Fig. 2B). Electron microscope examination also failed to detect any morphological difference between untreated (Fig. 3A) and 20-min-irradiated (Fig. 3B) virions. The following experiments, however, illustrate that marked changes in the capsid had already occurred during this time period of irradiation which lowered its stability and rendered the virion RNA sensitive to degradation by RNase. The RNA from nonirradiated virus was resistant to degradation by external RNase and could not be extracted by treatment with 0.02% sodium dodecyl sulfate. However, some of the virus RNA became RNase sensitive and extractable by 0.02% sodium dodecyl sulfate within 5 min of irradiation of intact virions (Fig. 4). By 20 min, when the virions still seemed morphologically intact, about 30% of the RNA became

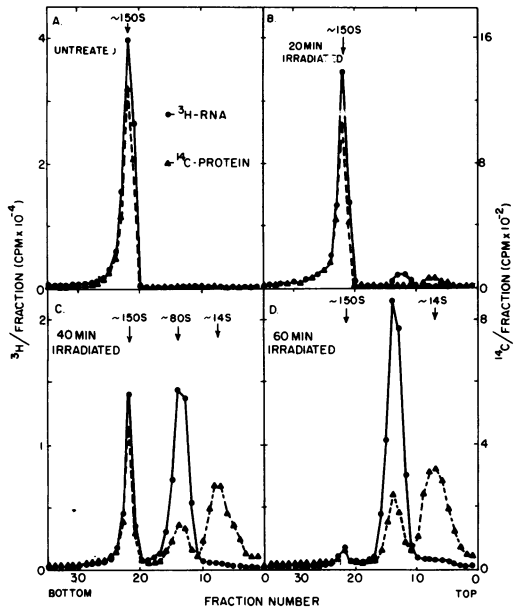

FIG. 2. *Velocity sedimentation analysis of nonirradiated and irradiated double-labeled mengovirus in sucrose density gradients. Samples of a suspension of mengovirus with [^{3}H]uridine-labeled RNA (●) and [^{14}C]amino acid-labeled protein (▲) were irradiated for 0 (A), 20 (B), 40 (C) and 60 min (D). The suspensions were then sedimented through 0.5 to 1.6 M sucrose gradients in buffer 13 for 8 h at 4 C, 22,000 rpm in an SW-27 rotor in a L-2 Beckman ultracentrifuge. One-milliliter fractions were collected from the top of the gradients and analyzed for ^{14}C and ^{3}H in acid-insoluble material. Sedimentation (S) coefficients were estimated by the method of Martin and Ames (14), assuming a sedimentation coefficient of 150S for intact mengovirus (28).*

degraded and released from the virions upon incubation with RNase, and about 60% was extractable by 0.02% sodium dodecyl sulfate. Further, 10-min-irradiated virus was converted during equilibrium centrifugation in a CsCl gradient to an RNP particle with a higher density (1.39 g/cm^3; Fig. 5B) than that of untreated virus (1.32 g/cm^3; Fig. 5A). This RNP particle was probably equivalent to the 80S RNP particle detected by zone sedimentation in sucrose density gradients (Fig. 2C and D). The protein that was released during breakdown of the irradiated virus equilibrated at a density of 1.28 g/cm^3 (Fig. 5B).

The appearance of unstable virions during irradiation coincided with marked changes in the electrophoretic mobility of the capsid proteins (Fig. 6). Leucine-labeled virus was irradiated for various time periods, and the 150S particles were isolated by sucrose density gradient centrifugation. The proteins were isolated from these particles and analyzed by polyacrylamide gel electrophoresis. The gel profiles of untreated virus (Fig. 6A) were similar to those reported for mouse Elberfeld virus by Rueckert and his co-workers (6, 27, 34) and the structural proteins were designated alpha to epsilon as suggested by these investigators. The distribu-

