NO EFFECT OF GUANIDINE ON AN IMPROVED IN-VITRO SYSTEM FOR VIRAL RNA POLYMERASE

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SUMMARY

Guanidine inhibits the replication of polio- and other picornaviruses in cell cultures. In an improved system in which ^{3}H-UMP is incorporated by poliovirus RNA polymerase in-vitro for two hours no inhibitory effect of guanidine was observed.
The result and its implications are discussed.

Guanidine inhibits the growth of wild strain polioviruses and other members of the picornavirus group at concentrations of between 0.5 - 3 mM (1, 2). Its mechanism of action is not yet known and numerous hypotheses have been discussed (3, 5, 6, 7, 8). It seems that particularly the synthesis of viral RNA is interrupted by guanidine (3, 4). According to Caliguiri and Tamm (9) the synthesis of viral RNA is more drastically inhibited by guanidine than that of viral proteins. We have obtained unequivocal evidence that guanidine does not interfere with the biosynthesis of viral structural proteins (manuscript in preparation). Thus it seems unlikely that guanidine directly inhibits the translation of viral RNA into

viral proteins. In keeping with this is the notion proposed by several authors (6, 7, 8) that the site of action of guanidine is to be sought at the viral RNA polymerase and its function.

In previous experiments, however,Baltimore et al. (5) have found no influence of guanidine on a system in which the activity of viral RNA dependent RNA polymerase is investigated in-vitro. In this system the incorporation of nucleotides proceeds for only 15 minutes (10). Therefore it is doubtfull if initiation of new viral RNA chains and their release from the complex takes place under these conditions.

As a result, the question remained whether or not guanidine failed to exert any measurable effect because it specifically inhibits the processes of initiation or release which could not have been analyzed in this case. In a recent publication Girard (11) described a considerably improved in-vitro system for the RNA dependent RNA polymerase of poliovirus. Here the incorporation proceeds for two hours or more. This system was used to re-investigate the question if guanidine acts directly on the viral polymerase particularly under conditions where initiation is likely to occur.

METHODS AND MATERIALS

In suspension 1-2 x 10^9 HeLa S 3 cells (3-5 x 10^5 cells per ml) (Flow Laboratories, Irvine, Scotland) were grown at 37° C with GBI-spinner medium containing 5 % calfserum.

These cells in GBI-spinner medium without calfserum were infected at high concentration (5 x 10^7 cells per ml) by addition of 30 - 50 PFU per cell of poliovirus type 1 (Mahoney) in the presence of 5 µg actinomycin D per ml. After 45 minutes (at 37° C) 460 ml of GBI-spinner medium with 5 % calfserum and 5 µg per ml actinomycin D were added and the suspension further incubated at 37° C. At 3 1/4 hours post infection the cells were harvested by low speed centrifugation (1000 rpm) in the cold.

The preparation of crude poliovirus RNA polymerase and its assay system has been described in detail by Girard (11).

Under the conditions given we obtain about 8-11 mg protein (Folin-method) from 2 x 10^9 infected cells in one preparation. Ten of such preparations were made and stored in liquid nitrogen for up to 8 weeks.

The various batches were combined, homogenized in a Douncehomogenizer and divided into aliquots to be used in the assay system. We followed the optimal conditions as given by Girard (11): 36.4° C; 1 ml of assay mixture described (11) contained 4 µmole KCl; 3,7 mg protein of the crude polymerase; 0,4 µmole each of ATP, GTP, CTP and 0.2 µmole ^{3}H-UTP (10 µ Ci per µmole). When the effect of guanidine was assayed the final concentration of the inh tor was 3 mmolar. All other conditions were exactly the same as described (11). The radioactivity of the samples was determined in a Packard-Scintillation-Spectrometer.

Following special chemicals and enzymes were used: ribonuclease free DNase (Worthington Biochemical Corp.), Phosphoenolpyruvate-cyclohexylammoniumsalt (Boehringer & Soehne, Mannheim, Germany), pyruvate kinase (Boehringer, Mannheim), nucleoside triphosphates (Boehringer, Mannheim), ^{3}H-UTP (Amersham, England), sodium deoxycholate (Serva GmbH, Heidelberg, Germany), other chemicals (Merck, Darmstadt, Germany) were of P.A. or ultrapure grade, Actinomycin D was a gift of Dr. Auhagen (Farbenfabriken Bayer, Elberfeld).

RESULTS AND DISCUSSION

In the complete system the incorporation was indeed found to proceed for at least two hours. As it is clearly shown in Fig. 1, guanidine had no inhibitory influence on either the rate or the final level of incorporation.

In contrast, in the control in which three of the four nucleotides were omitted the incorporation was reduced to 10 % during the first hour of incubation. During the second hour the rate of incorporation increased to about 45 % of that found in the complete system.

