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Deoxyribonucleic Acid Polymerase(s) of Rous Sarcoma Virus: Effects of Virion-Associated Endonuclease on the Enzymatic Product

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Purified preparations of Rous sarcoma virus (RSV) contain ribonuclease which is either a constituent of the virion surface or an adsorbed contaminant. Treatment of the virus with nonionic detergent to activate ribonucleic acid (RNA)-dependent deoxyribonucleic acid (DNA) polymerase renders the viral genome susceptible to hydrolysis by the external ribonuclease. The extent of this susceptibility can be substantially reduced by the use of limited amounts of detergent. At a concentration of detergent which provides a maximum initial rate of DNA synthesis, the degradation of endogenous viral RNA results in a reduced yield of high molecular weight DNA: RNA hybrid from the polymerase reaction. Attempts to detect virion-associated deoxyribonuclease, by using a variety of double helical DNA species as substrates, have been unsuccessful, but small amounts of nuclease activity directed against single-stranded DNA may be present in purified virus.

A deoxyribonucleic acid (DNA) polymerase associated with the virions of ribonucleic acid (RNA) tumor viruses (2, 26) utilizes the RNA of the viral genome as template (10, 19, 23). This RNA is single-stranded and therefore sensitive to hydrolysis by ribonuclease (18). Previous reports have suggested that purified preparations of RNA tumor viruses contain ribonuclease, possibly as integral components of the virion (20). If this enzyme were capable of acting upon endogenous viral RNA during the course of in vitro DNA synthesis by the virion polymerase, the consequent degradation of RNA template could have appreciable effects upon the nature of the DNA synthesized. We examined this possibility in the case of Rous sarcoma virus (RSV) and found that the conditions required for maximum in vitro DNA synthesis render the viral genome sensitive to hydrolysis by virion-associated ribonuclease.

The extent of this hydrolysis, its effect upon the structure of the DNA synthesized by virion polymerase, and the means by which we have been able to limit the effect of ribonuclease upon the DNA product are considered in the first portion of this communication. In addition, we examined purified virions for the presence of deoxyribonuclease activity in an effort to explain the exceptionally low molecular weight of the polymerase product. In contrast to the report of Mizutani et al. (16), we were unable to detect significant amounts of deoxyribonuclease active against double-stranded DNA, but purified preparations of the Schmidt-Ruppin strain of RSV do contain a limited amount of endonuclease specific for single-stranded DNA.

MATERIALS AND METHODS

Reagents. The following reagents were used: ^{3}H-thymidine triphosphate (^{3}H-TTP), 10 to 15 Ci/mM, New England Nuclear Corp.; ^{32}P-TTP, International Chemical and Nuclear Corp.; pancreatic ribonuclease A, Worthington Biochemicals, Inc.; Pronase, B grade, Calbiochem, self-digested at 37 C for 2 hr before use; deoxyribonucleoside triphosphates, deoxyadenosine triphosphate, deoxyguanosine triphosphate, and deoxycytidine triphosphate, Calbiochem; phenol, reagent grade, Mallinckrodt Chemical Works; Nonidet P-40 (NP-40), Shell Chemical Co.; Liquifluor, Pilot Chemicals Co.; ethylene glycol monomethyl ether, reagent grade, Mallinckrodt.

Propagation and purification of virus. The Schmidt-Ruppin strain of RSV was grown in chick embryo fibroblasts and purified as described previously (4).

Extraction and purification of viral RNA. RSV RNA was extracted from purified virus with sodium dodecyl sulfate and phenol (4). The 70*S* RNA was isolated by zonal centrifugation through density gradients of sucrose (4).

DNA polymerase reaction. Details of the enzyme

reaction mixture have been reported (11). ^{3}H-TTP was used as labeled precursor. Enzymatic activity was elicited by treating virus suspensions with various concentrations of NP-40. The detergent was included in the reaction mixture, which was warmed to 37 C before addition of appropriate amounts of purified virus. Under these conditions, DNA synthesis began immediately. Determination of acid-precipitable radioactivity was accomplished as described previously (11).

Extraction of enzymatic product. Reaction mixtures were treated with sodium dodecyl sulfate (0.5%, w/v) and Pronase (500 μg/ml) for 45 min at 37 C. The sample was then layered on density gradients of sucrose, either immediately or after storage at −20 C. The latter had no effect on the relative yield of the various forms of enzymatic product.

Rate-zonal centrifugation in density gradients of neutral sucrose. Rate-zonal centrifugation was performed as described previously (11). The gradients (15 to 30%) contained 0.1 M NaCl, 0.001 M ethylenediaminetetraacetic acid (EDTA), 0.02 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 7.4). Conditions of centrifugation are given with individual experiments.

Rate-zonal centrifugation in alkaline sucrose gradients. The gradients (5 to 20%) contained 0.9 M NaCl, 0.01 M EDTA, 0.6 N NaOH (1). Samples were adjusted to 0.6 N NaOH and were incubated at 37 C for 30 min prior to layering on the gradients.

Electrophoresis of nucleic acids in polyacrylamide. Electrophoresis in gels of polyacrylamide, cross-linked with ethylene diacrylate, was performed as described previously (4). Gels were fractionated into 1-mm slices (4). These were hydrolyzed in hydrogen peroxide at 65 C for 12 hr in sealed vials; hydrolysis was followed by the addition of a scintillation fluid containing two volumes of Liquifluor-toluene and one volume of ethylene glycol monomethyl ether (25).

