Mode of Replication and Temperature Characteristics of the RNA Polymerase of an Influenza Virus *in vitro*

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While the enzyme template complex of fowl plague virus (FPV) polymerase is rather stable during incubation *in vitro*, some of the newly synthesized RNA is degraded rapidly. At low temperatures a pronounced lag phase of RNA synthesis occurs, which can be prevented by incubation with substrate. The *in vitro* products are initially single-stranded RNA, which are converted to partially double-stranded molecules by phenol and SDS. The results are compatible with an open replicative intermediate structure, in which the newly synthesized RNA is bound to the complementary template only over a rather short region at the growing point.

A comparison of the temperature characteristics of the RNA polymerases of the highly virulent *FPV* with that of the low pathogenic virus *N* revealed that the lag phase is a virus-specific property. There appears to exist a correlation between virulence and heat stability of this enzyme, the activity of which is rate limiting at the normal temperature of the host (41 °C).

Introduction

After infection of chick cells with influenza viruses, an RNA-dependent RNA polymerase can be detected in the microsomal fraction of the host cells[1-5]. Concerning fowl plague virus (FPV) this enzyme synthesizes predominantly minus-stranded RNA, which has the base sequence complementary to the RNA isolated from virus particles[6].

The mode of action of the enzyme is not yet understood. In particular the extent by which double-stranded RNA molecules are involved in the biosynthesis of viral RNA is not known. It has been shown that only limited amounts of double-stranded RNA can be extracted by phenol from cells infected with FPV[7]. Contradictory results have been reported concerning the strandedness of the RNA synthesized by the viral RNA polymerase *in vitro*[5,6]. Therefore, in these studies we determined the amount of double-stranded RNA after incorporation of labelled GTP *in vitro*. It is known that partially double-stranded structures are formed from replicative intermediates by phenol and sodium dodecylsulfate (SDS)[8-10]. Therefore different RNA extraction procedures were investigated.

During these studies a pronounced lag phase of

[³H] GTP incorporation *in vitro* at low temperatures was observed. Therefore the temperature characteristics of the polymerase of the highly virulent FPV was compared with the enzyme of a low pathogenic avian influenza strain (virus N) in order to determine whether this property is virus specific and whether there is a correlation between virulence and the temperature characteristics of the enzyme.

Materials and Methods

Virus strains

The two avian influenza virus strains were the same as those used in a previous study[11]: Fowl plague virus (FPV), strain Rostock, and virus N[12].

Polymerase test

Virus RNA-dependent RNA polymerase was determined by the incorporation of [³H] GTP (1.0 Ci/mM; Schwarz BioResearch, Orangebourg, New York, USA) into trichloroacetic acid (TCA)-insoluble material using a cytoplasmic or microsomal fraction prepared from chick fibroblast cells 6 hours after infection[2]. Recently it was shown that the label appears almost exclusively in RNA with a base sequence complementary to virus particle RNA[6]. The standard incubation mixture contained in 1 ml: 2.75 μmoles creatine phosphate; 5,5 μg creatine phosphokinase; 1 μmole of each of the three non-labelled nucleoside triphosphates; 4 μmoles $MgCl_2$; 50 μmoles KCl; 5 μmoles 2-mercaptoethanol; 33 μmoles tris-HCl buffer (pH 8.0); 2.5 μCi [³H] GTP; and 0.5 ml of a cytoplasmic or microsomal fraction. These frac-

tions were prepared by washing the cell layers twice with minimal buffer (10 mM tris-HCl buffer, pH 7.4; 10 mM KCl; 1.5 mM MgCl$_2$; 1 mM 2-mercaptoethanol). The swollen cells were scraped off and homogenized in a tight-fitting Dounce homogenizer. The nuclei and cell debris were removed by centrifugation for 5 min at 3 000 x g. The supernatant contained the cytoplasmic fraction. From this fraction the mitochondria were removed by centrifugation at 10 000 x g for 10 min. From the supernatant the microsomes were spun down for 1 hour at 100 000 x g. In those experiments, in which a total exclusion of internal nucleoside triphosphates was desired, a microsomal fraction treated and washed with 0.02 M sodium acetate at pH 5 was used[13]. For comparative studies the high tris-buffer concentrations (0.2 M) in the incubation mixture as described by MAHY and BROMLEY[5] were investigated. The radioactivity of the TCA-insoluble material was determined in a Packard Tricarb scintillation counter as described before[6].

