IgG-neutralized Influenza Virus Undergoes Primary, but Not Secondary Uncoating in vivo

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SUMMARY

Even when neutralized by saturating amounts of monoclonal IgG directed against the haemagglutinin, influenza virus attaches to cells with kinetics similar to those of infectious virus. It then enters those cells and is uncoated; its RNA becomes localized within the nucleus and its lipid envelope and associated proteins remain in the cytoplasm. In this report we show that despite the apparent normality of these early stages of virus–cell interaction, neutralized virus underwent no detectable primary transcription. In contrast, there was only a slight inhibition of transcription by neutralized virus in vitro which was insufficient to account for the loss in infectivity, despite using mRNA to measure the production of capped oligonucleotides or to prime the elongation step. To test whether the absence of primary transcription in vivo resulted from non-accessibility of the genome rather than an effect on the transcriptase complex itself, we examined the susceptibility to RNase of virion RNA after inoculation of cells with neutralized virus. Data clearly show that, unlike RNA of infectious virus, RNA of neutralized virus did not become sensitive to RNase and we conclude that neutralization of influenza virus by IgG results in failure of virus to undergo a secondary uncoating process which is necessary for the activity of the virion transcriptase complex. Finally we show that by treatment of virions in vitro with detergent it is possible to produce a core structure which is stable and has some of the properties expected of a structure resulting from primary uncoating.

INTRODUCTION

The initial stages of infection by influenza virus are attachment, uptake, uncoating and transport of the virion RNA and associated proteins to the nucleus (Stephenson & Dimmock, 1975; Stephenson et al., 1978; Hudson et al., 1978). Then follow primary and secondary transcription and replication, all of which take place in the nucleus (Shapiro et al., 1987). For virus neutralized with saturating amounts of IgG, attachment and entry into CEF and BHK cells occur with approximately normal kinetics and to the same extent as infectious virus (Possee & Dimmock, 1981; Possee et al., 1982; Dimmock et al., 1984; Taylor & Dimmock, 1985b). In contrast virus neutralized with IgM or secretory IgA does not enter cells at all although about 50% attaches (Taylor & Dimmock, 1985a, b). These data also prove that IgG-neutralized virus enters cells since they were all obtained using the same techniques. Attachment of both infectious and neutralized virus is sensitive to pretreatment of cells with neuraminidase indicating that they use the same cell receptors (N. J. Dimmock, unpublished data). After entry into cells IgG-neutralized virus is uncoated as demonstrated by the presence of 94% of envelope choline radiolabel in the cytoplasm while virion RNA accumulates normally in the nucleus (Possee et al., 1982). However, there was no secondary transcription in cells inoculated with neutralized influenza virus indicating that neutralizing antibody interrupts some stage before this event (Possee et al., 1982). In order to locate the loss of function primarily responsible for neutralization we have examined the possibility that neutralized virus directs primary transcription but fails to make the transition to secondary transcription. To this end we have

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used cycloheximide to block the synthesis of proteins necessary for secondary transcription (Hay et al., 1977; Barrett et al., 1979) and a radiolabelled probe sensitive enough to detect the small quantities of mRNA synthesized. The latter is necessary as primary transcription of mRNA represents only 5 to 15% of that (+) RNA synthesis which occurs in the absence of inhibitors of protein synthesis (Mark et al., 1979).

These experiments (described below) demonstrate that neutralized virus is unable to direct primary transcription. We were then concerned to establish whether neutralizing antibody affected the transcriptase complex per se and investigated the ability of detergent-uncoated neutralized virus to undergo mRNA-primed transcription in vitro. In the course of synthesis of capped viral mRNA influenza virus obtains 5'-capped oligonucleotides from suitable heterologous mRNAs (Krug, 1985). The transcriptase complex binds to the 5' cap, endonucleolytically cleaves the mRNA 10 to 13 bases from the cap, and adds one nucleotide (initiation) and by repeated addition (elongation) synthesizes influenza virus mRNA with a short heterologous sequence making up the 5' end. These processes were only slightly affected by a neutralizing monoclonal IgG suggesting to us the possibility that although neutralized virus is uncoated of its lipid envelope, it fails to undergo primary transcription because of the absence of an event required for activation of the transcriptase enzyme complex.

