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In Vitro Transcription Catalyzed by Heat-Treated Human Rotavirus

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The in vitro characteristics of human rotavirus transcription have been examined. The virus has an associated RNA polymerase activity which was activated after a heat shock treatment. The enzyme required the presence of the four ribonucleoside triphosphates and a divalent cation (Mg^{2+}), and it required an optimum pH of 8.5. The polymerase was activated by monovalent salts and inhibited by Na PP_i. The addition of actinomycin D, α-amanitin, or rifampin did not inhibit the polymerase activity. After thermal shock of the virus, at least eight different RNA species were synthesized which may correspond to independent transcripts. Transcription also requires a hydrolyzable form of ATP. Analogs such as β,γ-imido ATP or β,γ-methylene ATP were inhibitory, whereas others, such as the β,γ-imido or methylene analogs of CTP, UTP, or GTP, were not inhibitory. This suggests that ATP is related to reactions other than polymerization, probably to initiation or elongation of RNA molecules, as has been described for vesicular stomatitis virus or vaccinia virus.

Rotaviruses have been associated with diarrheal disease in a variety of mammals. In humans, they are the major cause of acute gastroenteritis in infants and young children (11). Rotaviruses are members of the Reoviridae family, and their genome characteristically consists of 11 linear pieces of double-stranded RNA with molecular weights of 2.2×10^6 to 0.2×10^6 (9). The morphology of the virus is similar to that of other Reoviridae, such as orbivirus and reovirus. The mature particle is double-shelled and contains at least five different polypeptides with molecular weights of 14,000 to 133,000 (12). The nature of the viral particle, as well as evidence arising from histochemical and electron microscope studies, shows the cytoplasmic location of viral replication (11, 16). This suggests that the viral particle contains enzymes related to the early events of infection.

Several reports have described a virus-associated RNA-dependent RNA polymerase in strains of human and calf rotavirus (5, 8, 15). Cohen (5) has characterized the enzyme from calf rotavirus, showing by hybridization that the viral genome is fully transcribed under in vitro conditions. The RNA synthesized by this enzyme codes for viral polypeptides (6). Certain molecular aspects of RNA synthesis, such as regulation and RNA structure, remain unclear.

In this communication, we report in vitro properties of a human rotavirus RNA polymerase isolated from virus obtained from a single patient during an episode of diarrhea in the winter season in Chile (1979 to 1980).

The characterization of the viral RNA genome, the requirements for ATP hydrolysis during transcription, and the effect of inhibitors on RNA synthesis were studied. Our results show that the human rotavirus RNA-dependent RNA polymerase has some novel properties as compared with other polymerases (14).

MATERIALS AND METHODS

Viral purification. Stool samples (10 g each) from infants (0 to 6 months old) were diluted twofold with distilled water and treated as described by Espejo et al. (7). The polyethylene glycol pellet was suspended in 2 ml of 2 mM Tris-hydrochloride buffer, pH 7.5 (buffer A), and centrifuged in a Spinco type 65 rotor at 40,000 rpm for 2 h through a 3-ml layer of 10% sucrose in buffer A. The pellet thus obtained was resuspended in 500 μl of buffer A, and samples of 150 μl were layered onto a 20 to 35% sucrose gradient in buffer A and centrifuged in an SW65 rotor at 22,000 rpm for 60 min. Fractions (20 drops) were collected and analyzed by gel electrophoresis for the presence of rotavirus RNA (see Fig. 1). Briefly, 50-μl samples were adjusted to a final concentration of 3% sodium dodecyl sulfate-0.3% mercaptoethanol-18 μM EDTA and incubated for 30 min at 40°C before the addition of 5 μl of 0.01% bromophenol blue in 40% glycerol. The samples were then electrophoresed through a 0.5% agarose-2.5% acrylamide gel by the method of Adhya et al. (1). Gels were run at 35 mA for 3.5 h, stained

with a 1-μg/ml solution of ethidium bromide, and examined under UV light. Fractions of sucrose gradient which showed the characteristic pattern of RNA segments from human rotavirus were then examined by electron microscopy to determine the viral purity. The viral particles detected in the middle portions of the gradient consisted of a single morphological type of rotavirus and were free of bacterial contamination.

Fractions containing virus were pooled, diluted with three volumes of buffer A, and pelleted as described above. The viral pellet was resuspended in buffer A and stored at −20°C.

RNA polymerase assay. The standard reaction mixture (0.05 ml) contained 60 mM Tris-hydrochloride (pH 8.4), 6 mM $MgCl_2$, 0.9 mM each of ATP, CTP, and GTP, and 240 μM [^{3}H]UTP (70 cpm/pmol). To this reaction mixture, various amounts of virus that was heat activated by incubation in 10 mM Tris-hydrochloride buffer (pH 8.4) at 60°C for 1 min were added. The mixture was then incubated for 20 min at 37°C; reactions were halted by the addition of 0.3 ml of an ice-cold solution containing 130 mM Na PP_i and 0.3 mg of heat-denatured salmon sperm DNA per ml. Acid-insoluble material (5% trichloroacetic acid) was collected on glass-fiber filters, washed several times with 2% trichloroacetic acid and ethanol, dried, and counted in a Nuclear-Chicago Mark II liquid scintillation counter.