RNA extraction

RNA was isolated either by extraction with phenol-SDS[6] or with diethyl-pyrocarbonate (DEP = Baycovin from Bayer, Leverkusen, Germany)[14]. In the latter instance the sample was mixed thoroughly with 2 ml DEP. After separation of the two phases by centrifugation, the upper phase containing the RNA was decanted and treated again with 2 ml DEP. Further isolation of the RNA was done by precipitation with two volumes of ethanol as described for the phenol method.

Determination of double-stranded RNA

Labelled double-stranded RNA was determined by treatment with 0.1 mg/ml pancreas RNase in 2 x SSC (= 0.3 M NaCl, 0.03 M Na-citrate) for 20 min at 20 °C and precipitation with 6 % TCA containing 0.1 M pyrophosphate as described[6].

Results

A. RNA polymerase activity at different temperatures in vitro

At 28° C the incorporation of labelled GTP into FPV-specific RNA continued for about 120 min almost linearly (Fig. 1, left). Thereafter the corresponding curve levelled off. The cessation of incorporation at this time was not attributed to depletion or degradation of the labelled precursor, since in the presence of up to 10 μg/ml of non-labelled GTP the shape of the curves were identical[15]. By the addition
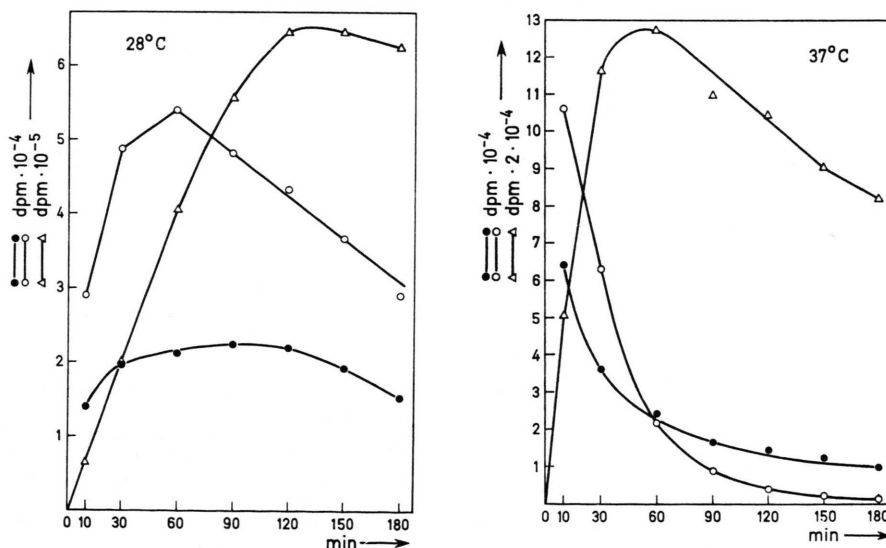

Fig. 1. Incorporation of [³H] GTP into virus-specific RNA in vitro at 28 °C (left) and at 37 °C (right). Microsomes of FPV-infected cells were homogenized in minimal buffer[6] (about 2.5 μg/ml protein) and were divided into 6 aliquots. △ = Prewarmed microsomes and reaction mixture containing 5μCi/ml [³H] GTP and 2μg/ml nonlabelled GTP[6] were mixed 1:1 and incubated either at 28 °C (left) or at 37 °C (right). At the times indicated on the abscissa 1 ml aliquots were removed and the TCA-precipitable radioactivity was determined (= total incorporation). ○ = Prewarmed microsomes and non-labelled reaction mixture containing 2 μg/ml GTP were mixed 1:1 and incubated at the temperature indicated. At the times indicated on the abscissa 1 ml aliquots were removed and incubated for 5 min at 28 °C or 37 °C, respectively, with 5 μCi/ml [³H] GTP. Thereafter the TCA-precipitable radioactivity was determined. ● = The microsomes were incubated without substrate at the indicated temperature. At the times indicated on the abscissa 0.5 ml aliquots were incubated for 5 min with the same volume of [³H] reaction mixture containing 2 μg/ml non-labelled GTP.

of fresh enzyme 120 min after the onset of incubation further incorporation was observed at normal rate[16].