Preparation of substrate DNA species. Replicative form (RF) DNA species of fd bacteriophage, extracted from infected *Escherichia coli* and purified by the procedure of Roulland-Dussoix and Boyer (21), were kindly provided by H. Boyer and J. Hedgpeth. RF-I (circular supercoils) was then purified by zonal centrifugation (see Fig. 5). DNA was extracted from chick embryo fibroblasts as described by Martin (14). The unfractionated extraction product had a molecular weight of 30×10^6 to 50×10^6 daltons as judged by comparative rate-zonal centrifugation (5). Purified lambda phage DNA was provided by H. Boyer.

Infectious RNA of poliovirus. Poliovirus RNA was extracted from purified virus with phenol at 37 C (3). Infectivity was measured as described previously (12).

RESULTS

Degradation of viral RNA by intrinsic ribonuclease. Virions of RSV display DNA polymerase activity only after treatment with a nonionic detergent (11, 26). A given concentration of viral protein requires a standard amount of detergent for maximum DNA synthesis (Table 1, and reference 11), and our early experiments with RSV (11) were carried out with detergent concentrations which appeared to be optimal for DNA synthesis, e.g., 0.05 to 0.10% in the case illustrated in Table 1. In the course of our investigations, we noted that the endogenous viral RNA in reaction mixtures of this sort is degraded to an acid-soluble state as DNA synthesis proceeds (Fig. 1). This observation raised the possibility that the properties of enzymatically synthesized DNA might be subject to substantial artifact as a consequence of the ongoing degradation of viral RNA template. We further noted that degradation as extensive as that described above is not observed when the enzymatic reaction is elicited with lower concentrations of detergent (0.01% in the case illustrated by Fig. 1; treatment of virus with this concentration of NP-40 yields approximately 30% of maximum polymerase activity measured over a period of 2 hr, as in Table 1). We therefore examined in greater detail the relative effects of these two detergent concentrations on the integrity of the viral genome and the nature of the enzymatic product.

Effect of detergent concentration on kinetics of

TABLE 1. *Relative enzymatic activity as a function of Nonidet P-40 (NP-40)*

Per cent NP-40 (v/v)	^{3}H-TMP incorporated[a] (counts/min)	Relative activity (%)
0	219	1
0.005	2,254	10.4
0.01	6,972	32
0.05	20,792	96
0.10	21,750	100
0.50	19,267	89
1.0	17,589	81
2.0	16,588	76

[a] ^{3}H-TMP, ^{3}H-thymidine monophosphate. Replicate enzyme reaction mixtures were prepared containing 10 μg of viral protein at a concentration of 200 μg/ml, 10^{-6} M ^{3}H-thymidine triphosphate, and the stated concentrations of NP-40. Incubation was carried out at 37 C for 2 hr, followed by acid precipitation and measurement of radioactivity (11). This experiment has been performed on repeated occasions with several virus preparations and with similar results. The concentration of virus protein and, consequently, the optimal concentrations of detergent are different from those reported previously (11). In addition, the relative loss of enzymatic activity at the higher concentrations of detergent is substantially less than that reported previously (11, 22).

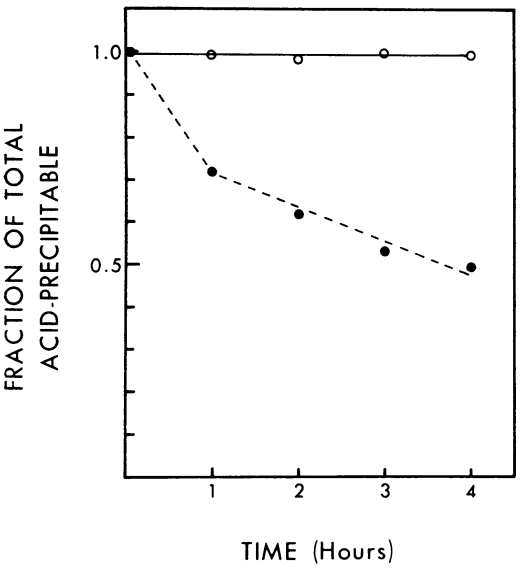

FIG. 1. *Degradation of endogenous viral RNA during the polymerase reaction. Replicate reaction mixtures were prepared with RSV labeled with ^{3}H-uridine, the four unlabeled deoxyribonucleoside triphosphates, and either 0.01 or 0.05% NP-40. The mixtures were incubated at 37 C, and samples were withdrawn at the indicated time points for measurement of acid-precipitable radioactivity. The acid-precipitable counts/minute at the onset of the reaction were used to compute the fraction of counts/minute remaining acid-precipitable after various periods of incubation. Symbols: ○, 0.01% NP-40; ●, 0.05% NP-40.*

DNA synthesis. The data contained in Table 1 represent the overall rate of DNA synthesis for the first 2 hr of the reaction and indicate that maximum activity by this criterion is elicited by 0.05 to 0.10% NP-40. However, a more detailed analysis of the reaction revealed the situation depicted in Fig. 2. As expected, the initial rate of DNA synthesis at 0.05% NP-40 is at least threefold more rapid than that at 0.01% NP-40. However, the rate of the former reaction slows appreciably after 60 to 90 min, whereas the latter reaction continues at a constant rate for a minimum of 4 hr. At 4 hr, both reactions have produced approximately the same relative amount of double-stranded DNA (ca. 30%) as determined by analysis on hydroxyapatite (8). The biphasic rate of DNA synthesis elicited by 0.05% NP-40 is not due to depletion of TTP (the limiting precursor in these reaction mixtures). Supplemental ^{3}H-TTP, added at 90 min, has no appreciable effect on the rate of the reaction (Fig. 2).