Analysis of the in vitro products of human rotavirus RNA polymerase reaction. Standard RNA polymerase reaction mixtures were increased twofold, and [^{3}H]UTP was replaced by [α-^{32}P]UTP (2,050 cpm/pmol). After incubation for 60 min at 37°C, the reaction was halted by the addition of a mixture of phenol and chloroform (1:1) saturated in 50 mM Tris-hydrochloride (pH 7.5) containing 0.1 mM EDTA. After extraction, the aqueous phase was precipitated with ethanol in the presence of 40 mM sodium acetate buffer pH 6.0 (buffer B) and 40 μg of *Escherichia coli* tRNA carrier. The precipitate was collected by centrifugation, resuspended in 100 μl of 50 mM Tris-hydrochloride buffer (pH 7.5), and reprecipitated with ethanol in the presence of buffer B. The pellet was collected by centrifugation and resuspended in 0.1 ml of 50 mM Tris-hydrochloride buffer (pH 7.5). The ethanol precipitation procedure was repeated twice. The final pellet was suspended in 25 μl of a 5 mM sodium borate buffer (pH 8.2) containing 1 mM EDTA and 10 mM sodium sulfate, and then 5 μl of 0.01% bromophenol blue and 1.25 μl of 0.1 M methylmercuric hydroxide were added. The mixture was applied to a 1.2% agarose horizontal gel containing 5 mM methylmercuric hydroxide (4) and electrophoresed at 50 mA for 20 h. The gel was cut into 1-mm slices, and the radioactivity in each slice was measured.

Protein determination. Protein concentrations were determined as described by Bradford et al. (3), using the Bio-Rad protein assay.

Materials. Unlabeled nucleotides were purchased from Sigma Chemical Co. Radioactive nucleotides were from New England Nuclear Corp. Imido or methylene ribonucleoside triphosphate analogs were from ICN. Pancreatic RNase and DNase were from Worthington Diagnostics.

RESULTS

Association of RNA polymerase activity with the viral particle. To show that the viral particle contains an associated RNA polymerase activity, sucrose density gradient centrifugation was done to isolate the virus, followed by assays of RNA polymerase activity in each fraction. The viral particles were located at the middle portions of the sucrose gradient (Fig. 1B), as detected by gel electrophoresis and ethidium bromide staining of the viral double-stranded RNA. Electron microscopic studies confirmed the above conclusion that the majority of the virus-like particles observed were located at the middle fractions of the gradient (data not shown). RNA polymerase activity was detected in the same fraction in which the majority of the virus sedimented (Fig. 1A).

No RNA polymerase activity was detected at the bottom or the top of the gradient, even though small amounts of virus could be detected in these fractions. It was difficult to detect enzyme activity in less purified viral fractions, probably due to the presence of inhibitors that may have been removed after sucrose gradient sedimentation.

RNA polymerase activity, as measured by [^{3}H]UMP incorporation, was linear with respect to increasing amounts of virus (Fig. 2). The time course of the reaction exhibited linear kinetics for at least 40 min (Fig. 3). Since no appreciable decrease was observed in the amount of acid-insoluble material formed after this time, the presence of significant amounts of active RNase was ruled out. The reaction products after phenol extraction and ethanol precipitation were 100% sensitive to a mixture of pancreatic RNase (50 μg/ml) and RNase T (100 μg/ml) and insensitive to pancreatic DNase (500 μg/ml), suggesting that the product of the reaction was RNA.

Divalent cation requirements. Viral preparations showed an absolute requirement for Mg^{2+} to catalyze the incorporation of [^{3}H]UMP into acid-insoluble material (Fig. 4). The optimum concentration of Mg^{2+} was 6 mM at a 3 mM concentration of ribonucleoside triphosphates.

Effect of monovalent salts. The rate of RNA synthesis catalyzed by human rotavirus-associated RNA polymerase was dependent upon the ionic strength. Salts, such as NaCl or KCl, at concentrations of 100 mM stimulated threefold the enzyme activity with respect to the control (Fig. 5). Higher concentrations of monovalent salts resulted in a diminution of the rate of the reaction.