The process of uncoating of influenza virus is poorly documented. The first stage appears to occur when the low pH of the endosomal vesicle in which the virus has entered the cell induces a conformational change in the haemagglutinin (HA) resulting in fusion of the viral and endosomal membranes and liberation of the subviral particles into the cytoplasm (reviewed by Patterson & Oxford, 1986). In the presence of low concentrations of the antiviral compound rimantadine, subviral particles containing membrane protein (M) and nucleoprotein (NP) [M1-ribonucleoprotein (RNP)] are found in the nuclear-associated cytoplasm and nucleus whereas in the absence of the drug, RNP complexes in which only NP is detectable are seen in the same location (Bukrinskaya et al., 1982). Thus there is a second stage of uncoating involving loss of M1 protein from subviral particles which results in the release of RNP complexes. Furthermore when cells are infected, virion RNA becomes sensitive to RNase digestion, but in the presence of rimantadine RNA remains resistant to RNase degradation (Koff & Knight, 1979). Similarly, rimantadine prevents the loss of light sensitivity seen when neutral red-labelled virus is inoculated onto cells (Kato & Eggers, 1969). Taken together these data suggest that a second stage of uncoating concerned with the relaxation of a core structure is necessary before the viral genome becomes functional.

Below we describe data which support the concept of secondary uncoating and demonstrate that neutralized virus does not undergo this process.

**METHODS**

**Virus.** We used the avian strain of influenza virus A/FPV/Rostock/34 (H7N1) and a reassortant FPV/RA:PR/8/8 (H7N1). These were obtained as infected allantoic fluids from embryonated hen's eggs. Infectivity was measured by plaque assay on primary chick embryo fibroblast (CEF) monolayers (Morser et al., 1973) and HA by agglutination of chicken red blood cells. Virus was purified by differential centrifugation followed by successive banding on sucrose velocity and density gradients (Kelly & Dimmock, 1974). Radiolabelled virus was grown in CEF cell monolayers (Dimmock et al., 1977).

**Antibodies.** We used HC2, a mouse neutralizing anti-HA (FPV/R) monoclonal IgG2a (kindly provided by J. J. Skehel and A. R. Douglas), an anti-neuraminidase IgG (A/PR/8/34; H1N1) 112/10/2R6 (from W. Gerhard) as a virion-binding, non-neutralizing antibody and a monoclonal antibody (MAb) (185/1) directed against an H3 HA as a non-specific IgG. Polyclonal IgG against the HA was prepared by H. P. Taylor by intravenous inoculation of rabbits with virus in phosphate-buffered saline (PBS) on days 0 and 21. Animals were bled on day 28. IgG was purified by affinity chromatography on Protein A-Sepharose (Sigma) (Ey et al., 1978).

**Detection of primary transcription.** BHK-21 cell monolayers (10⁶ cells/dish) were inoculated with virus-antibody mixtures in PBS (equivalent to 100 p.f.u./cell for non-neutralized virus), or mock-inoculated with PBS alone, and held on ice for 1 h. The cells were washed and incubated in tissue culture medium for 3 h at 37 °C. Cells were treated for 1 h at 37 °C before infection with cycloheximide at 200 µg/ml (Avery & Dimmock, 1975). Cycloheximide was also included at the time of inoculation. This dose of cycloheximide inhibited protein synthesis by 98.9%. Cells were harvested and RNA was extracted with guanidinium isothiocyanate according to Maniatis et al. (1982). RNA (18 µg per duplicate) was denatured by the method of White & Bancroft (1982), applied to
nitrocellulose (Andemann Chemicals) and hybridized by a method based on that of Maniatis et al. (1982). Transcripts were detected with virion RNA that had been partially digested with alkali (100 mM-NaCO3, at 50°C for 1 h) and 5' end-labelled with [α-32P]ATP and polynucleotide kinase (Bethesda Research Laboratories). Hybridization was carried out for 24 h at 42°C and then filters were washed three times for 5 min at room temperature in 2 x SSC and three times for 20 min in 0.25 x SSC at 65°C, air-dried and autoradiographed at -70°C using Fuji Rx X-ray film and an intensifying screen. Individual dots were cut from the membrane and immersed in scintillation fluid (Beckman) to determine the amount of radioactivity bound.