Five minute pulses were given at different times following the onset of the RNA synthesis in the presence of non-labelled substrates. It was demonstrated that after 2 hours synthesis of virus specific RNA continued, although no labelled RNA accumulated during long term incorporation. This indicated that concomitant with RNA synthesis, breakdown of the newly produced viral RNA occurred. When the template-polymerase complex was incubated at 28 °C for 3 hours without substrate, enzyme activity was not significantly affected (Fig. 1). The higher incorporation by the 5 min pulses during the incubation with substrate as compared with the incubation without substrate is attributed to the prevention of the lag phase, which was demonstrable using short incubation periods (see below).

At 37 °C the initial slope of the [3H] GTP incorporation curve of RNA was much steeper, however, degradation of newly synthesized RNA proceeded at a much faster rate than at 28 °C. At 37 °C the enzyme-template complex was inactivated relatively rapid (Fig. 1, right).

The degradation of newly synthesized RNA is clearly shown in Fig. 2. In this experiment chases with an excess of non-labelled GTP were started at different time intervals after the onset of the RNA synthesis. Initially some labelled RNA was rapidly degraded, while the residual RNA was relatively stable. Labelled cellular RNA added during incubation in vitro also was degraded under these conditions[15].

Preliminary characterization of the newly synthesized product in a sucrose gradient showed the RNA with a broad peak between 3 and 10S which is in agreement with the results of others[3,5].

B. Production of double-stranded RNA

In Fig. 2 it is shown that some of newly synthesized RNA was relatively stable against internal degrading enzymes. This could be attributed either to the presence of double-stranded RNA, which resists degradation, or the molecules were protected by any other mechanism. Therefore, we studied the RNase resistance of the newly synthesized products before and after deproteinization by phenol or DEP.

In Fig. 3 it is shown that during incubation at isotonic salt concentrations, the percentage of RNase-resistant RNA was rather low, when proteins were not removed. After phenol extraction, however, relatively high amounts of RNase-resistant labelled mate-
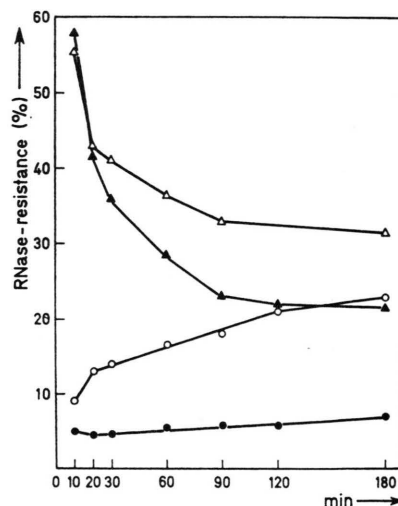
Fig. 3. Double-stranded RNA produced in vitro. A microsomal fraction as in Fig. 1 was divided into two parts. One was incubated at 28 °C with the standard incubation mixture as described in Fig. 1 (closed symbols), the other part was incubated at 28 °C according to Mahy and Bromley[5] with 0.2 m tris-HCl-buffer in the incubation mixture (open symbols). At the times indicated on the abscissa two 2 ml aliquots were removed from each incubation vial. The first aliquot was extracted with phenol plus SDS; and after dissolving the labelled RNA in 2 ml of 2 x SSC half of it was digested for 20 min with 0.1 µg/ml RNase. Thereafter the TCA-precipitable radioactivity was determined. The other half was processed without treatment with RNase. The percentage of RNase-resistant radioactivity was calculated (triangles). Half of the second aliquot was digested with RNase without prior deproteinization, while the other half was processed immediately. The RNase-resistant radioactivity was calculated as above (circles).
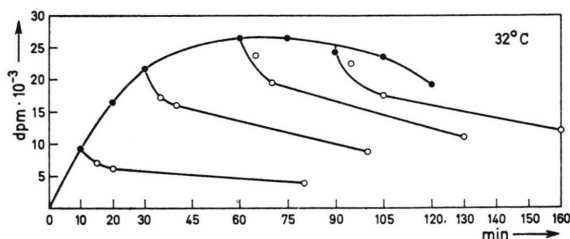
Fig. 2. Degradation of virus-specific RNA newly synthesized in vitro. A microsomal fraction as in Fig. 1 was incubated with [3H] reaction mixture at 32 °C. ● = At the times indicated on the abscissa 1 ml aliquots were removed and the TCA-precipitable radioactivity was determined. ○ = At different times of incubation aliquots were removed and further incubated at 32 °C with 1 mM non-labelled GTP (chase). At the times indicated on the abscissa 1 ml aliquots were removed and the TCA-precipitable radioactivity was determined.