Ribonucleoside triphosphate require-

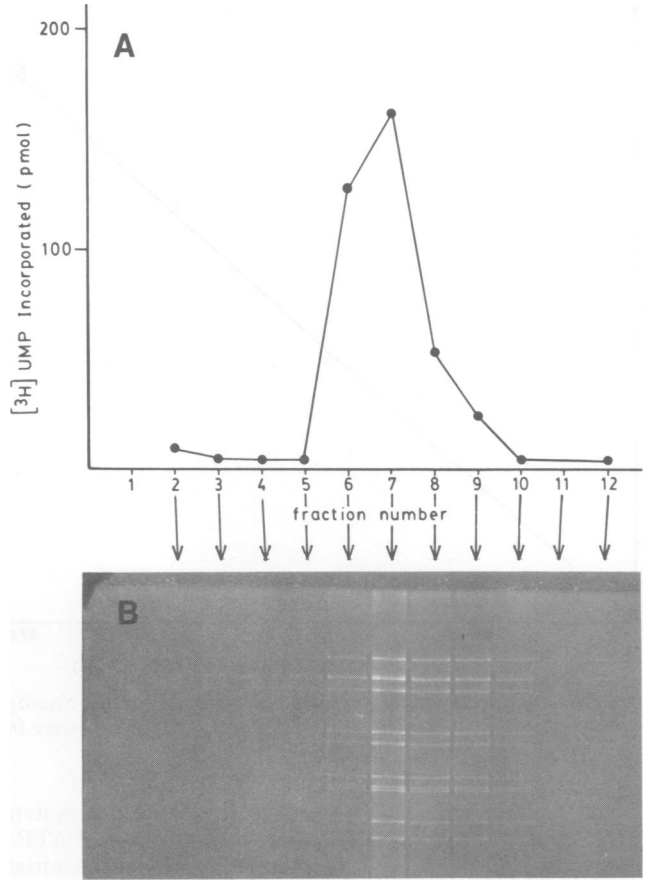

FIG. 1. *RNA polymerase activity of virus fractions after sucrose gradient centrifugation. (A) Viral fractions (10 μl) obtained from sucrose gradient centrifugation were assayed after thermal shock for RNA polymerase activity. (B) Fractions (60 μl) of the sucrose gradient described in (A) were subjected to gel electrophoresis to determine the location of virus. The arrows show the viral RNA pattern obtained with each fraction of the sucrose gradient.*

ments. Viral RNA synthesis required all four ribonucleoside triphosphates (Table 1). This suggests that the enzyme was not a terminal adding or polynucleotide phosphorylase type of activity.

A study with deoxyribonucleoside triphosphates showed that, of the four deoxyribonucleoside triphosphates, only dATP produced a 50% inhibition of the polymerization reaction when incubated in combination with all four ribonucleoside triphosphates. In the absence of a single ribonucleoside triphosphate, the corresponding deoxyribonucleoside triphosphate was unable to replace it.

In the presence of 6 mM $MgCl_2$, maximum RNA synthesis was obtained at a final ribonucleoside triphosphates concentration of 3.0 mM (Fig. 6).

The dependence of the rate of RNA synthesis on each of the four ribonucleoside triphosphates was examined, and the apparent K_m values were determined from double reciprocal plots. These values were 365 μM for GTP, 30 μM for ATP, 43 μM for CTP, and 45 μM for UTP (Fig. 7).

Effect of inhibitors. Actinomycin D at concentrations higher than 100 μg/ml had no effect on the viral RNA polymerase (Table 2). Rifampin (40 μg/ml) and α-amanitin (200 μg/ml) did not have any effect on the enzyme activity (Table 2).

pH optimum. The viral RNA polymerase displayed an optimal activity at a pH of 8.5 (Fig. 8). Higher or lower pH values decreased the activity at least 70%.

Effect of Na P_i and Na PP_i. The polymerase activity was insensitive to Na P_i at concentra-

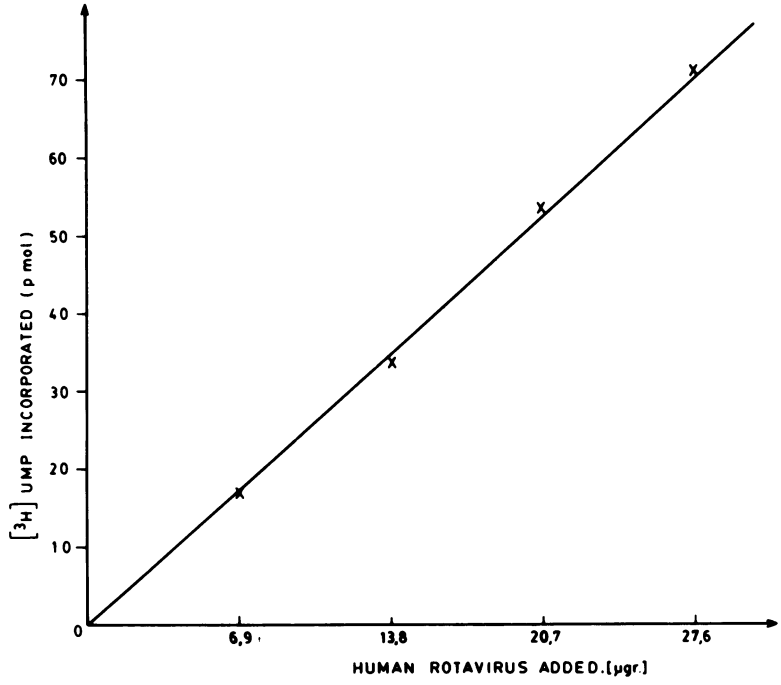

FIG. 2. *Dependence of RNA synthesis on the amount of human rotavirus. Standard reaction mixtures containing the indicated amounts of heat-treated virus (micrograms of protein) were incubated for 20 min at 37°C.*