Polyacrylamide gel electrophoresis of proteins. The gel system of Cook et al. (1979) containing 0.1% SDS and the discontinuous buffer system (Laemmli, 1970) were used. Gels were fixed in 50% methanol/7% acetic acid, soaked in Amplify (Amersham), dried and exposed to X-ray film. The intensity of bands on the autoradiograph was quantified using a Joyce-Loebl Chromoscan 3 densitometer.

Assay of influenza virus transcription in vitro

Elongation activity. The transcriptase activity of antibody-treated virus was assayed using the conditions described by Bouloy et al. (1979) with 0.25% NP40 to disrupt the viral envelope, but with cytoplasmic polyhedrosis virus (CPV) mRNA as primer. CPV mRNA was synthesized from CPV according to Smith & Furuichi (1980) but with 100 μg/ml bentonite in the reaction mixture. After removal of virus particles by centrifugation RNA was extracted with phenol. Ethanol-precipitated RNA was dissolved in 10 mM-Tris-HCl pH 8.0, 150 mM-LiCl, 2.5 mM-EDTA (TLE) containing 0.1% SDS and purified by chromatography on Sephadex G50.

To measure transcription, mixtures were made up on ice, adding virus last, and the reaction was started by raising the temperature to 30°C. Aliquots were assayed for TCA-insoluble [3H]UTP at the times shown. Consistent with its expected priming activity, we found that RNA synthesis was directly proportional to the amount of CPV mRNA added (data not shown). Its molar efficiency relative to ApG was 9500, comparing well with the figure of 7100 quoted by Kawakami & Ishihama (1983).

The transcriptase activity of viral cores isolated from gradients was assayed in the absence or presence of 0.25% NP40. The amount of TCA-insoluble radioactivity produced after 2 h incubation at 30°C was measured and the relative transcriptase activity (−NP40)+NP40) calculated.

Assays for initiation of influenza virus transcription. Reaction conditions were as described for elongation except that the only ribonucleoside triphosphate included was [32PGTP (Plotch et al., 1981) at a concentration of 1 μM (20 μCi/50 μl reaction mixture) and reactions were incubated at 30°C for 60 min. At the end of the reaction 0.5 μl of 2× TLE solution containing 0.2% SDS was added. Unincorporated [32PGTP was removed by spin column chromatography (Maniatis et al., 1982) using Sephadex G25 in 1× TLE containing 0.1% SDS. The eluate was phenol-extracted and ethanol-precipitated. Equal quantities were loaded onto slab gels of 16% acrylamide/0.8% bisacrylamide (Sanger & Coulson, 1978) and electrophoresed until the bromophenol blue marker had migrated 21 cm. Production of radiolabeled oligonucleotides was dependent upon the presence of both virus and CPV mRNA.

Assay for RNase sensitivity of influenza virion RNA after inoculation onto BHK-21 cells and in viral cores made in vitro. 32P-labelled influenza virus was inoculated onto BHK-21 cells held at 4°C and subsequently incubated for 90 min at 37°C. The RNase sensitivity was assayed following the method of Koff & Knight (1979) except that cells were frozen and thawed once only and further disrupted with ultrasound. The sonicate was incubated with RNases A (Sigma) (100 μg) and T1 (Sigma) (500 units) for 30 min at 37°C and samples were trapped onto filters. For each sample the fraction of radioactivity resistant to boiling in TCA was determined and subtracted.

Viral cores were produced by incubating virus for 4 min on ice with a mixture of detergents, usually 1 μl of 10% NP40 and 0.25% Triton X-100 per 1000 haemagglutination units (HAU) in a volume of 300 μl. Cores were purified by centrifugation at 12000 g for 16 h at 4°C on a 10:40:60% w/v sucrose step gradient and were treated with RNases A and T1 as described above.

