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# Influenza Virus-Induced Ribonucleic Acid Nucleotidyltransferase and the Effect of Actinomycin D on Its Formation*

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ABSTRACT: A ribonucleic acid (RNA) nucleotidyltransferase has been isolated from tissue infected with influenza virus PR-8. It shows a requirement for all four ribonucleoside triphosphates and $Mg^{2+}$. Guanidine and actinomycin D have no effect on the activity of this RNA nucleotidyltransferase preparation *in vitro*. However, actinomycin D prevents the appearance of viral RNA nucleotidyltransferase activity in tissues infected with influenza virus. The mode of action of actinomycin D is discussed.

Infection with RNA viruses causes the appearance of a new cytoplasmic RNA nucleotidyltransferase (nucleotide triphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) (Baltimore and Franklin, 1963; Baltimore *et al.*, 1963; Haruna *et al.*, 1963; Weissman *et al.*, 1963; August *et al.*, 1965a) and the conversion of the parental RNA into a double-stranded form which serves as template for the synthesis of progeny RNA (Ochoa *et al.*, 1964; Baltimore *et al.*, 1964; Burdon *et al.*, 1964; Fenwick *et al.*, 1964; Kaerner and Hoffmann, 1964; Kelly and Sinsheimer, 1964; Shipp and Haselkorn, 1964; Montagnier and Sanders, 1963; August *et al.*, 1965b). These processes are summarized by reactions 1 and 2, which might be catalyzed by viral RNA nucleotidyltransferase.

$$\text{viral RNA} \longrightarrow \text{RNA double-stranded} \quad (1)$$

$$\text{4 nucleoside triphosphate} \xrightarrow{\text{RNA double-stranded}} \text{4 pyrophosphate} + \text{viral RNA} \quad (2)$$

This report describes the isolation of virus-induced RNA nucleotidyltransferase from chorio-allantoic membrane (CAM)[1] infected with influenza virus, PR-8 strain, and the results obtained in the study of the mechanism of action of actinomycin D on influenza virus replication.

Influenza is one of the few RNA viruses inhibited by actinomycin D (Barry *et al.*, 1962). This antibiotic inhibits the formation of RNA by DNA-dependent RNA nucleotidyltransferase (Goodman and Rich, 1962; Reich *et al.*, 1962; Yankofsky and Spiegelman, 1963; Reich, 1964). Actinomycin-sensitive RNA synthesis has thus been suggested to be involved in the multiplication of influenza. However, it has been shown that actinomycin D inhibits only in the early part of the replication cycle (White *et al.*, 1965). It is suggested that influenza viral RNA replication is catalyzed by a viral RNA nucleotidyltransferase which is similar to that described by Baltimore *et al.* (1963), but is not synthesized in the presence of actinomycin D. We have found an RNA nucleotidyltransferase system in the cytoplasm of CAM infected with influenza and have carried out a study of the effects of actinomycin D on the appearance and activity of this enzyme system.

## Experimental Procedures

*Virus.* The PR-8 strain of influenza A virus, adapted to egg, was used throughout these studies. The virus was prepared by inoculating 10-day-old chick embryos by the allantoic route with 0.2 ml of infected allantoic fluid diluted in broth to $10^{-5}$. The infected eggs were incubated for 48 hr at 35°, and the allantoic fluid was collected. This virus solution was used immediately.

*Hemagglutination Test.* Serial twofold dilutions of the virus preparation were made in saline solution. To 0.5 ml of each dilution was added 0.5 ml of a 0.5% suspension of washed chicken red blood cells. The test mix-

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[1] Abbreviations used in this work: CAM, chorio-allantoic membrane; sucrose–Mg, 0.25 M sucrose containing 0.001 M $MgCl_2$; TCA, trichloroacetic acid; AMP, adenosine monophosphate; GMP and GTP, guanosine mono- and triphosphate.