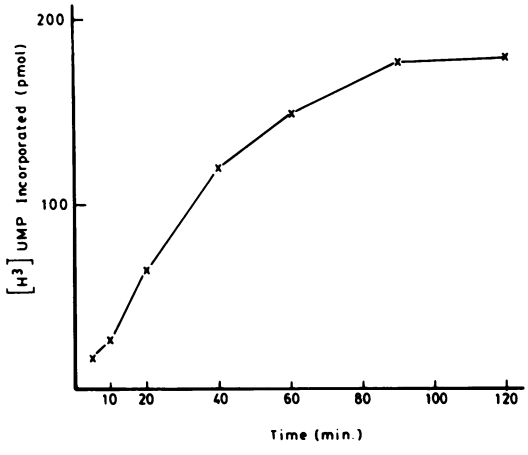

FIG. 3. *Time course of RNA synthesis. Standard reaction mixtures containing 4 µg of heat-treated virus were incubated for the indicated times at 37°C.*

tions as high as 40 mM; furthermore, at a concentration of 20 mM, there was a slight stimulation (Fig. 9). At a concentration of 2 mM, Na PP_i inhibited the reaction. However, at higher concentrations inhibition may reach 100%.

Effect of ribonucleoside triphosphate analogs. In several viral systems it has been shown that the hydrolysis of ATP to ADP and P_i was required for either the initiation or elongation of RNA molecules (13, 15). To test whether a similar situation exists in human rotaviruses, we examined the effect of ribonucleoside triphosphate analogs on viral transcription. Replacement of ATP by its analog β,γ-imido ATP resulted in a complete inhibition of RNA synthesis (Table 3). ATP cannot be replaced by the analog even in the presence of dATP, a nucleoside triphosphate which can be hydrolyzed to dADP and P_i but not incorporated into the RNA chain (Table 1). These results differ from those obtained with vaccinia virus, in which transcription could be supported by the addition of a mixture of dATP and β,γ-imido ATP (13). The analog effect seems to be specific for ATP since UTP could be replaced by its corresponding analog (β,γ-imido UTP) with a 72% reduction in the rate of incorporation. Similar results obtained for GTP and its analogs (β,γ-imido and β,γ-methylene GTP) were notable. When the imido and the methylene analogs were compared, striking results were obtained; the methylene analogs gave consistently less [^{3}H]UMP incorporation into RNA than did the imido analogs.

Analysis of the RNA product. In vitro

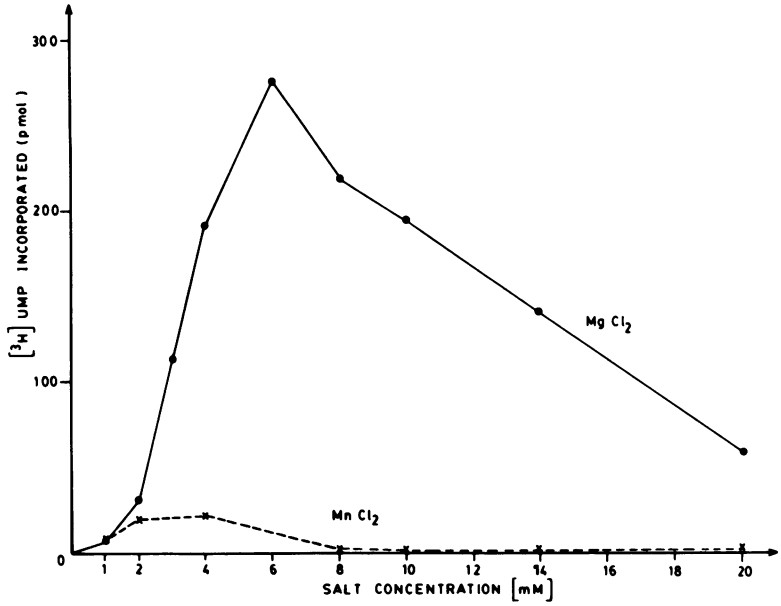

FIG. 4. *Effect of divalent cations on RNA polymerase activity. Standard reaction mixtures containing 4 μg of heat-treated human rotavirus were incubated for 20 min at 37°C. $MgCl_2$ and $MnCl_2$ were added as indicated.*