Effect of detergent concentration on the integrity of viral RNA. The integrity of viral RNA during the course of DNA synthesis was assessed by electrophoresis in polyacrylamide gels. This procedure has been used previously to delineate five classes of RNA associated with the virions of RSV: 4, 7, 18, 28 and 70*S* (4). The last is regarded as the viral genome (18) and provides the initial template for DNA synthesis by the virion polymerase (10, 11, 19, 23). Incubation of virus at 37 C in the presence of 0.01% detergent has no effect on either the absolute amounts or the relative proportions of the various classes of viral RNA (Fig. 3a, b). By contrast, use of 0.05% NP-40 results in extensive degradation of viral RNA (Fig. 3c, d). Only the 4*S* RNA can be identified with any confidence in the electropherogram although fragments of degraded RNA spread throughout the gel might obscure small amounts of residual 7, 18, and 28*S* RNA. It is clear, however, that the 70*S* viral RNA has been completely degraded by 1 hr after the onset of DNA synthesis. These results indicate that concentrations of detergent which yield a maximum initial rate of DNA synthesis also result in extensive degradation of endogenous viral RNA

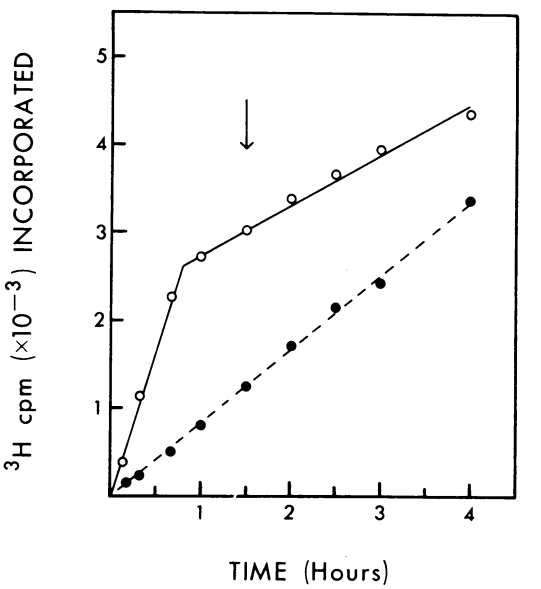

FIG. 2. *Kinetics of DNA synthesis at two concentrations of detergent. Replicate standard reaction mixtures were prepared containing 200 μg of viral protein per ml and 4 × 10^{-6} M ^{3}H-TTP. NP-40 was added to one at a concentration of 0.01% (●) and to the other at 0.05% (○); the mixtures were incubated at 37 C. Portions were withdrawn at the indicated time points for determination of acid-precipitable radioactivity (11). At 90 min (arrow), additional ^{3}H-TTP (4 × 10^{-6} M) was added to the reaction mixture containing 0.05% NP-40.*

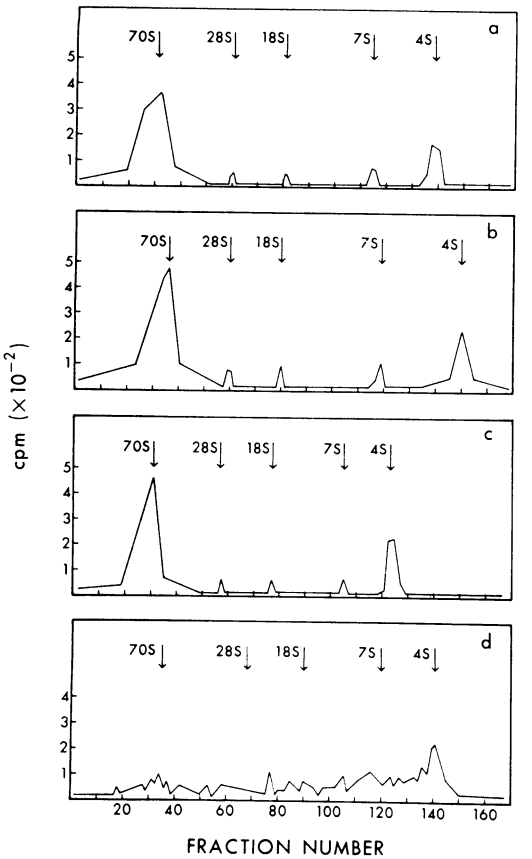

FIG. 3. *Electrophoresis of viral RNA extracted from enzyme reaction mixtures. Purified RSV, labeled with ^{3}H-uridine (4), was used to prepare replicate enzyme reaction mixtures as in Fig. 2. DNA synthesis was monitored in small portions with α^{32}P-TTP, and the results were essentially as illustrated in Fig. 2. At the onset of the reaction (T_0) and after 1 hr (T_{60}), samples were withdrawn, extracted with sodium dodecyl sulfate-Pronase-phenol (11), precipitated with ethanol, and analyzed by electrophoresis in 20-cm gels of 2.25% polyacrylamide. ^{32}P-labeled RNA, extracted from purified RSV, was included as marker. The arrows indicate the location of the recognizable classes of RSV RNA (4). (a) T_0, reaction containing 0.01% NP-40; (b) T_{60}, reaction containing 0.01% NP-40; (c) T_0, reaction containing 0.05% NP-40; and (d) T_{60}, reaction containing 0.05% NP-40.*

and that this problem can be obviated by utilizing somewhat lower concentrations of detergent.