rial were found. The percentage of double-stranded RNA detected after phenol extraction was significantly high after short incubation periods with [³H] GTP. When the ionic concentration was increased, the percentage of RNase-resistant material likewise increased markedly before deproteinization as well as after phenol extraction.

ÖBERG and PHILPSON[8] introduced the method of RNA extraction by DEP which permits the determination of double-stranded RNA initially present. DEP does not react with double-stranded RNA, however, it prevents the formation of double-stranded forms from complementary RNAs hold together by a polymerase molecule[8]. The latter structures form double-strands after treatment with phenol or SDS[9].
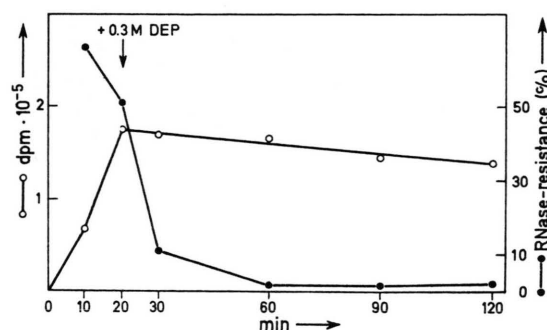

Fig. 4. Effect of diethylpyrocarbonate (DEP) on the synthesis of virus-specific RNA and on the production of double-stranded RNA. A microsomal fraction was incubated with [³H] reaction mixture at 28 °C as in Fig. 1. 20 min after the onset of incubation DEP (0.3 M end concentration) was added. ○ = 1 ml aliquots were removed and precipitated with TCA at the times indicated on the abscissa. ● = 1 ml aliquots were extracted with phenol plus SDS at the times indicated on the abscissa. The percentage of RNase-resistant material was determined.

As shown in Fig. 4 0.3 M DEP prevented incorporation of [³H] GTP into RNA, and shortly after its addition no double-stranded RNA could be isolated by phenol plus SDS. This single-stranded RNA,

Tab. I. Influence of different extraction procedures on the isolation of double-stranded RNA. A microsomal fraction was incubated with [³H]-reaction mixture at 28 °C as in Fig. 1. Thirty min later the RNA extraction procedures listed in the Table were employed.

Extraction procedure	% resistance against RNase
Phenol plus SDS	35.0
for 15 min SDS; thereafter phenol	35.5
phenol	32.0
DEP plus SDS	18.0
for 15 min SDS; thereafter DEP	20.5
DEP	2.0

however, still hybridized between 95 and 100% with an excess of non-labelled virus plus-strand RNA. Thus DEP separated strands which otherwise form partially double-stranded structures during phenol extraction. Results of experiments in which different extraction procedures were compared are summarized in Table I. At the end of the pulse no double-stranded structures were found, when the RNA was extracted by DEP without phenol.

C. Comparative temperature characteristics of the RNA polymerase of two fowl influenza viruses

Fig. 5 presents data which compare the incorporation of [³H] GTP into virus-specific RNA at different temperatures using enzymes of two different fowl in-