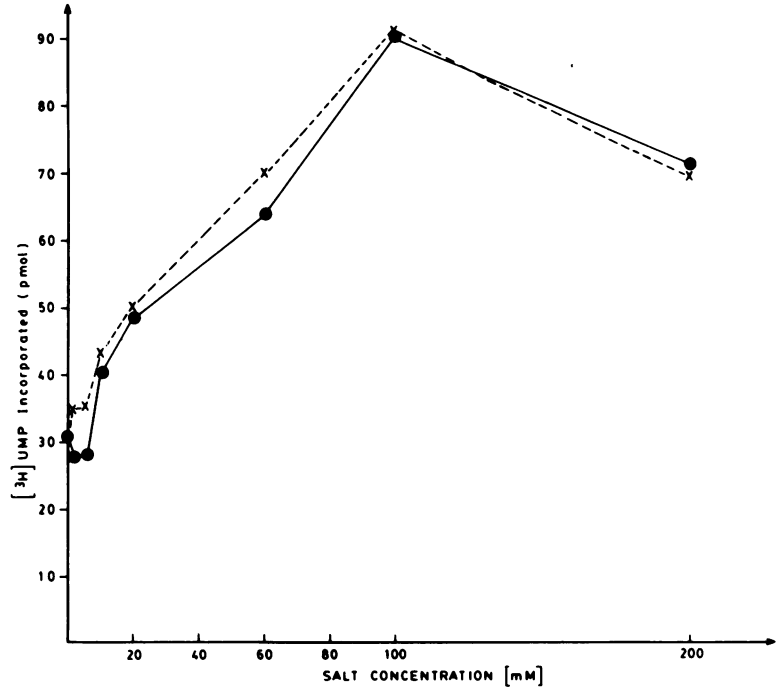

FIG. 5. *Effect of monovalent salts on RNA polymerase activity. Standard reaction mixtures contained 1.2 μg of heat-treated virus. Salts were added as indicated. Symbols: ●, NaCl; ×, KCl.*

RNA synthesized by heat-treated human rotavirus was analyzed under denaturing conditions by horizontal agarose-methylmercuric hydroxide electrophoresis. The distribution of the radioactivity in the gel suggests the presence of at least eight different RNA segments (Fig. 10).

DISCUSSION

We have studied in vitro viral RNA synthesis in human rotavirus which was isolated from infants with acute diarrhea during the winter season in Chile (1979 to 1980). The experiments reported here were performed with rotavirus purified from a single episode of diarrhea from one patient.

TABLE 1. *Ribonucleoside triphosphate requirements for the in vitro reaction catalyzed by human rotavirus RNA-dependent RNA polymerase*[a]

	Additions	[^{3}H]XMP incorporated into acid-insoluble material (pmol/20 min)
1.	ATP + CTP + GTP + UTP[b]	116
2.	CTP + GTP + UTP[b]	3
3.	ATP + GTP + UTP[b]	2
4.	ATP + CTP + UTP[b]	7
5.	ATP[c] + CTP + GTP + UTP	112
6.	ATP[c] + CTP + GTP	<1
7.	ATP + CTP + GTP + UTP[b] + dATP	58
8.	CTP + GTP + UTP[b] + dATP	1
9.	ATP + GTP + UTP[b] + dCTP	148
10.	ATP + GTP + UTP[b] + dCTP	1
11.	ATP + CTP + GTP + UTP[b] + dGTP	96
12.	ATP + CTP + UTP[b] + dGTP	2
13.	dATP + dCTP + dGTP + UTP	<1

[a] Standard reaction mixtures, as described in the text, contained 2.7 μg of heat-treated human rotavirus and a final concentration of nucleoside triphosphates of 0.8 mM, except that [^{3}H]UTP and [^{3}H]ATP were present at a concentration of 240 and 250 μM, respectively.

[b] [^{3}H]UTP had a specific activity of 70 cpm/pmol.

[c] [^{3}H]ATP had a specific activity of 240 cpm/pmol.

Incubation of purified virus with ribonucleoside triphosphates and Mg^{2+} was not sufficient to allow RNA synthesis. Several different treatments of the virus have been performed to stimulate transcription; among them are incubation of the virus with EDTA (5) and treatment with chymotrypsin (5) followed by reisolation of the virus. We used a thermal shock treatment in which the virus is incubated for 60 s at 60°C in 50 mM Tris-hydrochloride buffer (pH 8.4). This procedure, which has been used successfully with reovirus (2), is fast and circumvents a second reisolation of the treated virus. When this virus preparation was used to study RNA synthesis, analysis of the reaction products by gel electrophoresis as well as by nuclease digestion showed that the virus is able to synthesize at least eight different single-stranded RNA molecules (Fig. 10). These results indicate that the viral particle contains an associated RNA-dependent RNA polymerase. Mason et al. (10) showed that in simian rotavirus, eight groups of RNA species are synthesized by an associated RNA polymerase. These transcripts direct the synthesis of 11 polypeptides, indicating that posttranslational processing may occur or that