Effect of detergent concentration on the nature of enzymatic product. The virion polymerase synthesizes two classes of DNA with respect to sedimentation velocity: an initial product which cosediments with 70S viral RNA (11, 19), and a secondary product which sediments slowly (4 to 10S) in density gradients of sucrose. The former product represents short nascent chains of DNA hydrogen-bonded to 70S viral RNA (23; *unpublished data*). Its native sedimentation properties are identical to those of the viral RNA (11, 19). Consequently, degradation of the RNA would be expected to reduce the apparent yield of this DNA:RNA hybrid as detected by zonal centrifugation. This expectation derives from the fact that only a small portion of any given 70S RNA molecule is included in hybrid structure, the remainder being single-stranded and therefore ribonuclease-sensitive (11). The DNA:RNA hybrid which remains after ribonuclease treatment in high concentrations of NaCl has a low sedimentation velocity (4 to 10S) and cannot be distinguished from the other slowly sedimenting enzymatic products by zonal centrifugation (8; *unpublished data*).

The relative yields of rapidly sedimenting (ca. 70S) enzymatic product as a function of time, and at two different concentrations of NP-40, are illustrated in Fig. 4. It is apparent that the higher (and optimal with respect to initial rate of DNA synthesis) concentration of detergent leads to a rapid disappearance of 70S product. We ascribe this to the activation of virion-associated ribonuclease as demonstrated above (Fig. 3). By contrast, the limiting concentration of detergent (0.01%) allows prolonged synthesis of rapidly sedimenting hybrid (Fig. 4a–c). Two hours after the onset of DNA synthesis, more than 50% of the enzymatic product still cosediments with 70S viral RNA.

Localization of ribonuclease activity. The preceding data indicate that purified preparations of RSV contain ribonuclease which can attack the viral genome after partial disruption of the virion with nonionic detergent. In an effort to determine whether this ribonuclease is an internal component of RSV, we have exposed the single-stranded RNA of poliovirus to both intact and detergent-treated virions. In both instances, the infectivity of poliovirus RNA is rapidly inactivated (Table 2) although inactivation is less extensive in the presence of detergent than in its absence. We cannot presently explain the latter observation, but the experiment has been performed twice with similar results. No inactivation of infectious RNA is observed if RSV is omitted from the incubation mixture, confirming our supposition that the ribonuclease in question is associated with the RSV preparations. The fact that detergent treatment is not required to elicit ribonuclease activity external to the virions suggests that the enzyme is either a surface constituent of the virus or an adsorbed contaminant which is is not eliminated by the purification procedure.

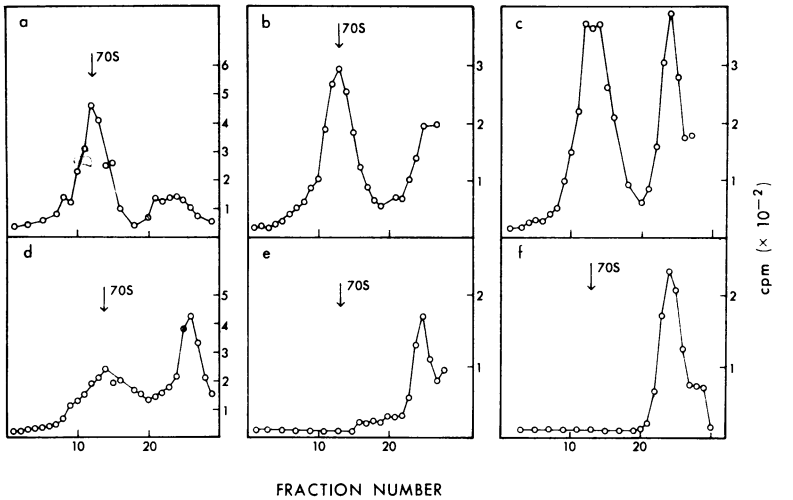

FIG. 4. *Effect of detergent concentration on the yield of rapidly sedimenting enzymatic product. Replicate reaction mixtures, containing 200 μg of viral protein and 4 × 10⁻⁶ M ³H-TTP, were prepared and incubated at 37 C. One contained 0.01% NP-40, the other 0.05% NP-40. Samples were withdrawn at the indicated times, extracted with sodium dodecyl sulfate-Pronase, and centrifuged through gradients of 15 to 30% sucrose as described in the text. Conditions of centrifugation: SW41 rotor, 40,000 rev/min, 180 min, 4 C. Purified ³²P-labeled 70S RSV RNA was included in all samples as a sedimentation marker (location indicated by arrow in all panels except c, where it was also coincident with the rapidly sedimenting enzymatic product). (a) 0.01% NP-40, 30 min; (b) 0.01% NP-40, 60 min; (c) 0.01% NP-40, 120 min; (d) 0.05% NP-40, 30 min; (e) 0.05% NP-40, 60 min; (f) 0.05% NP-40, 120 min.*

TABLE 2. *Ribonuclease activity associated with purified Rous sarcoma virus (RSV): inactivation of poliovirus RNA*

Prepn	Time of incubation (min)	Plaque-forming units/ml	Per cent survival[d]
Control[a]	0	10^7	
	60	1.5×10^7	100
Virus[b]	0	10^7	
	60	10^3	0.01
Virus with NP-40[c]	0	10^7	
	60	2×10^4	0.2

[a] Poliovirus RNA was added to a standard enzyme reaction mixture [(0.05% Nonidet P-40 (NP-40)] from which RSV had been omitted. Incubation was at 37 C.

[b] Reaction mixture contained RSV (200 μg/ml), but NP-40 was omitted.