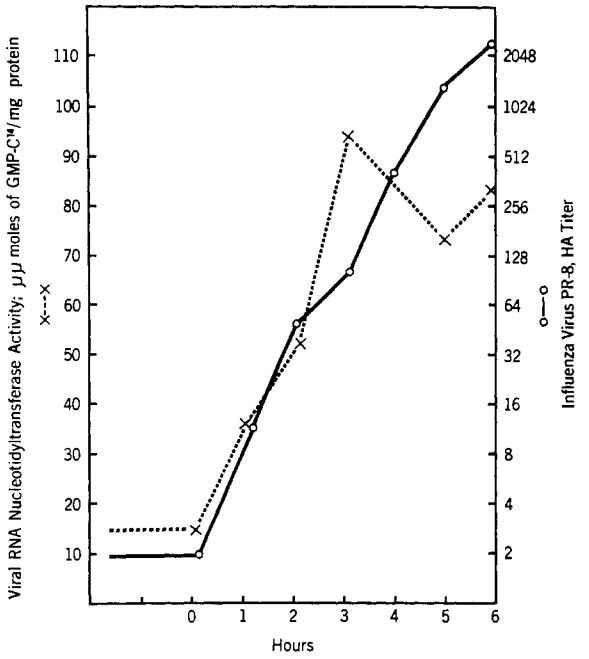


FIGURE 1: Specific activity of viral RNA nucleotidyltransferase after infection of CAM with influenza virus, PR-8; growth curve of virus. Enzyme preparations were made from infected CAM which had been incubated for various periods. Activity was assayed as described in Table I, with 0.2 μmole of GTP-$^{14}$C (1 μcurie/μmole) as precursor. Results are expressed as μμmoles of GMP-$^{14}$C incorporated per mg of protein.

TABLE I: Viral RNA Nucleotidyltransferase in CAM Cells Infected with Influenza Virus PR-8.[a]

| Additions | μμmoles incorp./mg protein | |
|---|---|---|
| | Infected | Uninfected |
| ATP-$^{14}$C | 205 | 7 |
| GTP-$^{14}$C | 93 | 0 |

[a] The assay mixture (0.5 ml) contained: 50 μmoles of Tris buffer, pH 8.5; 5 μmoles of phosphoenolpyruvate; 0.2 μmole of magnesium acetate; 50 μg of pyruvate kinase; 0.2 μmole of adenosine, cytosine, uridine, and guanidine triphosphates; and 0.2 ml of the microsomal suspension (10 mg of protein/ml). Labeled ribonucleotide (0.2 μcurie) was used as indicated. After 15-min incubation at 37°, the reaction mixture was chilled in ice and 0.5 ml of 0.1 M sodium pyrophosphate was added, followed by 5 ml of 10% TCA. The precipitate was collected on a Millipore membrane (25 mm in diameter, 8 μ in porosity) by filtration and thoroughly washed with 5% TCA containing 0.1 M sodium pyrophosphate. The dried membrane was counted in a Tri-Carb scintillation counter. Protein was determined by the method of Lowry *et al.* (1951). All results were corrected by subtracting the counting rate of an unincubated sample which is consistently below 60 cpm.

tures were incubated in the refrigerator at 4° for 2 hr, and the pattern formed by the red blood cells at the bottom of the tubes was recorded. The last tube giving partial but definite agglutination was considered the end point, which is expressed as the reciprocal of that dilution.

*Viral RNA Nucleotidyltransferase.* The chorioallantoic membranes were collected from 150 12-day-old embryonated eggs and were washed twice in cold sterile saline and once in Tyrode's medium (Parker, 1950). The membranes were then minced with scissors and were washed twice again with Tyrode's medium to remove blood. The tissue was then dispersed in 300 ml of medium and added to 800 ml of virus solution with a hemagglutination titer of 4096. After a 1-hr absorption period at 37°, the tissue was washed extensively and added to 800 ml of fresh medium, and incubation at 37° was begun (zero time). After 5 hr of incubation, the tissue was harvested by centrifugation, suspended in 0.25 M sucrose containing 0.001 M $MgCl_2$, and recentrifuged. The tissue was then suspended in 300 ml of sucrose–Mg with 20 g of acid-washed glass beads, and the mixture was homogenized for 1 min at high speed in a Virtis 45 homogenizer. The homogenate was centrifuged at 10,000 × *g* for 10 min to remove beads, cell debris, nuclei, and mitochondria. The supernatant fraction was centrifuged at 78,000 × *g* for 3 hr, and the resulting pellet was dispersed in sucrose–Mg at a concentration of 10 mg of protein/ml. The microsome pellet was usually stored frozen at −20° for 2–3 days with retention of full activity.

## Results

*Viral RNA Nucleotidyltransferase in CAM Infected with Influenza Virus, PR-8 Strain.* As is shown in Table I, microsomal preparations from influenza virus infected CAM incorporated 30 times more AMP-$^{14}$C than did similar preparations from uninfected tissues. In fact, the microsomal preparations from uninfected CAM show no incorporation at all with $^{14}$C-labeled GTP.