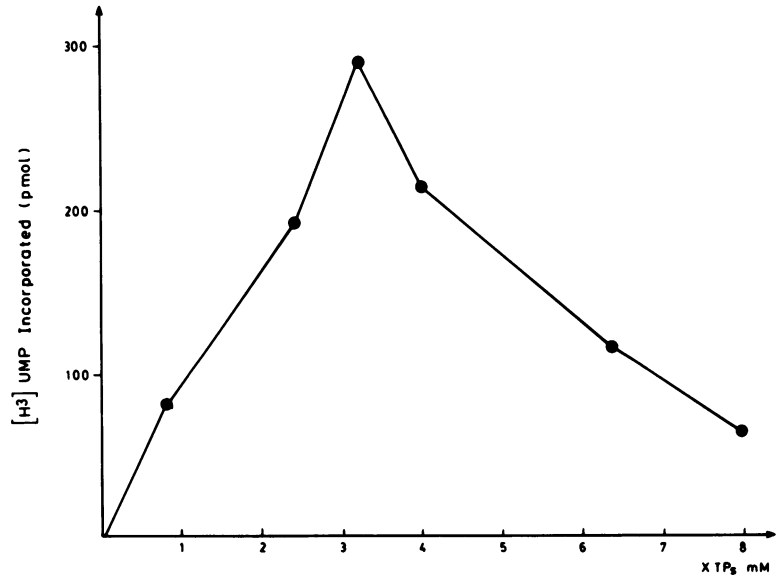

FIG. 6. *Dependence of the reaction catalyzed by the virus-associated RNA polymerase on the concentration of ribonucleoside triphosphates. Standard reaction mixtures containing 4 μg of heat-treated virus were incubated for 20 min at 37°C. The concentration of the ribonucleoside triphosphates was varied as indicated. The [^{3}H]UTP concentration was 0.24 mM (70 cpm/pmol).*

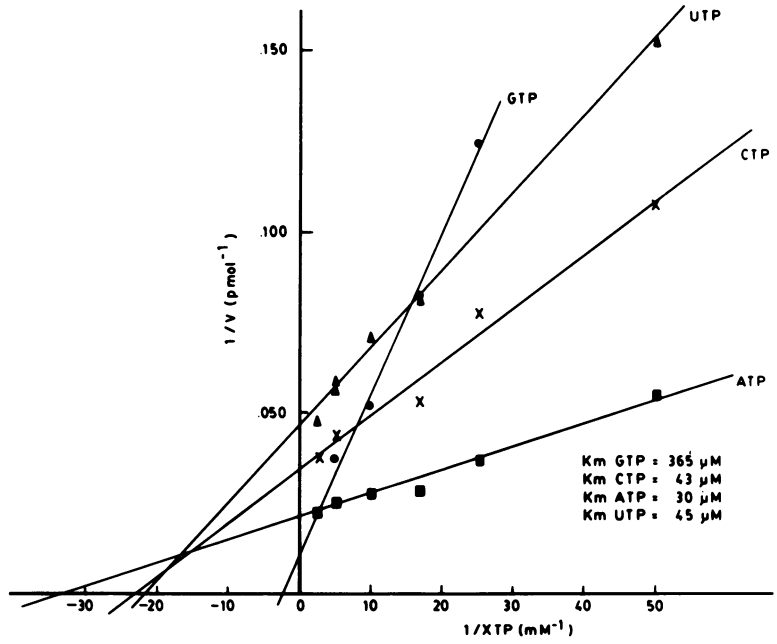

FIG. 7. *Determination of K_m values for the ribonucleoside triphosphates. Standard reaction mixtures contained 4 μg of heat-treated virus. [^{3}H]UTP was added to a final concentration of 0.24 mM except where indicated. The other ribonucleoside triphosphates were added at 0.8 mM except for the XTP, which was varied. The apparent K_m for each XTP is indicated. When UTP dependence was measured, [^{3}H]ATP was used as the labeled compound at a concentration of 0.25 mM (240 cpm/pmol).*

some RNA segments may code for more than one polypeptide. Whether RNA products isolated from human rotavirus represent mature mRNA or whether they require further processing, such as capping or polyadenylation, is not known and is under study.

The virus-associated RNA polymerase has an absolute requirement for all four ribonucleoside triphosphates, which cannot be replaced by the corresponding deoxyribonucleoside triphosphates. Viral transcription is also dependent on the presence of Mg^{2+} at an optimal concentration of 6 mM when the final ribonucleoside triphosphates concentration is 3 mM. In this reaction, Mn^{2+} cannot replace Mg^{2+} at any concentration. These properties, together with a peculiar optimal pH, the effect of monovalent salts and Na P_i, and the insensitivity to transcription inhibitors (Table 2) makes the human rotavirus enzyme different from other eucaryotic viral polymerases (14). Whether the characteristics of the human rotavirus enzyme different from those reported for calf and human rotavirus enzymes (5, 8) reside in differences in methods for enzyme activation or in the enzyme itself is not known.