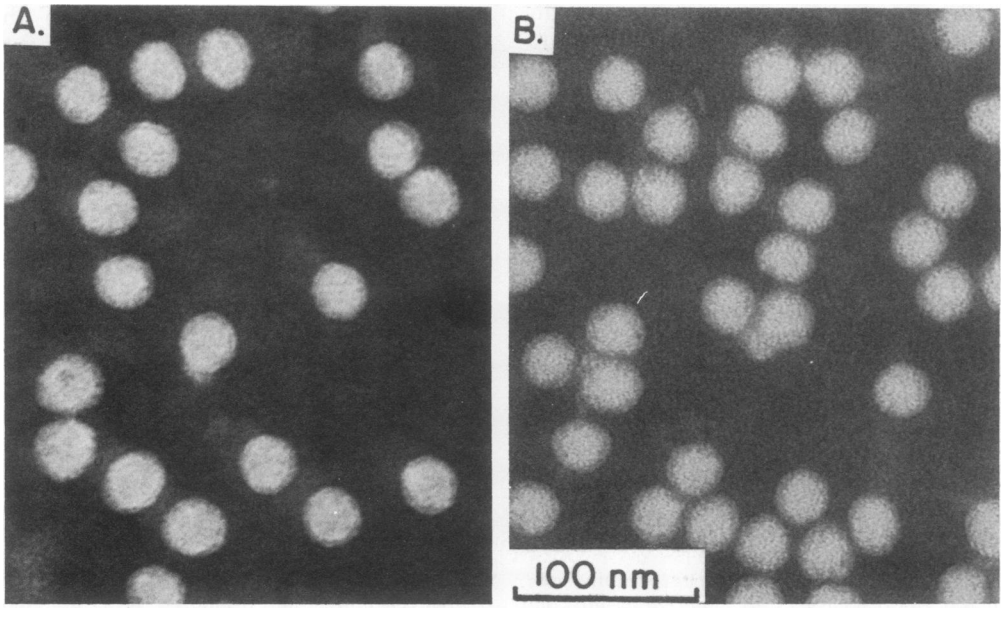

FIG. 3. *Electron micrographs of nonirradiated (A) and 20-min-irradiated mengovirus (B).* ×240,000.

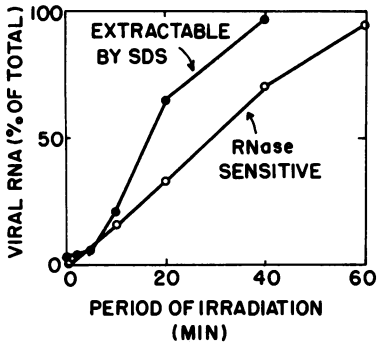

FIG. 4. *Effect of UV irradiation on stability of viral capsid. A suspension of [³H]uridine-labeled mengovirus was irradiated, and at various times of irradiation samples were analyzed for the following. (i) Amount of RNA in virion sensitive to RNase. The samples were supplemented with 10 μg of both RNase A and T1 per ml and incubated at 27 C for 30 min. The digests were sedimented through 0.5 to 1.6 M sucrose gradients as illustrated in Fig. 2A, and the 150S regions (intact virions) were analyzed for radioactivity in acid-insoluble material. (ii) Amount of RNA in virion extractable by treatment with 0.02% (wt/vol) sodium dodecyl sulfate. The samples were supplemented to 0.02% with sodium dodecyl sulfate and incubated with frequent mixing at 37 C for 10 min. The extracts were sedimented through 0.15 to 0.9 M sucrose gradients as illustrated in Fig. 7, and the 34 S regions (viral RNA) were analyzed for radioactivity in acid-insoluble material.*

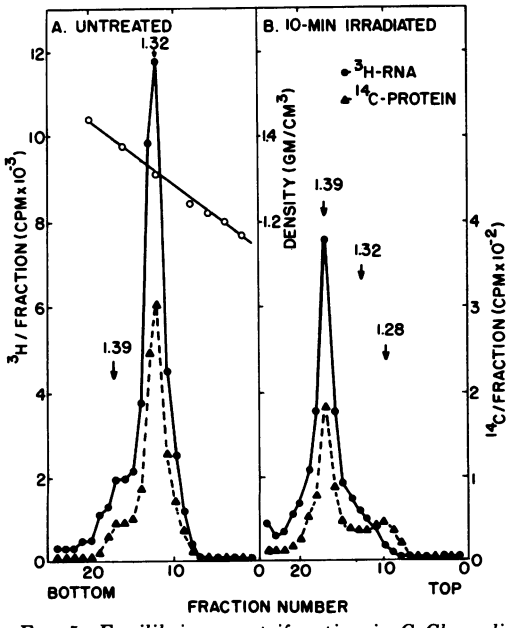

FIG. 5. *Equilibrium centrifugation in CsCl gradients of nonirradiated (A) and 10-min-irradiated virions (B). Samples of untreated and 10-min-irradiated double-labeled mengovirus ([³H]RNA and [¹⁴C]protein) were diluted with water to 5 ml and mixed with 2.417 g of CsCl (density = 1.30 g/cm³). The suspensions were centrifuged to equilibrium in an SW50 rotor at 35,000 rpm (40 h, 4 C). Fractions of 0.25 ml were collected and analyzed for density (○) by weighing 0.1 ml in a volumetric pipette and for ¹⁴C (●) and ³H in acid-insoluble material (▲).*