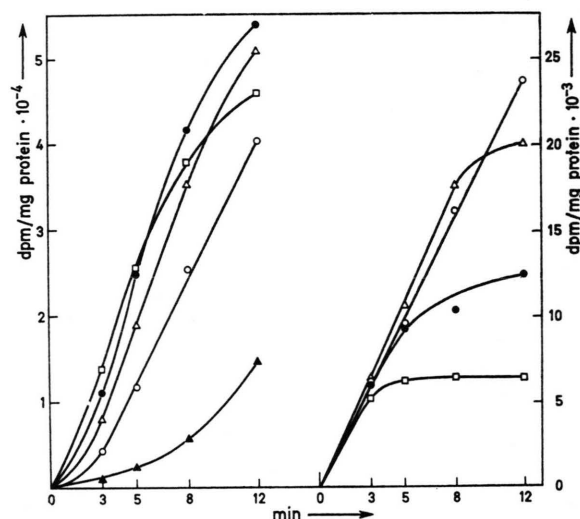

Fig. 5. Influence of temperature on the production of virus specific DNA in vitro. Comparison between fowl plague and virus N. Cytoplasmic fractions were prepared from FPV- or virus N-infected cells. Aliquots were incubated with [³H] reaction mixture as in Fig. 1 at different temperatures. At the times indicated on the abscissa 1 ml aliquots were removed and the TCA-precipitable radioactivity was determined. FP-Virus (left) ▲ 28 °C, ○ 34 °C, △ 36 °C, ● 38 °C, □ 40 °C. Virus N (right) ○ 34 °C, △ 36 °C, ● 38 °C, □ 40 °C.

fluenza viruses. With the FPV-polymerase at temperatures below 34 °C a pronounced lag phase in RNA synthesis was observed. In comparison with virus N the FPV-enzyme is more heat-stable. A corresponding lag phase was also found for the virus N enzyme at 27 °C or lower[15]. An Arrhenius plot was constructed (Fig. 6) in order to identify the temperature
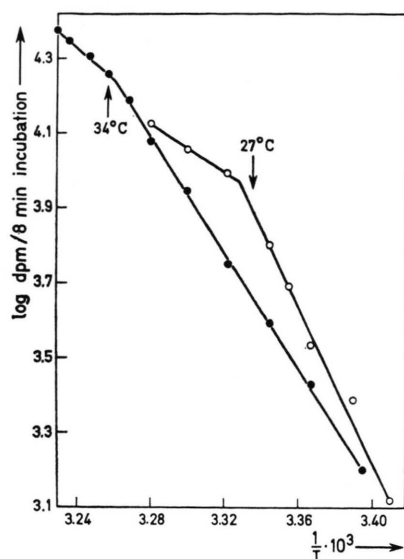
Fig. 6. A r r h e n i u s plot on the polymerase activity of FPV and virus N. Experimental conditions as in Fig. 5. The pulse length was 8 min. ○ = Virus N, ● = FP-virus.

more exactly at which the lag phase disappeared. For this purpose a pulse length of 8 min was chosen at which time the lag phase had a significant influence on the total incorporation. There was a difference between 6 and 7 degrees of the breaks in the two curves, representing to two viruses. This indicates that the lag phase is a virus-specific property.

D. Studies on the lag phase

As shown in Fig. 7 the lag phase of the FPV-enzyme does not seem to depend on the salt concentration tested. Furthermore, when the enzyme was incubated without substrate at 28 °C for 10 min and incubation was continued at 28 °C with labelled substrate, the lag phase was observed (Fig. 8). On the other hand, when the polymerization was started
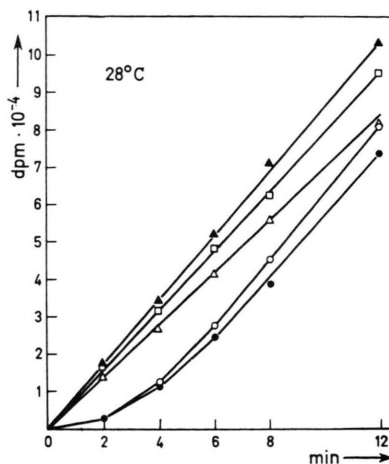
Fig. 8. Reversibility of the lag phase. A microsomal fraction as in Fig. 1 was precipitated with sodium acetate (20 mM end concentration, pH 5.0)[13]. The precipitate was taken up in the original volume of minimal buffer, and was divided into 5 aliquots. ○ = This aliquot was incubated for 10 min at 28 °C and mixed with [³H] reaction mixture. At the times indicated on the abscissa 1 ml aliquots were removed and the TCA-precipitable radioactivity was determined (dpm 0.5). ▲ = This aliquot was incubated for 10 min at 28 °C with nonlabelled reaction mixture containing 2 µg/ml GTP. Thereafter 5µCi/ml [³H] GTP was added (= time O). Further incubation at 28 °C. Aliquots were processed as above. ● = This aliquot was incubated for 10 min at 28 °C without reaction mixture and chilled for 3 min in an ice bath. After warming briefly to 28 °C the sample was mixed with [³H] reaction mixture (= time O) and further incubated at 28 °C. Aliquots were processed as above (dpm 0.5). □ = This aliquot was incubated for 10 min at 28 °C with nonlabelled reaction mixture containing 2 µg/ml GTP and thereafter chilled for 1 min in an ice bath. After warming for 15 s to 28 °C 5 µCi/ml of [³H] GTP were added (= time O) and the sample was further incubated at 28 °C. Aliquots were processed as above. △ = The same conditions as □, except that the time at 0 °C was 10 min.
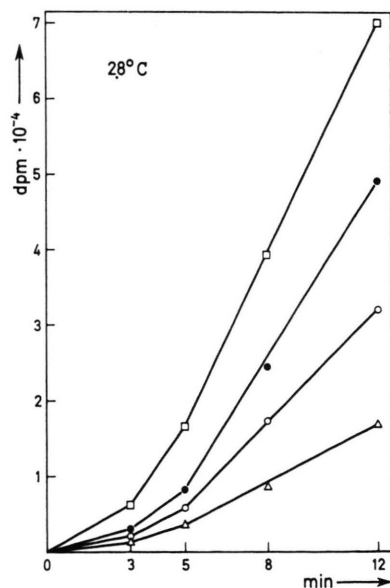