[c] Reaction mixture contained both RSV (200 μg/ml) and NP-40 (0.05%).

[d] Similar results were obtained if the reaction mixtures were extracted with sodium dodecyl sulfate-Pronase before dilution for the plaque test.

Search for virion-associated deoxyribonuclease. The DNA polymerase associated with RNA tumor viruses synthesizes low-molecular-weight polynucleotides, the chain length of which corresponds to approximately 1 to 5% of the apparent template 70S viral RNA (19). To date, no circumstance has been found which can induce the synthesis of high-molecular-weight DNA by virion polymerase. In an effort to explain these observations, we examined purified preparations of RSV for endonuclease activity directed against DNA. The initial tests were performed by using the supercoiled RF (form I) of bacteriophage fd as substrate for the putative endonuclease. This DNA molecule undergoes a considerable (and easily detectable) transition in tertiary structure after even a single cleavage in either of the two constituent polynucleotide chains (6, 21), with the supercoiled circles of form I RF (26S) being converted to the relaxed circles of form II RF (17S). Consequently, conversion of RF I to RF II constitutes an extremely sensitive assay for endonuclease.

RF I of fd bacteriophage was purified by zonal centrifugation (Fig. 5). Spontaneous conversion of RF I to RF II during storage (due to radioautolysis) necessitated the use of freshly prepared material. The assay for endonuclease was performed by addition of RF I to standard polymerase reaction mixtures, followed by incubation for various periods of time at 37 C. [In this experiment and the similar experiments which follow, substrate DNA was used at concentrations of 0.1 to 0.5 μg/ml, virus at 300 to 500 μg of protein/ml. The results of control experiments

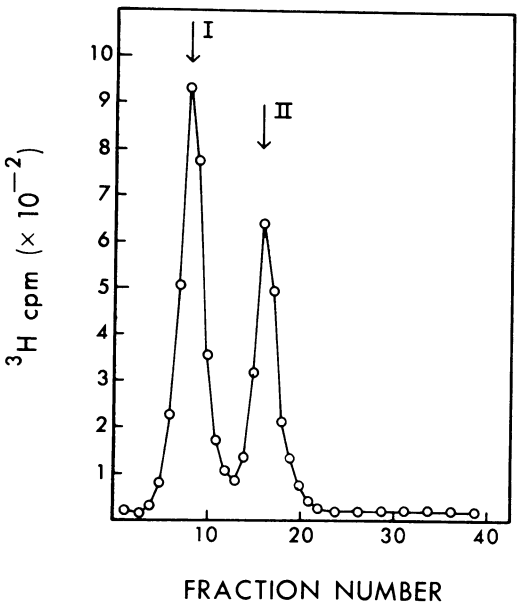

FIG. 5. *Purification of fd bacteriophage replicative forms. ^{3}H-labeled replicative form of fd bacteriophage was isolated from infected bacteria by the procedure of Roulland-Dussoix and Boyer (21). This procedure yields RF I which is 90% pure at the time of isolation. However, further purification is necessary after storage because of the spontaneous conversion of RF I to RF II. These can be separated by zonal centrifugation, as illustrated here. Centrifugation was in 5 to 20% sucrose gradients containing 0.1 M NaCl, 0.001 M EDTA, 0.05 M Tris-hydrochloride (pH 8.1). Condition of centrifugation: SW41 rotor, 28,000 rev/min, 16 hr, 10 C.*

indicate that all of the deoxyribonuclease assays (Fig. 6–9) are capable of detecting pancreatic deoxyribonuclease at a concentration of 0.001 μg/ml (unpublished data).] After deproteination of the reaction mixture, the extent of RF I degradation was assessed by zonal centrifugation. There was no detectable conversion of RF I to RF II in either the standard polymerase reaction or reaction mixtures containing a wide range of NP-40 concentrations (Fig. 6). These results suggest that neither the reagents nor the RSV virions contain an appreciable amount of endonuclease. The highest concentration (0.5%) of NP-40 used in these experiments is sufficient to disrupt completely RSV virions (*unpublished data*) and would, therefore, be expected to release intravirion endonuclease into a soluble state.

Three aspects of the phage DNA used in the preceding experiments might render it insensitive to a deoxyribonuclease of unusual substrate specificity: molecular topology (circularity), secondary structure, and species heterogeneity vis-a-vis avian viruses. Consequently, we elected to test RSV for deoxyribonuclease activity directed against a variety of other DNA species. (i) Linear double-stranded DNA of phage lambda. The DNA was sedimented in alkaline sucrose gradients after exposure to polymerase reaction mixtures. This procedure allows the detection of all single-chain endonucleolytic breaks rather than only double-strand breaks, thereby increasing the sensitivity of the assay (1). The results provide no evidence of endonuclease activity after 120 min of incubation at 37 C (Fig. 7). (ii) Single-stranded DNA, obtained by denaturation of lambda phage DNA before exposing the DNA to a polymerase reaction mixture. The substrate DNA was analyzed by rate-zonal centrifugation in neutral sucrose gradients (Fig. 8). A limited number of chain scissions are apparent after 2 hr of incubation. The enzyme in question appears to be an endonuclease because exonuclease would release mononucleotides, detectable as radioactivity at the top of the sucrose gradient. (iii) Homologous double-stranded DNA, extracted from chick embryo fibroblasts with sodium dodecyl sulfate-Pronase-phenol (14). This procedure yields DNA of relatively high molecular weight (30×10^6 to 50×10^6 daltons), but there is inevitably a considerable amount of molecular heterogeneity with respect to size. This circumstance renders sedimentation assays somewhat less discriminating than in the case of homogeneous DNA populations (such as the phage DNA species used above), but fractionation of the avian DNA prior to use was not performed to avoid the introduction of an unwanted selection factor. Again, the DNA was analyzed in alkaline sucrose gradients so as to detect single-chain breaks. The resulting sedimentation profiles are relatively broad and complex (Fig. 9), but under no circumstance were we able to detect a significant amount of endonuclease activity in either intact or detergent-disrupted virus.