tion of leucine-label among the structural proteins is indicated in the graph and was also similar to that reported for mouse Elberfeld virus (6, 34). No significant difference could be detected between the gel profiles of untreated virus and 5-min-irradiated virus (not shown). After 10 min of irradiation, however, a larger, new protein of about 70,000 daltons and heterogenous material migrating between the gamma and delta proteins had been formed (Fig. 6B). The nature and origin of these proteins is uncertain, but we have tentatively designated these proteins the large and small photoproducts, respectively. Further, the two structural proteins of similar molecular weight, alpha and beta, of the irradiated virions migrated as a sin-

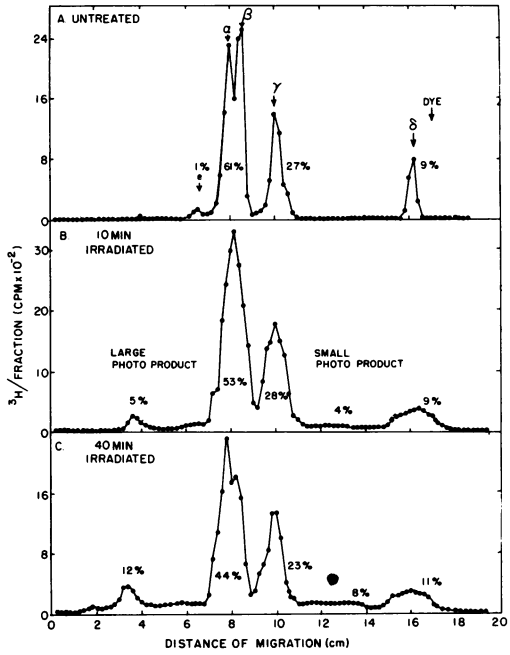

FIG. 6. *Polyacrylamide gel electrophoresis of proteins of irradiated and untreated virions. Samples of untreated and irradiated [³H]amino acid-labeled virus were sedimented through 0.5 to 1.6 M sucrose gradients as illustrated in Fig. 2. The 150S particles were isolated from the gradients by precipitation with 2 volumes of ethanol and disrupted, and the proteins were separated by electrophoresis on 10% acrylamide gels as described in Materials and Methods.*

gle broad band, and the proteins in the delta region migrated very heterogeneously (Fig. 6B) in contrast to the delta protein of nonirradiated virus which migrated as a sharp band (Fig. 6A). We do not know whether the heterogeneous proteins from the irradiated virus that migrated in the delta region represented modified delta protein or were degradation products of other proteins. The formation of altered proteins in the 150S particles continued with time of irradiation (Fig. 6C) and may have been responsible for the eventual disruption of the particles (Fig. 2C and D).

UV light-induced changes in sedimentation properties of viral RNA and covalent linkage of polypeptides to viral RNA. In addition to inducing changes in the properties of the structural proteins of the virus, UV irradiation resulted in an increase in the sedimentation velocity of the viral RNA. RNA extracted from 20-min-irradiated [^{3}H]uridine-labeled virus sedimented significantly faster (about 41S) in a sucrose density gradient than did the RNA from untreated virions (^{14}C-labeled, about 34S) (Fig. 7B). This increase in sedimentation rate of the viral RNA occurred only when whole virus was irradiated. Irradiation of phenol-extracted viral RNA had no effect on the sedimentation properties of the RNA (Fig. 7C), even though the formation of uracil dimers occurred as rapidly as when whole virus was irradiated (Fig. 1B). Thus, the increase in sedimentation rate of the RNA of irradiated virus was not due to the formation of uracil dimers per se.