In the polymerization reaction, the K_m for ATP was similar to that of UTP and CTP.

TABLE 2. *Effect of inhibitors on the in vitro activity of the human rotavirus RNA polymerase*[a]

Addition	Concn (μg/ml)	[^{3}H]UMP incorporated (pmol/20 min)
1. None		197
2. α-Amanitin	80	187
3. α-Amanitin	200	196
4. Actinomycin D	20	143
5. Actinomycin D	100	148
6. Rifampin	10	165
7. Rifampin	40	143

[a] Assays were done as described in Table 1, footnote *a*. α-Amanitin, actinomycin D, and rifampin were added after the thermal shock treatment; the reaction mixtures were then incubated for 5 min at room temperature, and ribonucleoside triphosphates were added to initiate transcription.

However, the K_m for GTP was nearly ninefold higher than the others. This high K_m suggests that GTP may be the initiator nucleotide. Similar conclusions were obtained in experiments in which a G cap type I has been identified at the 5′ end of the RNA molecule synthesized in vitro by the virus (manuscript in preparation). The calculated K_m values probably do not reflect the

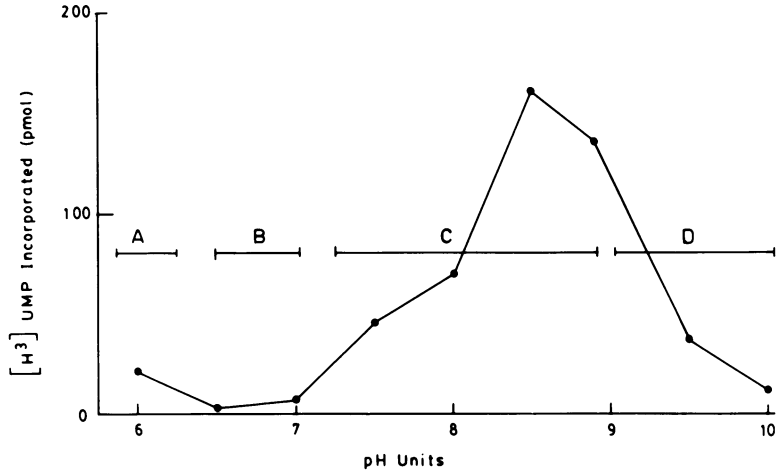

FIG. 8. *Influence of pH on RNA polymerase activity. Standard reaction mixtures containing 2.4 μg of heat-treated virus were used except that the Tris-hydrochloride buffer was replaced by buffer at the same concentration (60 mM), using the following: (A) sodium succinate; (B) imidazole; (C) Tris-hydrochloride; and (D) glycine.*

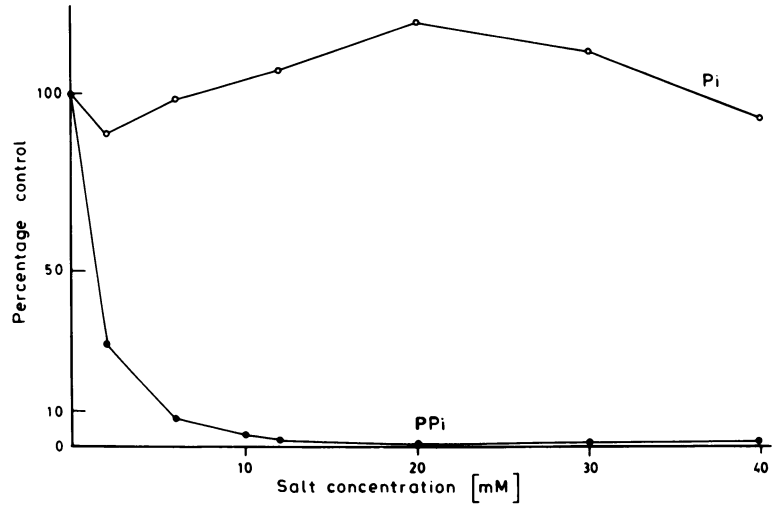

FIG. 9. *Effect of Na P_i and Na PP_i on the activity of viral RNA polymerase. Standard reaction mixtures were used except that P_i or PP_i was added with the heat-treated virus and then incubated for 30 min at 37°C. Here, 100% activity represents 130 pmol of [H^3]UMP incorporated into acid-insoluble material.*

exact K_m of the RNA polymerase, since these values may be affected by the permeability of the virus to the ribonucleoside triphosphates. Furthermore, differences in the K_m values for the four different nucleoside triphosphates have been observed after the addition of *S*-adenosylmethionine, which seems to diminish the requirement for high concentrations of ribonucleoside triphosphates (manuscript in preparation).