Absence of deoxyribonuclease activity from Pronase-treated virus. After completion of the experiments described above, we learned of the work of Mizutani et al. which suggests that purified RSV does, in fact, contain an endonuclease capable of degrading phage (T-7) DNA (16). In contrast to our results, these investigators found deoxyribonuclease associated with undisrupted virus. They considered this deoxyribonuclease to be a contaminant and eliminated it by treating their virus preparations with Pronase. A second endonuclease activity could then be elicited by disrupting the virus with nonionic detergent. Although there is no demonstrable deoxyribonuclease activity contaminating our

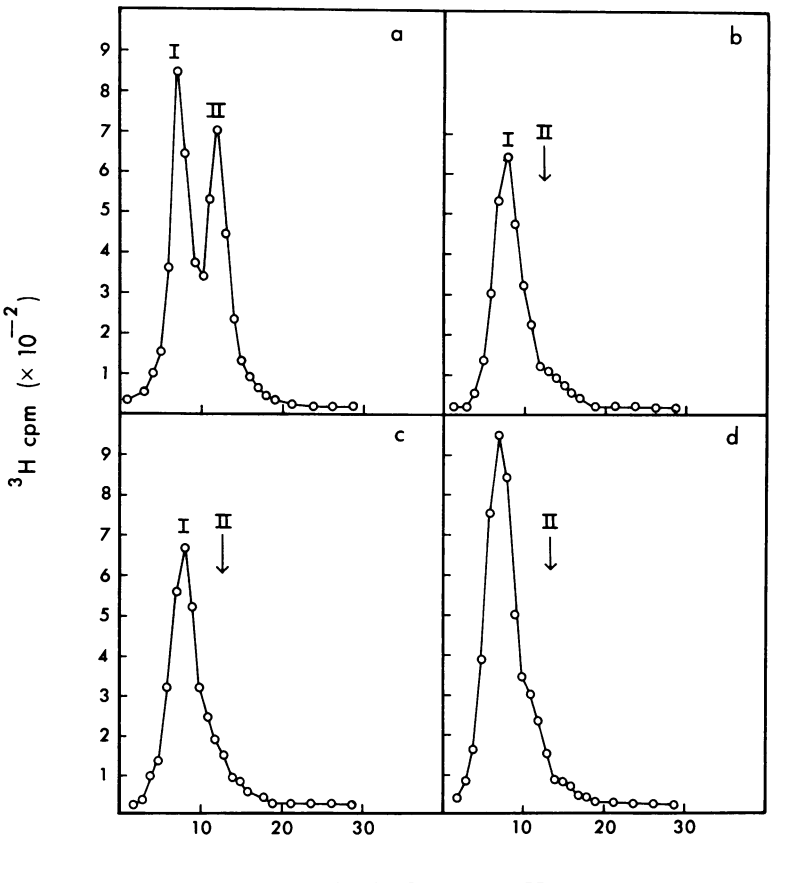

FIG. 6. *Rate-zonal centrifugation of fd bacteriophage RF after incubation in polymerase reaction mixture. A standard reaction mixture, containing 400 μg of viral protein per ml, was prepared as usual and incubated at 37 C (11). On separate occasions, NP-40 concentrations of 0.01, 0.05, and 0.5% were tested, as well as a mixture containing no detergent. The results were identical in every case to those illustrated. Purified RF-I (^{3}H-labeled, 15,000 counts/min) was included in each incubation mixture. Samples were withdrawn at the indicated times, extracted with sodium dodecyl sulfate-Pronase, and analyzed in 5 to 20% sucrose gradients as in Fig. 5. Conditions of centrifugation: SW56 rotor, 52,500 rev/min, 180 min, 10 C. Roman numerals indicate the anticipated locations of RF I and II, as determined from a simultaneous control centrifugation. (a) Mixture of RF-I and RF-II, to illustrate the resolution obtained, centrifuged simultaneously with the other samples; (b) T_0, onset of reaction; (c) T_{60}, after 60 min; (d) T_{120}, after 120 min.*

preparations of intact RSV (Figs. 6–9), we tested the possibility that pretreatment of virus with Pronase might render an intrinsic endonuclease more sensitive to activation by nonionic detergent. Purified RSV was incubated with Pronase as described by Mizutani et al. (50 μg/ml, 37 C, 20 min; reference 16) and then separated from the Pronase by isopycnic centrifugation (25 to 60% sucrose gradient, SW 41 rotor, 40,000 rev/min, 12 hr). The resulting band of virus again had deoxyribonuclease activity detectable only with single-stranded substrate (as in Fig. 8).