To determine whether the increase in sedimentation rate of the RNA was related to changes in secondary structure, we determined the sedimentation properties of RNA from irradiated and untreated virions in sucrose density gradients of varying ionic strength and compared them with the sedimentation properties of rRNA from Novikoff cells. Firstly, we found that the sedimentation rate of mengovirus RNA per se was significantly more affected by changes in ionic strength in the gradient than was that of rRNA. Whereas mengovirus RNA sedimented more rapidly than 29S rRNA in gradients of intermediate (15; Fig. 7A) or high ionic strength (Fig. 8B), it sedimented at about the same rate as 29S rRNA in gradients of very low ionic strength (Fig. 8A). A similar observation has been made with the RNA of encephalomyocarditis virus (16). Thus, caution must be exercised in calculating the S value and the molecular weight of a viral RNA based on its sedimentation rate relative to that of rRNA. Nevertheless, for reasons of simplicity, we will continue to refer to mengovirus RNA as having a sedimentation constant of 34S as estimated from its sedimentation rate relative to that of hot phenol-extracted 29S rRNA in gradients of

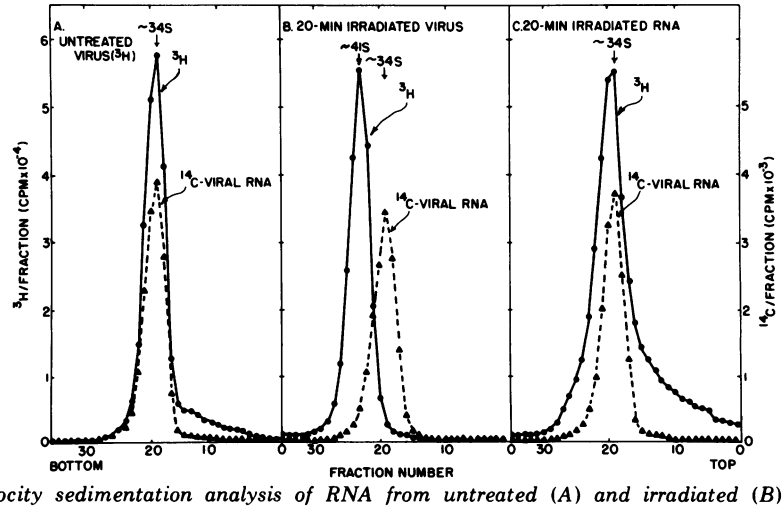

FIG. 7. *Velocity sedimentation analysis of RNA from untreated (A) and irradiated (B) virus and of irradiated viral RNA (C). Samples of untreated and 20-min irradiated [^{3}H]uridine-labeled mengovirus were supplemented with 2% (wt/vol) sodium dodecyl sulfate and incubated at 37 C for 10 min with frequent mixing. A sample of 20-min irradiated, phenol-extracted, [^{3}H]uridine-labeled viral RNA was similarly supplemented with sodium dodecyl sulfate. The samples were then mixed with a small volume of phenol-extracted, [^{14}C]uridine-labeled viral RNA added as a marker, and the mixtures were sedimented through 0.15 to 0.9 M gradients of sucrose in buffer 6 in an SW27 rotor at 22,000 rpm, 20 C, for 10 h. Fractions from the gradients were analyzed for ^{14}C (▲) and ^{3}H (●) in acid-insoluble material.*

intermediate ionic strength (15).

A comparison of the data in Fig. 8A and B and 8C and D indicates that the sedimentation rates of the RNAs from irradiated and nonirradiated viruses were similarly affected by changes in ionic strength. However, in both high and low-ionic-strength buffer, the RNA from irradiated virus sedimented significantly more rapidly than the RNA from nonirradiated virus.

The extent of secondary structure of the RNAs was further analyzed by determining the effect of pretreatment with 6% formaldehyde on the sedimentation rate of the RNAs (1, 36). Formaldehyde pretreatment lowered the sedimentation rate of RNA from nonirradiated virus from 34S to about 22S (Fig. 9A). The sedimentation rate of RNA from 20-min-irradiated virus was far less affected by formaldehyde treatment; its sedimentation rate was lowered from 41S to only about 34S (Fig. 9B). The relative difference in sedimentation rate between the formaldehyde pretreated RNAs from nonirradiated and irradiated virus was about the same whether or not the gradient solution contained 6% formaldehyde. The results suggest that the extent of unfolding due to formaldehyde treatment was considerably less for RNA from irradiated virus than for that from nonirradiated virus. Degradation of the RNAs during formaldehyde treatment (1) probably did not