RESULTS

Previous work has shown that neutralized influenza virus attaches to cells, enters cells and uncoats; the envelope remains in the cytoplasm and the RNA and associated proteins enter the nucleus (see Introduction). In none of these parameters could neutralized and infectious virus be distinguished either in the kinetics or final status. These data are reconfirmed in this study in which we found that equal amounts of neutralized or non-neutralized virus attached to BHK-21 cells (not shown), and that approximately 70% of cell-associated virion RNA from either neutralized or infectious inocula became nuclear (Fig. 1a) whereas over 85% of cellular RNA labelled with [3H]U over a 24 h period was cytoplasmic (Fig. 1b). The question as to the stage at which neutralized virus becomes unable to carry out the infectious process was addressed here by determining whether or not it could carry out primary transcription.
Failure of neutralized virus to direct primary transcription in vivo

Influenza virus transcription can be divided into two stages on the basis of the requirement for protein synthesis; only primary transcription occurs when protein synthesis is prevented. Neutralized virus was inoculated onto BHK-21 cells in the presence of cycloheximide and RNA extracted after incubation at 37 °C for the times shown in Fig. 2. Dot blot hybridization detected (+) RNA produced by primary transcription in cells inoculated with non-neutralized virus after 1 h and this accumulated with time. Detectable amounts of (+) RNA were not produced in cells inoculated with neutralized virus even after 4 h incubation. Thus neutralization directly or indirectly inhibits primary transcription, the first synthetic event of replication.

Failure of neutralizing antibodies to inhibit mRNA-primed transcriptase activity in vitro

Possee et al. (1982) reported that the elongation part of the transcriptase reaction was inhibited in vitro by neutralizing polyclonal antibody directed against the HA of strain FPV/R or by MAb 171/7 against the HA of A/X49 (H3N2) but not by non-neutralizing antibodies against the neuraminidase. However, the extent of inhibition (up to 89%) was much less than the extent of neutralization of infectivity (>99.9%). In addition to an elongation activity, the polymerase enzyme complex of influenza virus possesses activities which recognize and endonucleolytically remove 5'-capped oligonucleotides from heterologous mRNAs (Krug, 1985). Thus, if antibody were inhibiting the cap recognition or endonuclease reactions that yield the primer, infectivity could be reduced without marked inhibition of the in vitro elongation activity. Fig. 3 shows that 99% neutralization by HC2 IgG was accompanied by an inhibition of mRNA-primed
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(a) Time (h)

(b) Filter-bound radioactivity (cpm \times 10^{-7})

Fig. 2. Neutralized virus does not direct primary transcription in vivo. This is shown by analysis of influenza (+) RNA extracted from BHK-21 cell monolayers which had been pretreated with cycloheximide and inoculated with antibody-treated virus in the presence of cycloheximide. The reassortant FPV/R/PR/8 (1440 HAU) was incubated with 15 μg neutralizing (N; 0) anti-HA IgG (HC2) or an equivalent amount (μg) of non-neutralizing (C; 0) anti-NA IgG (2R6) at 25 °C for 1 h. MI, mock-infected cells. After inoculation cells were incubated at 37 °C for the times shown. RNA was then extracted and viral (+) RNA was detected as a dot blot (a) by hybridization to fragmented 5' end-radiolabelled virion (−) RNA. Dots were excised and radioactivity was counted (b).

transcription of less than 50%. In an experiment where the utilization of capped mRNA was tested directly, neutralization had no detectable effect upon the generation and addition of the first nucleotide (32P-labelled GTP) to primer oligonucleotides derived from CPV mRNA (Fig. 4). Similar results were obtained using IgG from a polyclonal anti-HA IgG (data not shown).

Insensitivity of the genome of neutralized virus to RNase in vivo

Others have shown that, after infection in vivo, the RNA of infectious influenza virus becomes progressively more sensitive to digestion by RNase thus reflecting its involvement in active transcription (Koff & Knight, 1979). This gives an ideal system to determine whether neutralized virus, which we know loses its lipid envelope in vivo with its RNA becoming nuclear (Possee et al., 1982; Taylor & Dimmock, 1985a; this study, data not shown), enters that RNase-sensitive state which is presumably a necessary preliminary to, or corollary of, primary transcription. We first confirmed the data of Koff & Knight (1979) by showing that after inoculation onto cells on ice for 90 min (Fig. 5, right-hand hatched column) the virion RNA of infectious (non-neutralized) virus was still essentially RNase-resistant but that after warming to 37 °C for 90 min, the majority (58%) of the genome of non-neutralized virus became RNase sensitive, an increase of over sevenfold (Fig. 5, right-hand open column). In contrast, when neutralized virus was inoculated virion RNA remained RNase-resistant even though, as stated above, such virus loses its lipid envelope and the genome becomes located in the nucleus. The technique is relatively insensitive but the differences are significant (P = 0.05 with n − 1 degrees of freedom). At the three highest concentrations of antibody (all neutralizing to at least 99.9%) virion RNA remained resistant to RNase even after incubation of inoculated cells at 37 °C, at a level which did not differ significantly from non-neutralized virus inoculated onto cells and held on ice. The RNA of 79% neutralized virus, however, was more susceptible to RNase, but still less susceptible than that of non-neutralized virus. There is thus a good correlation between
neutralization and absence of an increase in RNase sensitivity of the virion genome suggesting that neutralization prevents events which in the normal infectious process lead to relaxation of structure(s) associated with, and necessary for, the expression of virion RNA.