DISCUSSION

The present data confirm previous reports that purified preparations of RNA tumor viruses contain ribonuclease (20). The enzyme appears to be either a surface constituent of the RSV virion or an adsorbed contaminant which is not eliminated by purification of the virus. Consequently, at least partial disruption of the virion is necessary before the enzyme can gain access to the viral genome. This would account for the fact that 0.01% NP-40 does not elicit extensive degradation of intravirion RNA (Fig. 3) and conforms to previous observations that this

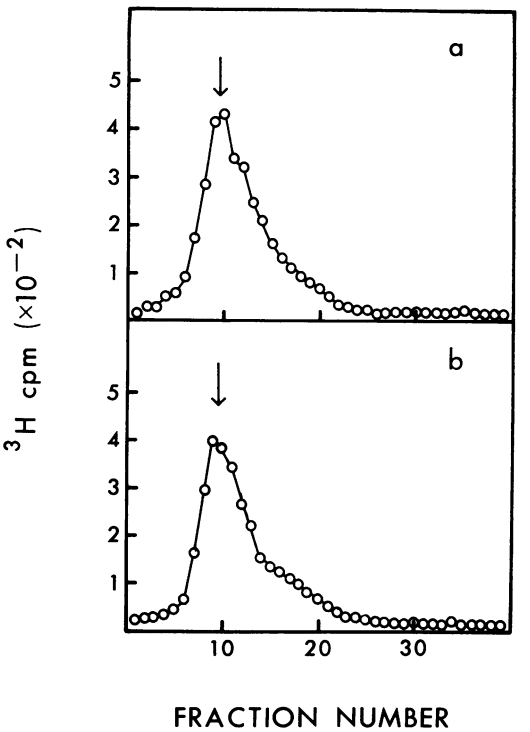

FIG. 7. *Centrifugation of lambda phage DNA in alkaline sucrose after exposure to the polymerase reaction mixture. ^{32}P-labeled lambda phage DNA was incubated in polymerase reaction mixtures under conditions identical to those given for Fig. 6, extracted with sodium dodecyl sulfate-Pronase, adjusted to 0.6 N NaOH, incubated at 37 C for 30 min, and then centrifuged in 5 to 20% sucrose gradients containing 0.9 M NaCl, 0.6 N NaOH, 0.01 M EDTA (1). Conditions of centrifugation: SW 65 rotor, 64,000 rev/min, 110 min, 25 C. Gradients were collected dropwise directly onto filter paper squares. These were dried and counted in Liquiflour-toluene. (a) T_0, sample taken at onset of the reaction; (b) T_{120}, sample taken after 120 min of incubation. The arrow indicates the location in a separate gradient of lambda DNA which had not been exposed to virus.*

concentration of detergent does not appreciably disrupt virions (22; *unpublished data*).

The presence of ribonuclease in purified RSV may introduce at least two major artifacts into the study of the virion-associated DNA polymerase: (i) rapid degradation of 70S hybrid to a slowly sedimenting, equimolar DNA:RNA hybrid and (ii) degradation of the latter, yielding single-stranded DNA. The first artifact is readily apparent in the data presented above (Fig. 4). The second is more subtle, but, in view of the relatively low concentration of salt in the reaction mixtures (generally limited to ca. 0.01 M Mg^{2+}), it must be considered a likely possibility if extensive amounts of ribonuclease activity are present. Release of single-stranded DNA from hybrid structures in this manner could confuse efforts to interpret the events which mediate the synthesis of double-stranded DNA, the final product of the enzymatic reaction (8, 9). The present data indicate that it may be possible to study the mechanism of DNA synthesis within intact virions in the absence of artifacts due to ribonuclease if DNA synthesis is elicited with relatively low concentrations of detergent. Studies of this sort may profitably supplement experiments with template-dependent, purified polymerase.

The biphasic kinetics of DNA synthesis elicited by 0.05% NP-40 are similar to those reported for avian myeloblastosis virus (9) although the rate of DNA synthesis changes much earlier (ca. 4 min) in the latter instance. It has been proposed (9) that kinetics of this sort reflect the fact that two DNA polymerase activities are present in the virions of RNA tumor viruses: RNA-dependent synthesis of single-stranded DNA (8; *unpublished data*) and DNA-dependent synthesis of double-stranded DNA (9, 15, 24). According to this hypothesis, the initial, rapid reaction represents RNA-dependent synthesis of DNA which terminates at the point where the overall reaction rate changes. The ensuing slower rate of synthesis would then represent the DNA-dependent reaction. We presently have no conclusive evidence that this is correct. Nevertheless, endogenous RNA template has been extensively degraded by 1 hr after the onset of DNA synthesis (Fig. 3d), and it seems reasonable to presume that this degradation will eventually interrupt (or greatly depress) the RNA-dependent reaction. Alternatively, the change in reaction rate may reflect a failure to reinitiate synthesis of single-stranded DNA on the RNA template. Resolution of this issue will require further study of DNA synthesis in the presence of 0.05% NP-40.

We cannot presently explain the discrepancy between the results of our efforts to detect virion-associated deoxyribonuclease and those of Mizutani et al. (16). Moreover, the significance of the small amount of apparent nuclease activity directed against single-stranded DNA (Fig. 8) is presently indeterminate. Single strand-specific endonucleases have been described previously (17), and it appears likely that RSV con-

