Green,[5] and Pereira,[6] two possibilities were suggested, one of which was that the newly formed virus associated tightly with cells and were not released into the fluid phase of a culture until later, thus the virus could hardly infect the surrounding cells. Another one is that the cytophatic effect might be caused by some of the toxic substances produced by the virus in the process of replication, and the delayed cytophatic effect in the infection of adeno virus suggests that such toxic substances were poorly produced. On the basis of inadequate findings on the mechanism of cytophatic effect of adeno virus, it was difficult at present to offer any concrete explanation as to the effect of arginine on cytophatic effect of adeno virus in both KB[1] and Hep. No. 2 cells. Further inquiry into this explains the usual delayed appearance of cytophatic effect. Present results showed that the long incubation time required for adeno virus to obtain cytophatic effect could be shortened by using an arginine-rich medium, and that the use would be highly beneficial in the screening test of anti-adeno compounds.

Summary

The accelerating effect of arginine on cytophatic effect of adeno virus was investigated by using such cell lines as HeLa wild type, HeLa S_3 strain, FL and Hep. No. 2 cells. The effect of arginine in promoting a cytophatic effect was demonstrated in the Hep. No. 2 cells-arginine-rich maintenance medium system. In the investigation on the effect of 3-(alkoxyphenoxy)-1,2-propandiols on adeno virus, the use of the Hep. No. 2 cells-arginine-rich maintenance medium system was found to be most promising for the screening test of anti-adeno compounds.

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6) H. Pereira, A.C. Allison : *Ibid.*, **7**, 300 (1959).

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2. Shigeshi Toyoshima, Takeo Ueda, Tadakazu Tsuji, Yoshiko Seto, and Junko Nomoto : Inhibitory Effect of Guanidine on Several Viruses Including Polio and Measles.

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In the preceding report,[1] it was described by Ueda and Toyoshima that 2-imino-5-methylhexahydro-*s*-triazine exerted an inhibitory effect on the growth of polio virus in HeLa cells. However, from the further research it was inferred that the effect of this compound should not be due to its own property, but should be ascribed to an action of one of decomposed substances from the above compound, guanidine. According to this inference, this work was prompted by the present authors to investigate.

As to the antiviral effect of guanidine, Bawden and Pirie[2] reported that it showed a virus inactivating effect on tabacco mosaic virus only at a high concentration of 2.7*M*.

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1) T. Ueda, S. Toyoshima : Nippon Yakurigaku Zasshi, **56**, 145 § (1960).
2) F.C. Bawden, N.W. Pirie : Biochem. J., **34**, 1278 (1940).

No report, however, has been published concerning its inhibitory effect on other viruses since then.

The purpose of this report is to describe the inhibition of guanidine on the growth of several viruses, particularly polio and measles in tissue culture and its action site.

Experimental

Materials and Methods

1) Viral materials: The following viruses were employed for the experiments; polio virus (Mahoney, MEF_1 and Saukett strains), Japanese B Encephalitis virus (Nakayama strain), adeno virus types 1~7 strains) and measles virus (Edmonstan strain).

2) Host cells: The following cell lines were employed; HeLa cells (wild type), FL cells (wild type) and Hep. No. 2 cells (wild type).

3) Media: The following media were used; for the growth medium, YLA medium supplemented with 15% bovine serum, and for the maintenance medium, YLA medium supplemented with 5% bovine serum or 5% horse serum were employed. The horse serum containing maintenace medium was only for the cultivation of adeno virus, and all other employed viruses were cultivated using the bovine serum containing maintenance medium.

4) Procedures: 2×10^5 cells/cc. of each employed cell line were added into tubes, and then these tubes were incubated at 37° for 4 days to obtain the monolayer cell sheet. After the establishment of sheets, growth medium was removed from these tubes, and they were washed three times with phosphate buffer solution (pH 7.6), and then used for the experiments. Tissue culture infective dosis fifty*2 of each viral material was estimated by Reed and Muench's method[3] and represented by the value per 0.1 cc.

Results

1) Inhibitory Effect of Guanidine Nitrate on the Growth of Several Viruses

$10^{-3}M$ of guanidine nitrate at the final concentration was added into tubes in which the monolayer of each cell line had been established, and immediately after this, various dilutions of each viral material were inoculated into these tubes. After the incubation at 37° for 10 days, the effectiveness of guanidine nitrate, on all tested viruses except measles virus were critisized, on the other hand, all tubes in the experiments with measles virus were incubated at 37° for 21 days. After the daily microscopic observation, $TCID_{50}$ of both the control and the treated group was calculated. The virus-host cells systems employed in these experiments were as follows; polio

Table I. Effect of Guanidine Nitrate on Several Viruses

Strain of Virus	Host Cells	$TCID_{50}$ (−log)	
		Control	Treated
Polio			
Mahoney	HeLa	7.5	4.0
MEF_1		6.5	4.0
Sukett		8.0	5.5
Measles			
Edmonstan	Hep. No. 2	6.5	3.5
	FL	4.0	1.5
Japanese B Encephalitis			
Nakayama	HeLa	3.5	3.5
Adeno			
From type 1 to type 7	FL	3.5	3.5

$10^{-3}M$ of guanidine nitrate was added into tubes, soon after, each of viral materials was inoculated.