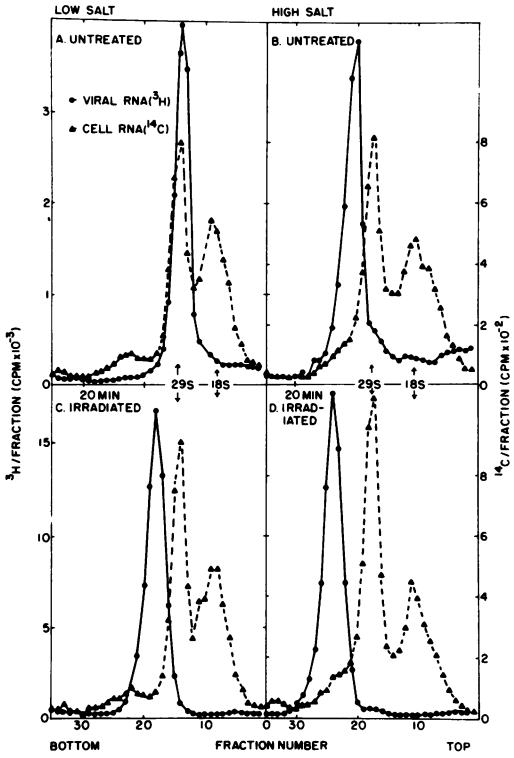

FIG. 8. *Velocity sedimentation analysis of RNA from irradiated and nonirradiated mengovirus on high- and low-ionic-strength gradients. Duplicate samples of untreated (A and B) and 20-min-irradiated (C and D), [^{3}H]uridine-labeled mengovirus (●) were supplemented with 2% sodium dodecyl sulfate, incubated at 56 C for 10 min, and then mixed with a small amount of phenol-extracted. [^{14}C]labeled cellular RNA (▲). One sample of each virus preparation (A and C) was sedimented through a 0.15 to 0.9 M gradient of sucrose in low-ionic-strength buffer (1 mM EDTA, 1 mM Tris-chloride, pH 7.4, and 0.5% sodium dodecyl sulfate), and the other sample (B and D) was sedimented through a 0.15 to 0.9 M gradient of sucrose in high-ionic-strength buffer (20 mM EDTA, 20 mM Tris-chloride, pH 7.4, 100 mM NaCl, and 0.5% sodium dodecyl sulfate). Centrifugation was in an SW27 rotor at 22,000 rpm, 20 C, for 10 h. Fractions from the gradients were analyzed for ^{14}C and ^{3}H in acid-insoluble material.*

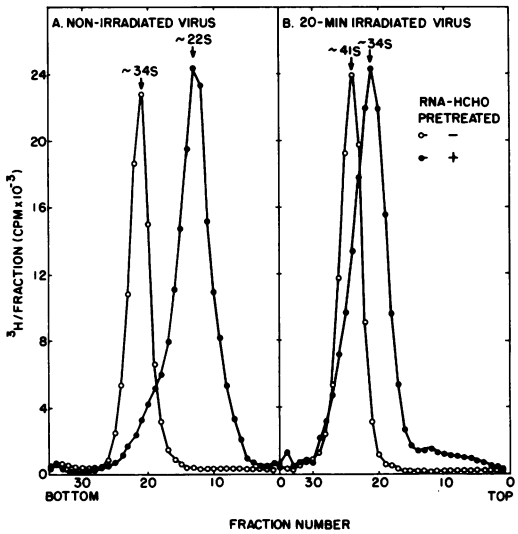

FIG. 9. *Effect of formaldehyde pretreatment on the sedimentation rate of RNA from nonirradiated (A) and 20-min-irradiated (B) virus. Duplicate samples of nonirradiated and 20-min-irradiated [^{3}H]uridine-labeled mengovirus were supplemented with 2% sodium dodecyl sulfate and incubated at 56 C for 10 min. One sample of each virus preparation was then left untreated (○), and the other sample was supplemented with 6% (vol/vol) formaldehyde and incubated at 63 C for 15 min (●). The mixtures were quickly cooled and then mixed with [^{14}C]viral RNA as marker and sedimented through 0.15 to 0.9 M gradients of sucrose in buffer 6 as illustrated in Fig. 7. Fractions from the gradients were analyzed for ^{3}H and ^{14}C in acid-insoluble material. Each frame is a composite of the gradient profiles of the formaldehyde-treated and untreated ^{3}H-labeled RNA. The ^{14}C-profiles of viral RNA were approximately identical to that of the ^{3}H-labeled RNA from nonirradiated virus (A) and have been omitted for clarity.*

play a major role in lowering the sedimentation rates, since treated RNA sedimented as relatively sharp bands in the gradients. That the difference in sedimentation rate between the RNA from irradiated and nonirradiated virus was not due to the presence of uracil dimers in the RNA from irradiated virus was indicated by the finding that the sedimentation rate of viral RNA that was irradiated after isolation by phenol extraction was altered by formaldehyde treatment in the same manner as RNA from nonirradiated virus (see Fig. 9A; results not shown).