The kinetics of viral RNA nucleotidyltransferase appearance in influenza virus infected CAM are shown in Figure 1. The nucleotidyltransferase activity appeared shortly after incubation, rose to a maximum at 3 hr, and then plateaued. The appearance of viral RNA nucleotidyltransferase correlates well with the increase in virus (Figure 1). However, the results suggest that maximal amounts of viral RNA nucleotidyltransferase are made before a full yield of virus particles is produced. It is also possible that the increased activity is due not to enzyme increase, but to accumulation

TABLE II: RNA Nucleotidyltransferase Activity in Microsomal Preparation of Uninfected CAM.[a]

| | GMP-$^{14}$C ($\mu\mu$moles incorp/mg protein) | | | | | |
|---|---|---|---|---|---|---|
| | A | | | B | | |
| Additions | 4 Ribonucleotides | Single ribonucleotide | Net | 4 Ribonucleotides | Single ribonucleotide | Net |
| None | 18 | 18 | 0 | 13.5 | 2.5 | 11 |
| RNA | 18 | 18 | 0 | 14 | 0 | 14 |
| DNA | 12.5 | 13.5 | 1 | 14 | 0 | 14 |

[a] The assay conditions for column A are those of viral RNA nucleotidyltransferase, while the assay conditions for column B are those of DNA-dependent RNA nucleotidyltransferase (Balandin and Franklin, 1964). The microsomal fraction (12 mg of protein/ml) was prepared from CAM as described in the text. The net incorporation represents the enzymatic activity of the 4-ribonucleotides-dependent reaction.

TABLE III: Requirements for Four Nucleoside Triphosphates Reaction.[a]

| System | AMP-$^{14}$C incorporated ($\mu\mu$moles/mg protein) | GMP-$^{14}$C (incorporated $\mu\mu$moles/mg protein) |
|---|---|---|
| Complete | 210 | 95 |
| Omit CTP | 105 | 37 |
| Omit UTP | 87 | 37 |
| Omit GTP | 88 | |
| Omit ATP | | 12 |
| Omit ATP, CTP, UTP | | 10 |
| Omit $Mg^{2+}$ | 0 | 25 |
| Add ribonuclease (10 $\mu$g) | 0 | 6 |
| Add deoxyribonuclease (10 $\mu$g) | 200 | 82 |
| Add actinomycin D (2 $\mu$g/ml) | | 75 |
| Add guanidine (1 mM) | | 88 |
| Add DNA (100 $\mu$g) | | 100 |
| Add RNA (100 $\mu$g) | | 80 |

[a] The conditions of experiment were identical with those described in Table I. ATP, UTP, GTP, CTP are adenosine, uridine, guanidine, and cytosine triphosphates, respectively.

of cytoplasmic viral RNA as a template for a preexisting cellular enzyme. Since it is unlikely that a cellular enzyme would only specifically utilize viral RNA as a template, the preexisting cellular RNA nucleotidyltransferase should also be capable of using DNA or RNA from other sources as template. There was no stimulation in the enzymatic activity when calf thymus DNA or yeast RNA was incubated with the microsomal preparation of noninfected cells (Table II) or the viral RNA nucleotidyltransferase (Table III). It thus excludes the possibility of the presence of an RNA nucleotidyltransferase in the microsomal preparation of noninfected cells.

Further evidence for the induction of this enzyme system was provided when a similar microsomal preparation of noninfected cells was incubated with the viral RNA nucleotidyltransferase. There was no significant change in the enzymatic activity. This experiment, then, rules out the presence of inhibitors in normal tissue and their depression during infection.

*Requirements for Four Nucleoside Triphosphates.* The formation of RNA by viral RNA nucleotidyltransferase requires all four ribonucleoside triphosphates and a divalent metal ion (Table III). The addition of RNAase results in no appreciable product formation, while the addition of DNAase and actinomycin D causes no significant decrease of enzyme activity. In the absence of a single ribonucleotide, the extent of reaction is greatly decreased. The observed incorporation of a single ribonucleotide into the acid-insoluble material may be due to nonspecific reaction or homopolymerization. This single ribonucleotide reaction does not occur in the microsomes of uninfected CAM (Table I). However, to correct for any nonspecific incorporation of labeled ribonucleotide into the acid-insoluble material, further enzymatic assays were performed using an identical reaction mixture, except without unlabeled ribonucleotides as the experimental blank.