*2 Abbreviation $TCID_{50}$.

3) L. J. Reed, H. Muench, Am. J. Hyg., **27**, 493 (1938).

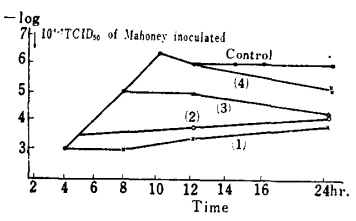
virus-HeLa cells, Japanese B Encephalitis virus-HeLa cells, adeno virus-FL cells and measles virus-Hep. No. 2 and FL cells.

As can be seen in Table I, guanidine nitrate had inhibitory effect on the growth of both polio and measles viruses, but was ineffective on adeno and Japanese B Encephalitis viruses. The difference between the effect on polio virus and that on measles virus is found not so great, so it may be said that guanidine nitrate is effective on both of polio and measles viruses in the same degree.

2) Effect of Guanidine Nitrate on the Various Phases of the Multiplication of Measles and Polio Viruses in Cells

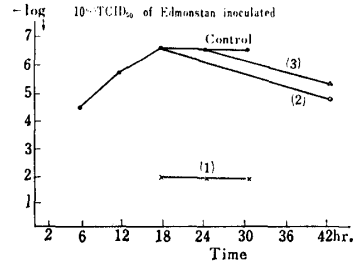
On the basis of the above findings, the inhibitory effect of guanidine nitrate on the multiplication of measles and polio viruses in the host cells was investigated.

$10^{6.5}$ $TCID_{50}$ of Edmonstan strain or Mahoney strain was inoculated into tubes, in which the monolayer sheet of each of Hep. No. 2 cells and HeLa cells had been established, then these tubes were incubated at 37° for 1 hour, washed three times with phosphate buffer solution, added with the maintenance medium, and further incubated. After that, $10^{-3}M$ of guanidine nitrate was added into these tubes at various viral growth phases after the inoculation. After the additions of guanidine nitrate, the medium was removed from the tubes at various intervals, and then the cells were washed three times with phosphate buffer solution, and original volume of the maintenance medium was added into each of the tubes. The cells were frozen and thawed five times, then the fluid was centrifuged at 2000 r.p.m. for 15 minutes. The viral amounts of the supernatant were estimated by using the newly monolayer cell sheet established tubes. For measles experiments, Hep. No. 2 cells were used and for polio experiments, HeLa cells were employed.



(1) Guanidine was added 2 hours after the viral inoculation
(2) Guanidine was added 5 hours after the viral inoculation
(3) Guanidine was added 8 hours after the viral inoculation
(4) Guanidine was added 12 hours after the viral inoculation

Fig. 1. Inhibitory Effect of Guanidine Nitrate on the Multiplication of Mahoney Strain in HeLa Cells



(1) Guanidine was added 2 hours after the viral inoculation
(2) Guanidine was added 18 hours after the viral inoculation
(3) Guanidine was added 24 hours after the viral inoculation

Fig. 2. Inhibitory Effect of Guanidine Nitrate on the Multiplication of Measles Virus in Hep. No. 2 Cells

As can be seen in Figs. 1 and 2, guanidine nitrate inhibited the intracellular growth of both polio and measles viruses, and its effect was found particularly marked when guanidine nitrate was added into tubes 2 hours after the inoculation in the experiments of both of polio and measles viruses. Though the effect was observed even when guanidine nitrate was added after the viral growth arrived at the stationary phase, the effect was found less than that of the group in which guanidine nitrate was added at

the latent phase. From these results, it may be said that the action site of the inhibitory effect of guanidine nitrate on both polio and measles viruses in the host cells should be in the latent period of the viral growth.

3) Virus Inactivating Action of Guanidine Nitrate against Polio Virus

The inhibitory effect of guanidine nitrate on the intracellular growth of both of polio and measles viruses as described above might be due to the virus inactivating action. Accordingly, to make this point clear, the inactivating effect of guanidine nitrate against polio virus was examined.