The following results indicate that viral protein became covalently linked to the viral RNA molecule as a result of UV irradiation. This may have been responsible for the increased sedimentation rate of the viral RNA. The RNA from untreated and irradiated [^{3}H]leucine-labeled virus was isolated and analyzed by sucrose density gradient centrifugation. About 0.5% of the total leucine radioactivity remained associated with the RNA from untreated virus (fractions 20–25), but the amount of protein associated with the viral RNA increased to 1.5% of the total upon irradiation for 20 min (fractions 24–28) (Fig. 10). The protein label associated with the RNAs of irradiated and nonirradiated viruses exhibited the same sedimentation rates as the corresponding uridine-labeled RNAs (Fig. 7B). To rule out nonspecific binding of protein to viral RNA, we isolated the RNA from the gradient fractions by precipitation with ethanol and recentrifuged it in a second gradient. Practically all of the leucine label remained associated with the RNA (Fig. 10B), though it became released upon alkaline hydrolysis of the RNA (Fig. 10C). The leucine-labeled material released by alkaline hydrolysis was acid insoluble (Fig. 10C), whereas uridine-labeled viral RNA was completely hydrolyzed to acid-soluble material under these conditions. This indicates that the leucine label was present in polypeptides and not nucleotides of the viral RNA. Polyacrylamide gel electrophoresis of the alkaline digest showed that the polypeptides associated with the viral RNA from irradiated virus were very heterogeneous in size and in general of

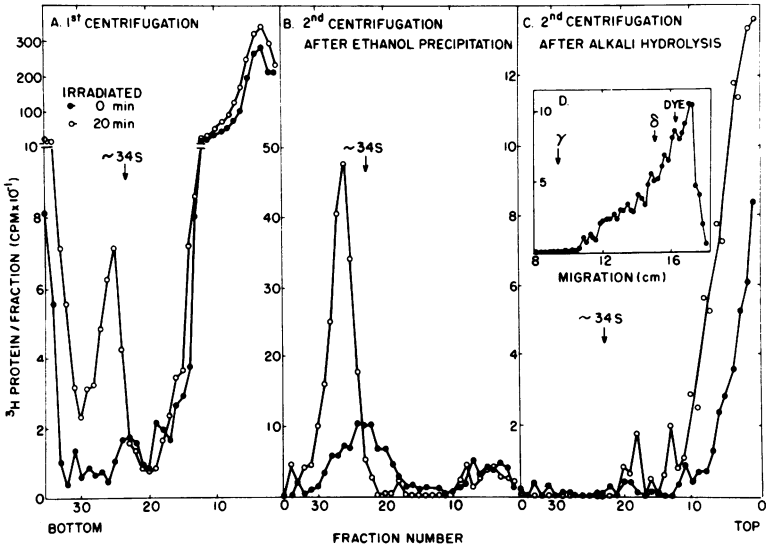

FIG. 10. *Association of viral protein with mengovirus RNA. (A) Samples of untreated and 20-min-irradiated, [^{3}H]leucine-labeled mengovirus were supplemented with 2% sodium dodecyl sulfate and incubated at 56 C for 10 min. The extracts were sedimented through 0.15 to 0.9 M sucrose gradients in buffer 6 in an SW27 rotor at 22,000 rpm, 20 C, for 10 h. Fractions (1 ml) were collected, and a 0.1-ml portion of each fraction was analyzed for radioactivity in acid-insoluble material. The remainder of gradient fractions 21–25 and 24–27 of the untreated and irradiated virus samples, respectively, were pooled, and the macromolecules were precipitated by addition of 2.5 volumes of ethanol at −20 C. The precipitates were collected by centrifugation and resuspended in 10 mM Tris-chloride (pH 7.4), and each was divided into two equal samples. (B) One sample of each RNA-protein complex isolated from the gradients in A was resedimented through sucrose gradients as described in A, and fractions from the gradients were analyzed for radioactivity in acid-insoluble material. (C) The other sample was supplemented with 0.2 N NaOH and incubated at 37 C for 18 h. A portion of this digest was neutralized and sedimented through sucrose gradients as described in A, and fractions from the gradients were analyzed for radioactivity in acid-insoluble material. (D) Another portion of the alkaline digest prepared in C was electrophoresed in a 10% acrylamide gel as described in Materials and Methods.*

low molecular weight, though they ranged in size up to about 20,000 daltons (Fig. 10D).