Fig. 7. Influence of different incubation conditions on the lag phase of the FPV-polymerase. A cytoplasmic fraction as in Fig. 5 was incubated at 28 °C. At the times indicated on the abscissa 1 ml aliquots were removed and the TCA-precipitable radioactivity was determined. ● = Standard incubation. ○ = The cytoplasmic fraction was mixed 1:1 with minimal buffer before use. △ = The cytoplasmic fraction was mixed 1:3 with minimal buffer before use. □ = The cytoplasmic fraction was incubated at high ionic strength as employed by MAHY and BROMLEY[5].

with non-labelled substrates at 28 °C and after 10 min [³H] GTP was added, no lag phase could be seen. If the samples were chilled for 10 min in an ice bath after incubation with non-labelled substrate, the lag phase was not observed.

Discussion

The incorporation of [³H] GTP by RNA polymerase at 28 °C continues almost linearly for about 120 min. Concomitant with the synthesis of virus-specific RNA significant breakdown of this RNA occurs (Fig. 1 and 2). Thus, it is not surprising that the molecular weight of the in vitro product is relatively low[3-5]

After addition of DEP to the product synthesized in vitro at low salt concentration, double-stranded RNA could not be extracted. This is in contrast to labelled RNA obtained by the phenol-SDS technique without DEP. Thus during synthesis of complementary RNA, double-stranded molecules are not formed in significant amounts. The partially double-stranded structures obtained by phenol extraction must be laboratory artifacts.

Our in vitro results on the synthesis of minus-strand RNA (plus-strand RNA is not being synthesized in our system[6]) are in agreement with the model of viral RNA replication as proposed by WEISSMANN and his colleagues[9,10] and by ÖBERG and PHILIPSON[8]. They suggest an open structure as replicative intermediate, in which only a short segment of double-stranded RNA occurs at the growing point held together by the polymerase molecule. In this model polymerase has the additional function of displacing the newly synthesized RNA from the template.

At relatively low temperatures a pronounced lag phase of [³H] GTP incorporation has been observed. If the temperature is raised above a specific point the lag phase disappears. Comparing two different avian influenza viruses this temperature point differs by 6 to 7 degrees. Therefore, it is concluded that the lag phase is strain specific. The significance of the lag phase is not yet known. After extended purification of the enzyme-template complex this lag phase is abolished[13]. This type of lag phase reminds one on a corresponding effect of RNA synthesis on a double-stranded DNA template, which has been discussed as a melting process of the template prior to the initiation of RNA synthesis[17].

A comparison of the temperature characteristics of the RNA polymerases, associated with two different fowl influenza viruses, has revealed that the highly pathogenic FPV induces an enzyme which is more heat-stable than that induced by the low pathogenic virus N. In a previous study[18,19] it was suggested that at infra-optimal and probably also at supra-optimal temperatures virus-induced RNA polymerase activity becomes rate limiting during influenza virus multiplication. Thus an apparent correlation exists between virulence and temperature sensitivity of influenza virus RNA polymerases.

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