5×10^5 $TCID_{50}$ of Mahoney strain and $10^{-1}M$ of guanidine nitrate were mixed in a tube, in which any host cell was not contained, and then this tube was allowed to stand at 22° for 24 hours, then this mixture was diluted by using YLA medium with ten fold dilution method. After that, these dilutions were added into the newly monolayer cell sheet established tubes, then these tubes were incubated at 37° for 7 days. For the host cells, HeLa cells were employed. As the results, $TCID_{50}$ of the control was $10^{-6.5}$, and that of the treated group was $10^{-6.0}$. Thus, it may be said that any significant difference between $TCID_{50}$ of the control and that of the treated group could not be found from these results. According to these findings, it may be suggested that guanidine nitrate did not possess any inactivating action against polio virus.

4) Influences of Guanidine Nitrate on the Adsorption of Polio Virus onto Host Cells

As can be seen above, the possibility to be produced the inhibitory effect of guanidine nitrate on polio virus through the virus inactivating action was denied. The next possibility should be concerned with the influence of guanidine nitrate on the viral adsorption onto the host cells. To clarify this point, the effect of guanidine nitrate on the adsorption of polio virus onto HeLa cells were examined.

$10^{-3}M$ of guanidine nitrate was added into tubes, in which the monolayer of HeLa cells had been established, and these tubes were incubated at 37° for 24 hours. After that, the medium of these guanidine nitrate containing tubes was removed, and the cells were washed three times with phosphate buffer solution, then the dilutions of Mahoney strain were inoculated. For the control, phosphate buffer solution was employed instead of guanidine nitrate solution. After the incubation for 10 days, $TCID_{50}$ of both of the control and the treated group was determined.

$TCID_{50}$ of the control was $10^{-6.5}$, and that of the treated group was just the same. Thus, it may be said that the inhibitory effect of guanidine nitrate should not be due to the inhibition of the adsorption of polio virus onto the host cells.

Discussion

In the experiments described here, it was shown that guanidine nitrate had inhibitory effect not only on polio virus but also measles virus. Both of polio and measles viruses belong to RNA containing virus, on the other hand this compound was ineffective on adeno virus, one of DNA containing virus. These results suggest that guanidine nitrate should exert inhibitory effect on some of RNA containing virus. The results of Bawden and Pirie[2] as to the effect of guanidine on tabacco mosaic virus showed that its effect was due to the virus inactivating action, while in the results of the authors it was shown that guanidine nitrate neither posseses the virus inactivating action nor the inhibitory effect on the viral adsorption onto host cells. This compound was found most effective when it was added into tubes at the latent phase of viral growth and the intracellular multiplication of virus was clearly suppressed by the addition of guanidine nitrate. Consequently, it may be suggested from the results desc-

ribed in this report that the effect should be caused by the inhibition of the process for the viral reproduction, and its action site should be in intracellular site. The study as to the mode of action of the inhibitory effect of guanidine nitrate is now going on. The results of this work will be reported in future. At any rate, to find highly effective antiviral agants, the findings described in this report concerning the effect of guanidine should be a milestone. Thus, the research regarding the relationship between the antiviral property and the chemical structure of guanido group containing compound is going on, too. These results also will be described in another paper.

Summary

The inhibitory effect of guanidine nitrate on the growth of several small animal viruses was investigated. It was found that guanidine inhibited the growth of both poliomyelitis and measles viruses. The findings presented in this report suggest that guanidine inhibits the intracellular multiplication of these viruses and block the replication mechanism of the viruses.

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3. Hisao Tsukamoto, Hidetoshi Yoshimura, and Hiroyuki Ide : Metabolism of Drugs. XXXII.*1 The Metabolic Fate of Secobarbital [5-Allyl-5-(1-methylbutyl)barbituric Acid].

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In the previous paper[1] the metabolic fate of thiamylal, thio-analogue of secobarbital [5-allyl-5-(1-methylbutyl)barbituric acid] was reported and a caboxylic acid derivative was isolated and characterized as a major metabolite. However further studies on this metabolic fate seemed to be rather difficult, because the paper chromatogram of the urine extract of rabbits to which this drug was administered was so complicated that the resulted product might be of simultaneous formation of desulfuration and oxidation reaction. However, a comparison of paper chromatograms of urine extracts treated by both Thiamylal and secobarbital suggested that some of the metabolites of the former could be identical with that of the latter.

It has therefore been undertaken to elucidate firstly the metabolic fate of Secobarbital which has not been known so far.

Secobarbital [5-allyl-5-(1-methylbutyl)barbituric acid] is one of the common sedatives and hypnotics of short duration. It seems to undergo similar oxidation processes and lose its activity in animal body as the other short-acting barbiturates do.[2] It possesses however allyl and 1-methylbutyl side chains, and so it is very interesting to know which is more susceptible to biological oxidation.

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