That the polypeptides were covalently linked to the RNA from irradiated virus is also indicated by the results from additional experiments (not shown) which indicated that the [^{3}H]leucine-labeled polypeptides remained associated with the viral RNA upon incubation at 60 C for 1 h in solutions containing 6% (vol/vol) formaldehyde. We also repeatedly lost most of the RNA from irradiated virus into the interphase during phenol extraction whether or not the phenol-virus mixture contained 1% sodium dodecyl sulfate or whether the extraction was conducted at room temperature or at 60 C. We contribute the accumulation of the viral RNA in the interphase to the presence of the covalently linked polypeptides.

Composition of 80S RNP and 14S protein particles released from irradiated virus. The 80*S* and 14*S* particles were isolated from sucrose density gradients of 40-min irradiated uridine or amino acid-labeled virus (see Fig. 2D). The RNA and proteins of these particles were then analyzed by sucrose density gradient centrifugation and acrylamide gel electrophoresis, respectively. The results showed that the 80*S* particle contained intact viral RNA which exhibited the sedimentation properties typical of RNA from irradiated virus (Fig. 11B), and at least the main structural proteins of the capsid (alpha, beta, and gamma; Fig. 11A). There was, however, a preponderance of large and small photoproducts and little, if any, delta protein present. The 14*S* particles had a similar protein composition (Fig. 11C) and were devoid of RNA (see Fig. 2D).

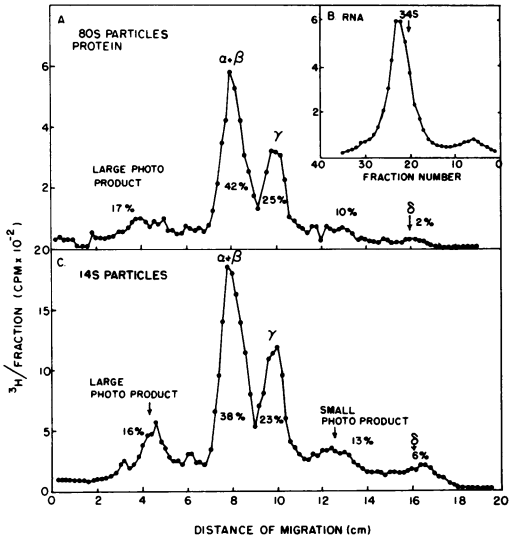

FIG. 11. *Protein and RNA content of 80S and 14S particles released from irradiated virions. Samples of [^{3}H]uridine-labeled or [^{3}H]amino acid-labeled virions were irradiated for 40 min and centrifuged through sucrose gradients as illustrated in Fig. 2C. The 80S and 14S material was isolated from the gradients by precipitation with ethanol and resuspended in 10 mM Tris-chloride (pH 7.4). (A and C) The [^{3}H]amino acid-labeled 80S and 14S material was electrophoresed in 10% polyacrylamide gels as described in Materials and Methods. (B) The [^{3}H]uridine-labeled 80S material was treated with sodium dodecyl sulfate and centrifuged in a sucrose density gradient as illustrated in Fig. 7.*

DISCUSSION

We have distinguished three stages in the effect of UV irradiation on mengovirus. The infectivity of the virus is lost very rapidly, and this coincides with and is probably causally related to the formation of uracil dimers in the viral RNA. The number of uracil dimers formed per PFU of virus inactivated remained relatively constant through the first 2 min of irradiation, averaging 1.7 dimers per PFU inactivated (Fig. 1).