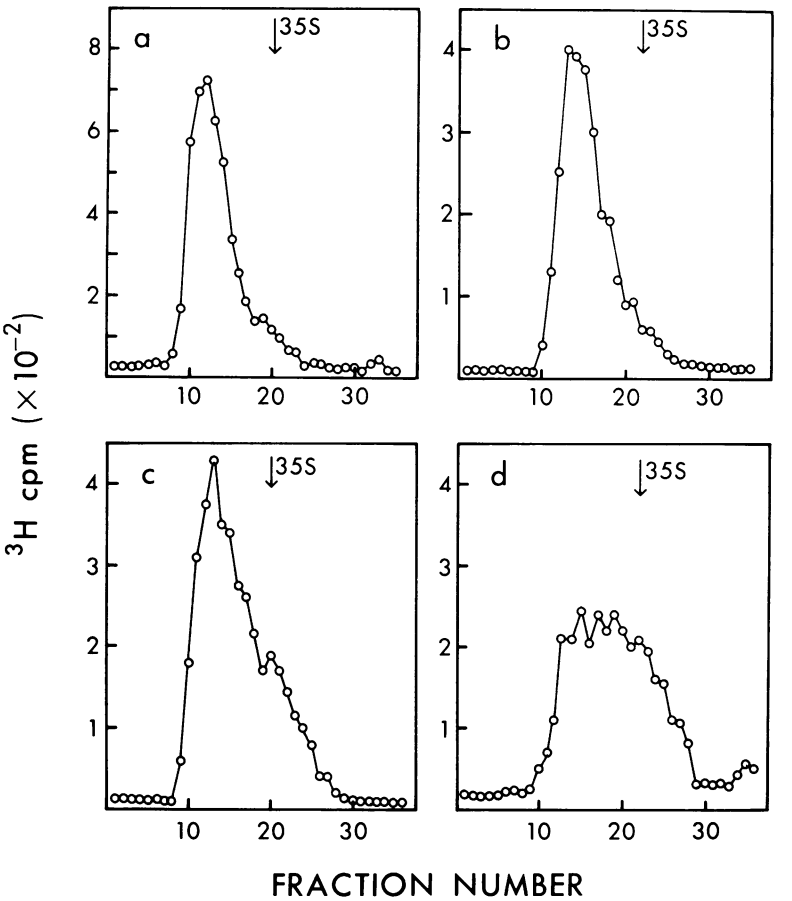

FIG. 8. *Centrifugation of denatured lambda phage DNA after exposure to polymerase reaction mixture. ^{3}H-labeled lambda phage DNA was denatured by incubation in 0.6 N NaOH at 37 C for 30 min. After neutralization, intact single strands (56S) were isolated by centrifugation in neutral sucrose gradients and stored at 4 C. Samples of this material were added to polymerase reaction mixtures as described for Fig. 6, incubated at 37 C, extracted with sodium dodecyl sulfate, and centrifuged through neutral sucrose gradients as described for Fig. 5. ^{32}P-labeled poliovirus RNA (35S) was added as a sedimentation reference. Conditions of centrifugation: SW65 rotor, 64,000 rev/min, 75 min, 4 C. Arrows indicate the position of the 35S poliovirus RNA marker. (a) T_0, sample taken at onset of incubation; (b) T_{120}, control, incubated 120 min in absence of virus but with all other components of the reaction mixture present; (c) T_{60}, virus, incubated 60 min in presence of virus; (d) T_{120}, virus, incubated 120 min in presence of virus. Similar results were obtained if virus was added but the detergent omitted.*

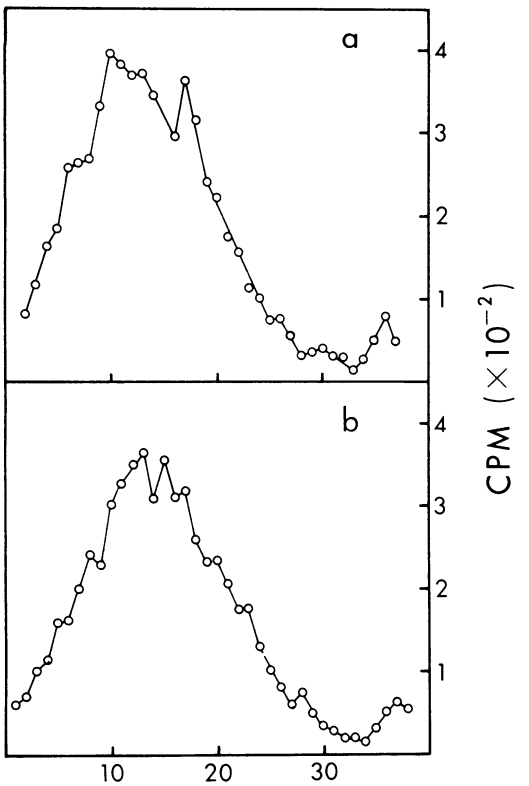

FIG. 9. *Centrifugation of avian DNA in alkaline sucrose after exposure to polymerase reaction mixture. Standard enzyme reaction mixtures were prepared with 500 μg of viral protein per ml and several concentrations of NP-40 (0, 0.01, 0.05, 2%) and incubated at 37 C. ^{3}H-labeled chick embryo fibroblast DNA was included in all of the mixtures. Samples were withdrawn at the indicated time points, treated with sodium dodecyl sulfate-Pronase for 45 min at 37 C, adjusted to 0.6 N NaOH, incubated at 37 C for an additional 30 min, and then centrifuged in alkaline sucrose gradients as described for Fig. 7. Conditions of centrifugation: SW65 rotor, 64,000 rev/min, 90 min, 25 C. Gradients were fractionated as in Fig. 7. The results were identical for all of the detergent concentrations tested. (a) Control, sample withdrawn at onset of reaction; (b) T_{90}, sample withdrawn after 90 min of incubation.*

tains such an enzyme as either an integral virion component or an adsorbed contaminant. The enzyme in question could conceivably play a role in the synthesis of virus-specific DNA by attacking the single-stranded DNA intermediate described by Manly et al. (13). This would provide an explanation for the small size of the final enzymatic product, but we presently have no evidence that such an explanation is, in fact, justified.

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