The results obtained with the ribonucleoside triphosphate analogs suggest that human rotavirus transcription has a requirement for a form of ATP which can be hydrolyzed to ADP and P_i. Other viral systems also require ATP hydrolysis for transcription; for example, vesicular stomatitis virus and vaccinia virus utilize ATP hydrolysis for initiation and elongation of RNA chains, respectively (13, 15). In these viral models, the K_m for ATP is higher than the calculated values for other ribonucleoside triphosphates. However, in the case of human rotavirus, it seems that large amounts of ATP hydrolysis are not required to allow transcription to occur, suggesting that ATP may be re-

TABLE 3. *In vitro utilization of ribonucleoside triphosphate analogs by human rotavirus RNA polymerase*[a]

	Additions	[^{3}H]XMP incorporated (pmol/20 min)
1.	ATP + CTP + GTP + UTP	121
2.	ATP + CTP + GTP + UTP + AMPPCP	65
3.	CTP + GTP + UTP + AMPPCP	1
4.	CTP + GTP + UTP + AMPPCP + dATP	1
5.	ATP + CTP + GTP + UTP + AMPPNP	126
6.	+ CTP + GTP + UTP + AMPPNP	3
7.	ATP + CTP + GTP + UTP + GMPPCP	64
8.	ATP + CTP + UTP + GMPPCP	58
9.	ATP + CTP + GTP + UTP + GMPPNP	137
10.	ATP + CTP + UTP + GMPPNP	29
11.	ATP + CTP + GTP + UTP	112
12.	ATP + CTP + GTP + UMPPNP	31

[a] Assays were done as described in Table 1, footnote *a*. The ribonucleoside triphosphate analogs were added to a final concentration of 1 mM. AMPPCP, β,γ-Methylene ATP; AMPPNP, β,γ-imido ATP.

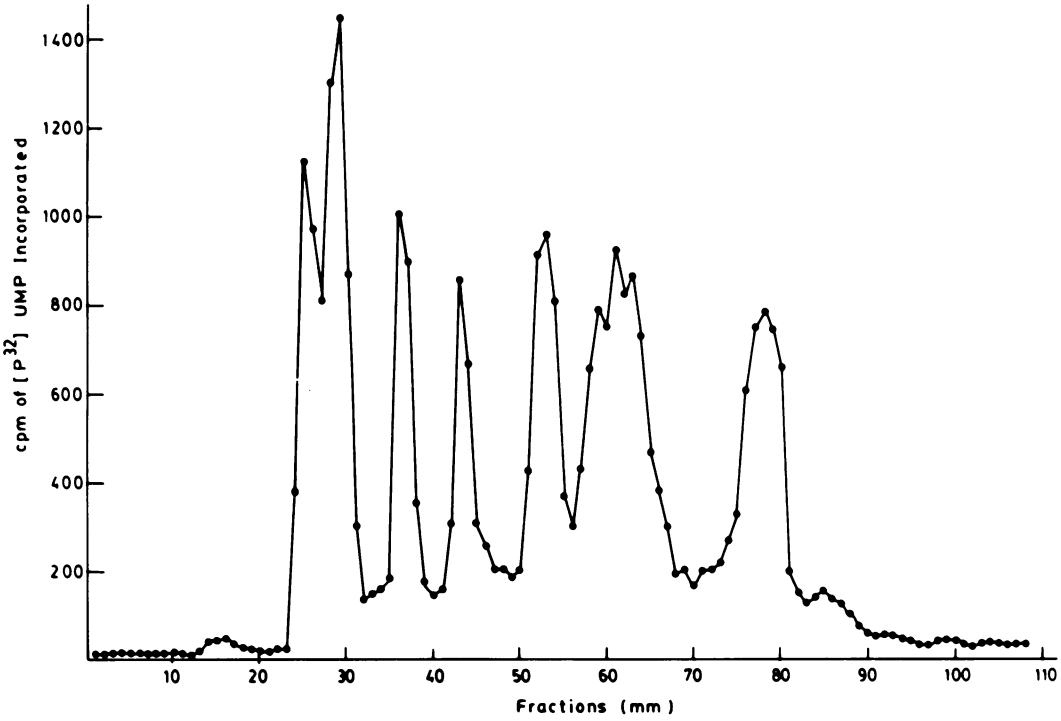

FIG. 10. *Analysis of the RNA product of the human rotavirus-associated RNA polymerase. Reaction mixtures (0.1 ml) containing [^{33}P]UTP (2,030 cpm/pmol) at a final concentration of 240 μm were incubated for 60 min at 37°C. The reaction products were processed as described in the text. Fraction 1 corresponds to the top of the gel.*

quired in catalytic amounts in the reaction associated with transcription. At the present time, the exact role of ATP in human rotavirus RNA synthesis is unclear. Using this viral preparation, we have not detected protein phosphorylation measured after [γ-^{32}P]ATP incorporation into viral protein, suggesting that protein phosphorylation is not clearly involved in transcription (manuscript in preparation).

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