More slowly, UV irradiation also causes structural modifications of the capsid proteins resulting in the formation of large and small photoproducts. The nature and origin of these products is uncertain, but the relatively high molecular weight of the larger photoproduct(s) (50,000–90,000 daltons) suggests that it is formed by covalent linkage between two or more of the viral proteins. Comparison of tryptic digests of the various products with those of the structural proteins may aid in identification of their origin. The formation of large photoproduct proteins has also been reported for irradiated reovirus (35). Particularly interesting is the rapid loss or modification by irradiation of the delta protein which, if analogous with VP4 of coxsackievirus, is probably responsible for the interaction of the virus with host cell receptors during adsorption (4). The UV light-induced changes in viral proteins are probably responsible for the early change in conformation of the viral capsid. Although the virus particles seem to remain intact for at least 20 min of irradiation as indicated by sedimentation analyses as well as by electron microscope studies, structural changes in the capsid must have occurred during this time period. This is indicated by the fact that the RNA of the virions has become sensitive to degradation by external RNase and is extractable by low concentrations of sodium dodecyl sulfate, and that the virions break down during centrifugation in CsCl gradi-

ents. The structural changes, or possibly the loss or modification of the delta protein, are probably also responsible for the loss of HA activity of the virions. Studies are in progress to determine whether antigenic changes accompany these structural changes in the capsid.

In addition to causing modifications of the proteins, UV irradiation of complete virus also results in the covalent linkage of viral polypeptides to the viral RNA. It seems likely that specific proteins located in close proximity to the viral RNA in the capsid are involved in this interaction. The heterogeneity and low-molecular-weight of the polypeptides associated with viral RNA, however, preclude any conclusion as to the protein(s) of their origin. Further work is required to answer this question and to elucidate the nature of the covalent bond between protein and RNA. UV-induced covalent bonds between cysteine and the uracil of RNA have been described (32). Association of protein subunits with RNA as a result of UV irradiation has also been observed with tobacco mosaic virus, but the proteins were not covalently linked because they were removable by treatment of the RNA with phenol, acetic acid, or guanidine-hydrochloride (8).

The finding that irradiation of free viral RNA does not affect its sedimentation properties whether or not it is treated with formaldehyde before sedimentation analysis suggests that the covalent linkage of polypeptides to the RNA of irradiated virus is responsible for the increased sedimentation rate of this RNA. This finding also indicates that the presence of uracil dimers per se is not the cause of the increased sedimentation rate of this RNA. We cannot explain at present, however, how the association of such a small amount of protein with the viral RNA can so markedly affect its sedimentation rate. The sedimentation constant of the viral RNA is increased from 34*S* to about 41*S*. This would be equivalent to an increase in molecular weight of approximately 10^6 (33), assuming the molecule is single stranded. The amount of protein covalently linked to the viral RNA, on the other hand, represents not more than 1.5% of the total protein capsid (about 6×10^6 daltons; 34) and thus not more than 9×10^4 daltons of protein per RNA molecule. The increased sedimentation rate of the RNA from irradiated virus is probably due, at least in part, to an increased compactness of the RNA molecule in solution. The reason for the increased compactness of the RNA molecule is not known. It might be due to cross-linking of normally hydrogen-bonded loops in the RNA molecule by the covalently linked polypeptides. Such cross-links would also impair denaturation of the RNA and could account for the relatively small effect of formaldehyde treatment on the sedimentation rate of the RNA from irradiated virus.

It seems likely that the progressively increasing changes of the viral proteins ultimately result in the disintegration of the virions even under normal experimental conditions with the release of 80*S* RNP particles and 14*S* protein particles. The 14*S* particles resemble those observed in poliovirus-infected cells (18, 20) or formed upon dissociation of various picornaviruses (5, 6). These probably represent pentamers of the basic trimer composed of one of each of the structural proteins, alpha, beta, and gamma (6, 34). The nature of the 80*S* RNP particles remains to be elucidated. The results show that they are composed of intact viral RNA, though in a form accessible to RNase, and all three of the larger proteins which are present in about the same proportion as in the complete virion. Similar structures have been reported to be formed during in vitro reassociation of poliovirus RNA and protein (5) and may represent assembly complexes. The 80*S* particles are certainly not empty capsids which, on the basis of studies with poliovirus (18, 20), would exhibit a similar sedimentation rate. In fact, empty capsids of mengovirus have never been observed by electron microscopy or gradient analysis in preparations of irradiated virus or in lysates of mengovirus-infected N1S1-67 cells. Empty capsids, on the other hand, are regularly formed in poliovirus-infected cells (18, 20), in in vitro reaction mixtures of poliovirus proteins, or during irradiation of poliovirus (11). Evidence has been presented to indicate that empty capsids are intermediates in the assembly of poliovirus (10, 20). The failure to find empty capsids of mengovirus under equivalent experimental conditions suggests that empty capsid intermediates of this virus are formed only very transiently or that a different mechanism is responsible for the assembly of the virus.

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