*pH Optimum and Metal Ion Requirement.* The optimum pH for RNA synthesis is 8.5 (Figure 2). As mentioned previously, a divalent ion is required for viral RNA synthesis. $Mg^{2+}$ is the most effective metal ion tested, although $Mn^{2+}$ also stimulates slightly (Figure 3). At concentrations higher than 4 mM, magnesium inhibits the reaction. Thus, this enzyme differs from the normal DNA-dependent RNA nucleotidyltransferase in that the latter system requires $Mn^{2+}$ at low concentrations, but $Mg^{2+}$ at higher concentrations (Fox and Weiss, 1964; Furth and Ho, 1965).

*Inhibition by Actinomycin D of Viral RNA Nucleotidyltransferase Appearance in Infected CAM.* Actinomycin D has no effect on the activity of viral RNA nucleotidyltransferase preparation *in vitro* (Table III). In contrast to the lack of direct effect on the nucleotidyltransferase, actinomycin D prevents the appearance of the viral RNA nucleotidyltransferase activity in CAM infected with influenza virus when the tissue was treated with actinomycin D at zero time (Figure 4). It was of interest to determine the effect of actinomycin D when added during the exponential increase phase in virus multiplication.

CAM was inoculated with influenza virus and actinomycin D (40 $\mu$g/ml) was added at intervals thereafter.

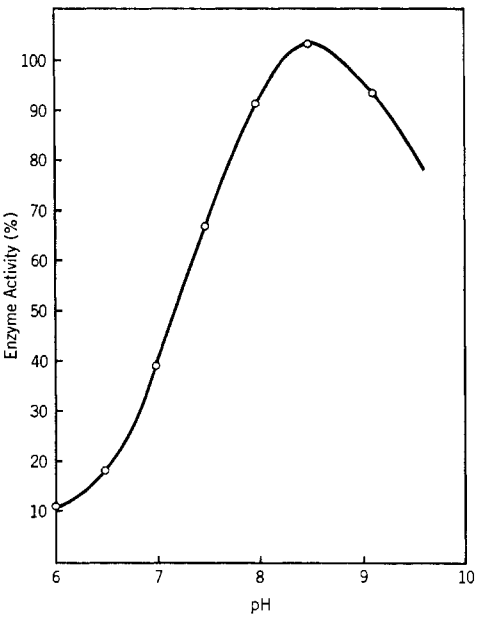


FIGURE 2: Effect of pH on the activity of viral RNA nucleotidyltransferase. The enzyme assay was carried out as described in Table I, except that an identical reaction mixture omitting unlabeled ribonucleotide was run as the experimental blank. The enzyme activity is expressed as per cent of activity against that at pH 8.5.

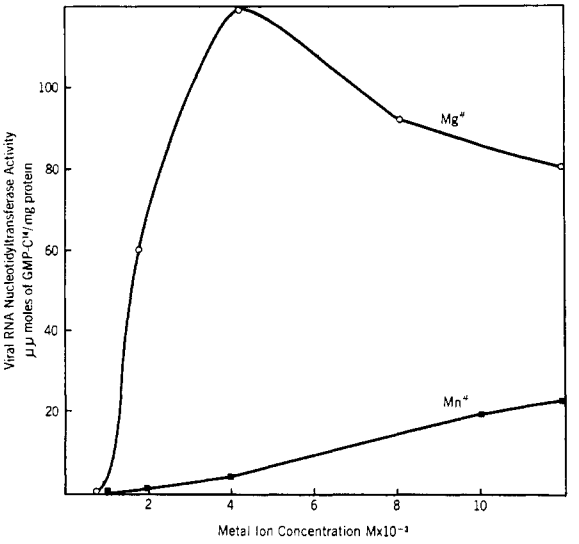


FIGURE 3: Effect of metal ion concentration on the activity of viral RNA nucleotidyltransferase. The reaction conditions were as for Figure 2 except that the enzyme activity is expressed as $\mu\mu$moles of GMP-$^{14}$C incorporated per mg of protein against that without the other three nucleotides. The reaction was carried out at its optimum pH, 8.5.