Properties of influenza virus cores produced in vitro

Data above suggest that after removal of the lipid envelope in vivo, the virion genome resides in a core structure in which it is inaccessible to RNase. Such structures have not been unequivocally demonstrated to exist although it is widely assumed from electron microscopic evidence that the M1 protein forms a layer surrounding the RNP (Apostolov & Flewett, 1969; Bäch et al., 1969; Compans & Dimmock, 1969) and structures such as these have been seen under the electron microscope after various delipifying treatments (Nermut, 1970; Skehel, 1971; Schulze, 1972; Reginster & Nermut, 1976). Brief exposure of unlabelled virus to an NP40/Triton
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Fig. 5. Virion RNA of neutralized virus remains resistant to RNase in vivo. The effects of different dilutions of neutralizing antibody on the RNase sensitivity of the genome of influenza virus after inoculation onto BHK cells are shown. 32P-labelled FPV/R was incubated for 1 h at 25 °C with dilutions of neutralizing monoclonal IgG (HC2), which lowered infectivity as indicated, or with non-specific IgG at the relative concentration shown. Virus–antibody mixtures were cooled and 100 μl was inoculated onto six replicate monolayers of BHK-21 cells (6 × 10⁶ cells/dish). Each 100 μl initially contained 6.8 × 10⁷ p.f.u., 2.7 × 10² HAU and 2.1 × 10⁶ c.p.m. Two monolayers were sampled after 90 min on ice (hatched sections of bars) and the remaining four dishes were transferred to 37 °C (unshaded) and incubated in tissue culture medium for a further 90 min before RNase sensitivity was assayed. The ordinate shows the mean values and a bar indicates the standard error of the mean (P = 0.05 with n - 1 degrees of freedom). Values shown along the abscissa are as follows: (i) neutralizing antibody, (ii) 1/relative antibody concentration, (iii) percentage neutralization.

X-100 mixture and centrifugation on a sucrose density gradient produced a uniform band, visible by eye, which had a higher density than virions (Fig. 6a). When analysed for polypeptides, cores produced from [35S]methionine-labelled virus had the characteristics expected for a core structure being composed of the majority of NP and M1 present in the gradient, but with only traces of HA and NA (Fig. 6b). P proteins were not visible at this exposure even in whole virions, nevertheless cores contained 76% of the maximum transcriptase activity (Fig. 6c). Only traces of lipid (defined here as TCA-soluble 32P) were associated with cores, most of it being found at the top of the gradient (data not shown). Cores and virus banded at approximate densities of 1.35 and 1.22 g/ml, respectively. Glycoproteins form a discrete band of lower density than the cores at the position marked in Fig. 6(a) although this is never visible by eye. The procedure was much more efficient than treatment of virus with deoxycholate (Skehel, 1971) which converted only a minority of virus into cores with the rest being reduced to constituent components. The cores are stable at 4 °C and can be rebanded without loss. With a longer treatment at ×1 detergent concentration the cores disintegrate.