It is possible that here, owing to nuclease activity, nucleotides were released which subsequently became phosphorylated by the regenerating systems present in the mixture.

It is concluded that even in the improved in-vitro system of Girard (11) for assaying the RNA dependent RNA polymerase derived

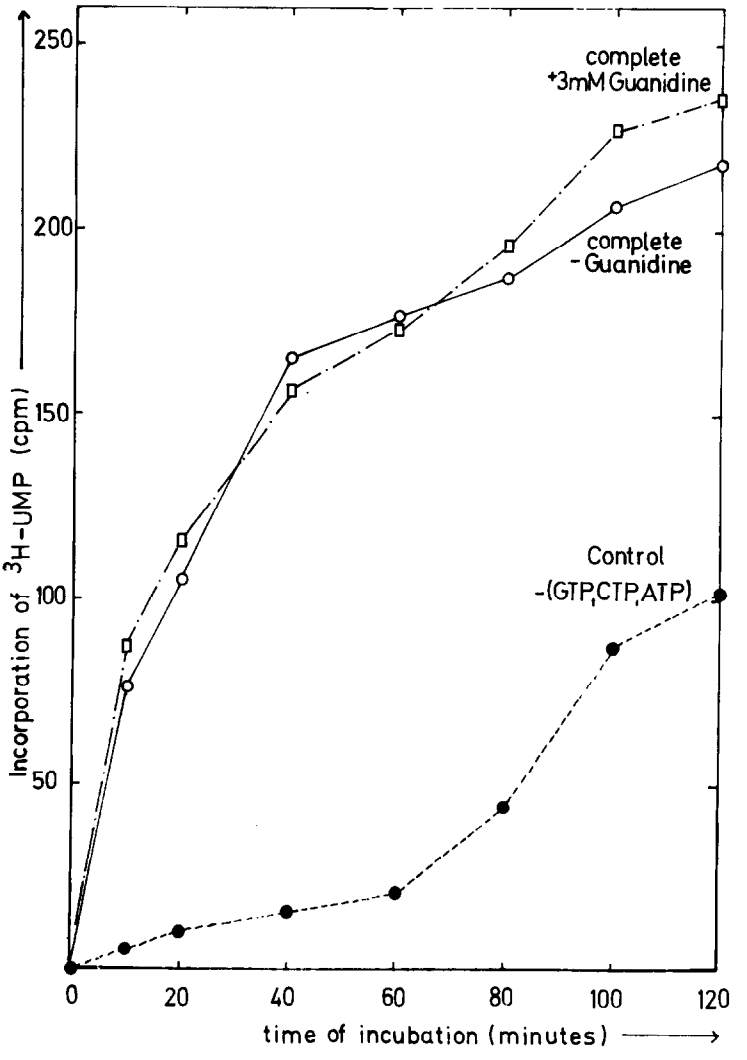

Fig. 1: Kinetics of the poliovirus RNA-polymerase reaction in vitro (Incorporation ^{3}H-UMP).
o——o Complete system <u>without</u> guanidin
□—·—□ Complete system with guanidine (3mmolar)
●------● incomplete system (- ATP, GTP, CTP)
Each point represents the average acid-precipitable radioactivity of two 0.5 ml samples.
100 cpm ^{3}H-UMP incorporated = 42 µµMole (zero-time values substracted = 15 cpm)

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from polio-infected cells guanidine has absolutely no effect

upon the incorporation of precursors into the newly produced RNA. According to Girard's findings the system synthesizes new RNA resembling viral single- and doublestranded forms. Our results suggest therefore that guanidine does not interfere with the synthesis and the subsequent release from the replicative intermediate complex of newly formed viral RNA chains.

The fact that the system continues to incorporate precursor into RNA over a period of two hours does not in itself prove that initiation of new RNA replication takes place. However, the chase experiments described by Girard (11) strongly suggest that this is indeed the case. Twenty minutes after beginning of the chase the label has disappeared from the replicative intermediate complex. The time required for the complete synthesis of one new RNA chain would accordingly appear to be 20 minutes. Since incorporation proceeds for about two hours this strongly favors the notion that initiation actually does occur.

In that case, guanidine would not appear to interfere with either the initiation, the chain prolongation or the release of finished viral RNA chains under the conditions applied in these in-vitro experiments.

A really definite answer, however, cannot be given as yet. Even if one could demonstrate no effect of guanidine upon a system in which truly isolated polymerase replicates RNA upon addition of viral RNA templates, the difficulty inherent in comparing in-vivo and in-vitro systems would still remain. For example it is

noteworthy that in-vitro the polymerase is active for two hours while in-vivo it was found to be functional for only 10 minutes (5). Nevertheless, the findings described here provide additional reasons to search for the mechanism of guanidine inhibition at levels other than the functional viral RNA polymerase.

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