The total yield of viral RNA nucleotidyltransferase was determined after 5 hr and was expressed as a percentage of the 5-hr yield from untreated controls (Figure 4). Infected CAM produces a yield of 15% that of untreated controls when actinomycin D is added at 1 hr after post infection, while the drug added at 2 hr after infection has no inhibitory effect at all. It would appear that actinomycin D inhibits the formation of viral RNA nucleotidyltransferase which reaches full activity about 3 hr after infection.

Guanidine has been shown to prevent the appearance of viral RNA nucleotidyltransferase activity in HeLa cells infected with poliovirus (Baltimore *et al.*, 1963). We have also tested the effect of guanidine on the appearance and activity of influenza virus-induced RNA nucleotidyltransferase system. In contrast to actinomycin D, guanidine has no effect on the formation of viral RNA nucleotidyltransferase nor on its enzymatic activity (Table III and Figure 4). This finding is to be expected since guanidine inhibits only the multiplication of picornavirus (Melnick *et al.*, 1963) but has not been shown to affect the multiplication of viruses which belong to other major groups, such as influenza virus (Eggers and Tamm, 1961, 1962; Rightsel *et al.*, 1961).

## Discussion

From the foregoing results, it appears that influenza virus, like poliovirus and Mengovirus (Baltimore *et al.*, 1963; Baltimore and Franklin, 1963), induces the appearance in infected CAM suspension of an RNA nucleotidyltransferase activity which is not present in the uninfected cells. The time course of enzyme appearance and the correlation of activity with the growth of influenza virus implicate the enzyme in the process of viral RNA synthesis.

Although viral RNA nucleotidyltransferase is similar to DNA-dependent RNA nucleotidyltransferase in that the reactions require ribonucleoside triphosphate as substrate, they differ in the metal requirement. At lower concentrations of metal ion (*e.g.*, 4mM), the synthesis of normal cellular RNA requires $Mn^{2+}$, while the viral RNA nucleotidyltransferase requires $Mg^{2+}$.

It has been reported by Smith *et al.* (1955) that the mature influenza viruses contain some of the host antigens. The action of actinomycin D on influenza multiplication has thus been first postulated to interfere with the synthesis of the host antigen necessary for the maturation of viruses. However, an electron microscope study on influenza replication by Duc-Nugyen *et al.* (1965) has indicated that little or no host antigen is associated with mature virus particles. Moreover, recently actinomycin D has been shown to block only the early stage of influenza viral multiplication cycle (White *et al.*, 1965; Cohen and Maassab, 1965). By the time the synthesis of viral RNA and structural protein begins, actinomycin D is totally inactive. It seems unlikely then that actinomycin D would interfere in the maturation of virus which occurs at a later stage of the multiplication cycle.

On the other hand, it has been shown recently by Honig and Rabinovitz (1965) that actinomycin D

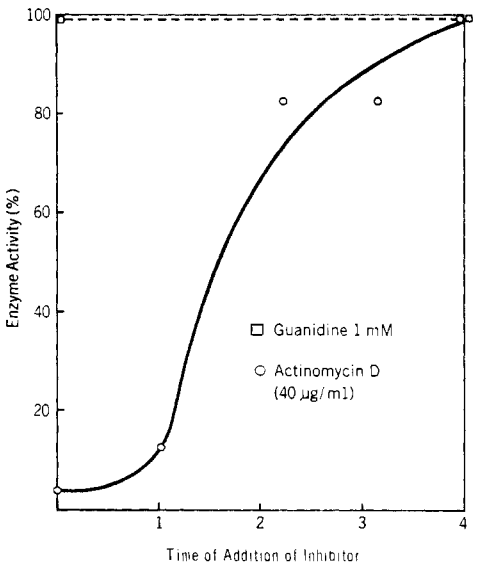


FIGURE 4: Inhibition of viral RNA nucleotidyltransferase formation by the late addition of actinomycin D or guanidine. After inoculation with influenza virus, PR-8, actinomycin D (40 µg/ml) or guanidine (1 mM) was added at the time shown. The total yield of viral RNA nucleotidyltransferase was determined after 5 hr and is expressed as a percentage of the 5-hr yield from untreated controls.

exerts an inhibition of protein synthesis unrelated to the effect on template RNA synthesis. Based on our results, it can be suggested then that the antibiotics inhibit virus multiplication by blocking the synthesis of viral RNA nucleotidyltransferase. It is noteworthy that guanidine also inhibits the formation of viral RNA nucleotidyltransferase in polio virus infected cells. However, the exact mechanisms of these inhibitions are not clear at the present time.

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