The RNase sensitivity of the RNA in cores produced using a range of detergent concentrations was assayed and was compared to that in structures obtained from neutralized virus in vivo as described above. A relative detergent concentration of 0.3 removed the lipid (Fig. 7a) but the cores remained RNase-resistant (Fig. 7b). A threefold higher concentration of detergent was required before any sensitivity to RNase was seen, suggesting that this much was required to modify the structure of the core particle. In parallel experiments, mRNA-primed transcriptase activity and the protein composition of the cores were determined. Transcriptase activity of cores was measured without adding more detergent or with the addition of 0.25% NP40 which gives a maximum value. As the quantity of detergent used to prepare cores was increased the transcriptase activity of the cores in the absence of any free detergent also
Fig. 6. Preparation of influenza virus cores in vitro. Virus was mixed with 0.03% NP40 and 0.001% Triton X-100 (called arbitrarily a detergent concentration of 1.0) in ice for 4 min and immediately centrifuged at 112000 g for 16 h at 4 °C on a 10:40:60% (w/v) sucrose step gradient. The optimum proportions were 1 μl of a 10% NP40, 0.25% Triton X-100 mixture per 10^7 HAU of virus in a total volume of 300 μl PBS. (a) Positions of visible bands of virus (V) and cores (C); m is the meniscus; gp marks the position of the viral glycoproteins although these were never visible. (b) PAGE analysis of [35S]methionine-labelled virus (lane 1), glycoproteins (lane 2) and cores (lane 3) pooled from positions of peak radioactivity on the gradients. (c) Distribution of transcriptase activity from two separate gradients used to purify virions or to prepare cores as described above. Arrows mark the peak of core (●) or virion (○) protein determined separately. Gradient fractions were assayed for transcriptase activity with the addition of 0.25% NP40 and [3H]UTP in transcriptase buffer. No primer was used and the incubation time was 30 min.
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Fig. 7. RNase sensitivity and transcriptase activity of cores purified by sucrose density gradient centrifugation of virus treated with increasing amounts of detergent. In (a) and (b) $^{32}$P-labelled influenza virus was treated with detergent for 4 min on ice and centrifuged as in Fig. 6. A relative detergent concentration of 3-0 contains 0-1% NP40 and 0-0025 Triton X-100. The gradients were fractionated and the total amount of $^{32}$P at the top of the gradient (representing mainly viral lipid) is plotted in (a) and the RNase sensitivity of cores is presented in (b). (c) The transcriptase activity of cores produced by detergent treatment of unlabelled influenza virus. These were assayed in triplicate for transcriptase activity using CPV mRNA as primer either without the further addition of detergent or in the presence of 0-25% NP40 which gives a maximum value, and expressed as a percentage of the maximum value.

Analysis of cores by SDS-PAGE (Fig. 8) followed by quantitative scanning of the resulting autoradiograph showed that the proportion of M1 relative to NP decreased by over twofold as more detergent was used in their preparation. At this exposure P proteins could not be seen although longer exposures showed that they were not diminished at any detergent concentration.

DISCUSSION

Previous data have shown that saturating amounts of neutralizing IgG specific for influenza virus HA do not significantly affect any of the early stages of infection in vivo including attachment, penetration, removal of the envelope or accumulation of the viral genome plus associated proteins in the nucleus (Possee & Dimmock, 1981; Possee et al., 1982; Dimmock et al., 1984; Taylor & Dimmock, 1985b; Taylor et al., 1987). However, the virus was unable to
undergo secondary transcription (Possee et al., 1982). In this report we show that virus neutralized with monoclonal anti-HA IgG undergoes no primary transcription in vivo. Is this an effect on the transcriptase activity directly or the result of some indirect influence? To answer this question we returned to the in vitro system in which Possee et al. (1982) showed that there was some inhibition of transcriptase by neutralizing antibody, although always at a level over 100-fold lower than the amount of neutralization. One possible explanation was that neutralization did not affect the elongation part of the transcriptase reaction but the initial stages of cap recognition and endonucleolytic cleavage of the mRNA primer (Krug, 1985). However, we have been unable to see any substantial inhibition of mRNA-primed transcription or capped oligonucleotide production after neutralization with the HC2 MAb or with a neutralizing polyclonal rabbit IgG (data not shown). Nonetheless as Shimizu et al. (1985) have found, the result might be different with monoclonal antibodies directed against other antigenic sites on the HA. [HC2 produces escape mutants with a single mutation in HA1 (Gly 133→Glu) in site A (J. McCauley, A. S. Carver, H. P. Taylor and N. J. Dimmock, unpublished data).] This is equivalent to residue 143 and antigenic site A on the H3 structure (Wiley et al., 1981).

Thus as the in vitro system does not appear to reflect accurately the situation in vivo we have re-examined the uncoating of neutralized virus in vivo to determine whether this was affected by neutralization. Since earlier work had shown that the lipid envelope, HA and NA of neutralized virus were found in the cytoplasm whereas virion RNA accumulated in the nucleus (Possee et al., 1982; A. S. Carver & N. J. Dimmock, unpublished data), it was thought that uncoating of neutralized virus occurred normally. However, when we examined the susceptibility of viral RNA to digestion by RNase (Fig. 5) we found that neutralization inhibited the extent to which virion RNA became RNase-sensitive. Thus it seems that there is a second stage of uncoating which occurs after the lipid and associated envelope proteins have been removed, an event which is suggested by studies on the inhibition of uncoating by rimantadine (Koff & Knight, 1979; Bukrinskaya et al., 1982). It follows that the stage of infection compromised by neutralization occurs after removal of the viral envelope and at, or prior to, secondary uncoating. We now postulate that neutralization prevents secondary uncoating which in turn results in failure of the virus to direct transcription. Thus transcription may not take place because the transcriptase complex is inaccessible to small molecules needed for RNA synthesis. Alternatively it may be that continued presence of M1 is directly inhibitory to transcription (Zvonarjev & Ghendon, 1980; Mikheyeva & Ghendon, 1983; Kato et al., 1985; Ye et al., 1987).

Fig. 8. (a) SDS-PAGE analysis of the polypeptide composition of cores produced by detergent treatment of [35S]methionine-labelled virus as described in Fig. 5 showing the progressive loss of M1 protein relative to NP with increasing detergent concentration (concentrations are 0.1, 0.3, 1 and 3 in lanes 2 to 5, respectively). Lane 1, untreated virus. A scan of autoradiograph tracks showing virus treated with the lowest (a) and highest (b) relative detergent concentrations is shown in (b).
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In an effort to gain some understanding of the nature of the de-enveloped, RNase-insensitive, transcriptionally inactive particle produced by neutralization with IgG we treated virus with critical amounts of detergent. Unlike a previous study with deoxycholate (Skehel, 1971) this produced a stable 'core' of greater density than virus and which lacked the majority of lipid and envelope proteins. Of particular interest was the fact that with a relative detergent concentration of 0.3, even though there was maximum release of $^{32}$P-labelled presumptive lipid, virion RNA was almost totally resistant to degradation by RNases, a situation which exactly parallels that of neutralization. Increasing the detergent concentration rendered the genome RNase-sensitive in a manner analogous to secondary uncoating. Failure to obtain RNase sensitivity of greater than 50% probably reflects the fact that we used TCA precipitation as a measure of the integrity of virion RNA rather than, for example, by running it on a denaturing gel. In general there was also good correlation between the transcriptase activity of viral cores taken from their characteristic position on the sucrose density gradient and the amount of detergent used to release them from virions. However the fact that their activity could always be increased by adding detergent showed that they were not maximally activated. The slightly greater transcriptase activity (30% of maximum) of cores prepared at a detergent concentration of 0.3 compared with RNase sensitivity (6% of maximum) may reflect the greater permeability of the cores to RNA precursors than to RNases or indicate that RNA is being precipitated by TCA in association with other core components.

Although we must be cautious in comparing cores prepared with detergent to structures which result from secondary uncoating of virus in vivo, our data lead us to suggest that the early events of infection are as follows. Primary uncoating in vivo consists of the removal of the lipid envelope and associated glycoproteins, resulting in a spheroidal core structure which is bounded by M1 protein and contains all the other components of the virion. Further we suggest that this is transcriptionally inactive until it undergoes the events of secondary uncoating which increase its permeability before or after it gets to the nucleus. Much more remains to be done. If cores from neutralized virus are inactive solely because of an inability to undergo secondary uncoating we would predict that they should be capable of being activated by detergent in vitro. So far we have failed to obtain an answer to this question. In summary we see IgG neutralization as the transmission across the viral envelope of a signal which is initiated by anti-HA IgG as first suggested by Possee et al. (1982). However from the data described above we conclude that the simplest interpretation of the effect of this signal is upon the secondary uncoating process rather than the transcriptase activity per se.

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