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Effect of 8-Azaguanine on Encephalomyocarditis Virus Multiplication.* (31500)

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The purine analog 8-azaguanine (azaG) inhibits the growth of mammalian cells and of several microorganisms. It has been suggested that the inhibitory effect of this analog is due to its conversion to 8-azaguanilic acid by inosine monophosphate (IMP) and/or guanosine monophosphate (GMP) pyrophosphorylases(1) and to its subsequent incorporation into RNA(1-3). When azaG-sensitive (azaG-S) mouse L cells are grown in the presence of the analog for varying periods of time, resistance may be established(4,5). Since resistance is associated with a loss of IMP-GMP pyrophosphorylases(5,6), it could be anticipated that in azaG-resistant (azaG-R) cells, azaG would not be converted to azaguanilic acid and would not, therefore, inhibit the growth of intracellular parasites. However, the analog has an inhibitory effect on psittacosis agent(4), vaccinia, and encephalomyocarditis (EMC) virus(7) in both azaG-S and azaG-R cells. The present study was carried out to investigate some aspects of the inhibitory effect of azaG on the multiplication of EMC virus.

Materials and methods. Cell cultures. Earle's strain L of mouse cells, clone 929, was obtained from Dr. H. Eagle, and grown in 12-ounce bottles with Eagle's Minimal Essential Medium (MEM)(8) containing 10% inactivated horse serum, 100 units of penicillin and 100 μg of streptomycin/ml. Analog-resistant cells were obtained by exposing monolayer cultures in logarithmic phase of growth to increasing levels of azaG.† Eventually, cells were obtained which were resistant to 100 μg/ml of the analog. However, no toxic effect on the cells, that is no change of cell morphology or rate of cell growth, was apparent once a level of resistance to 20 μg/ml had been attained.

Virus. The strain of EMC virus used in this study was originally obtained from Dr. K. K. Takemoto and has been grown in L cells. Most of the experiments were carried out with a purified small plaque variant(9) which allowed higher plaque counts per plate.

Virus assay. All virus assays were carried out on L cells using the plaque method as described by Takemoto and Liebhaber(9).

Cell growth assays. Falcon plastic Petri dishes, 60 $\times$ 15 mm, were seeded with 5 $\times$ 10^4 cells. After 1 day, azaG was added at a final concentration of 100 μg/ml to half of the plates. The cells in a measured area at the center of the plates were counted daily for a period of 4-5 days.

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† Nutritional Biochemicals.

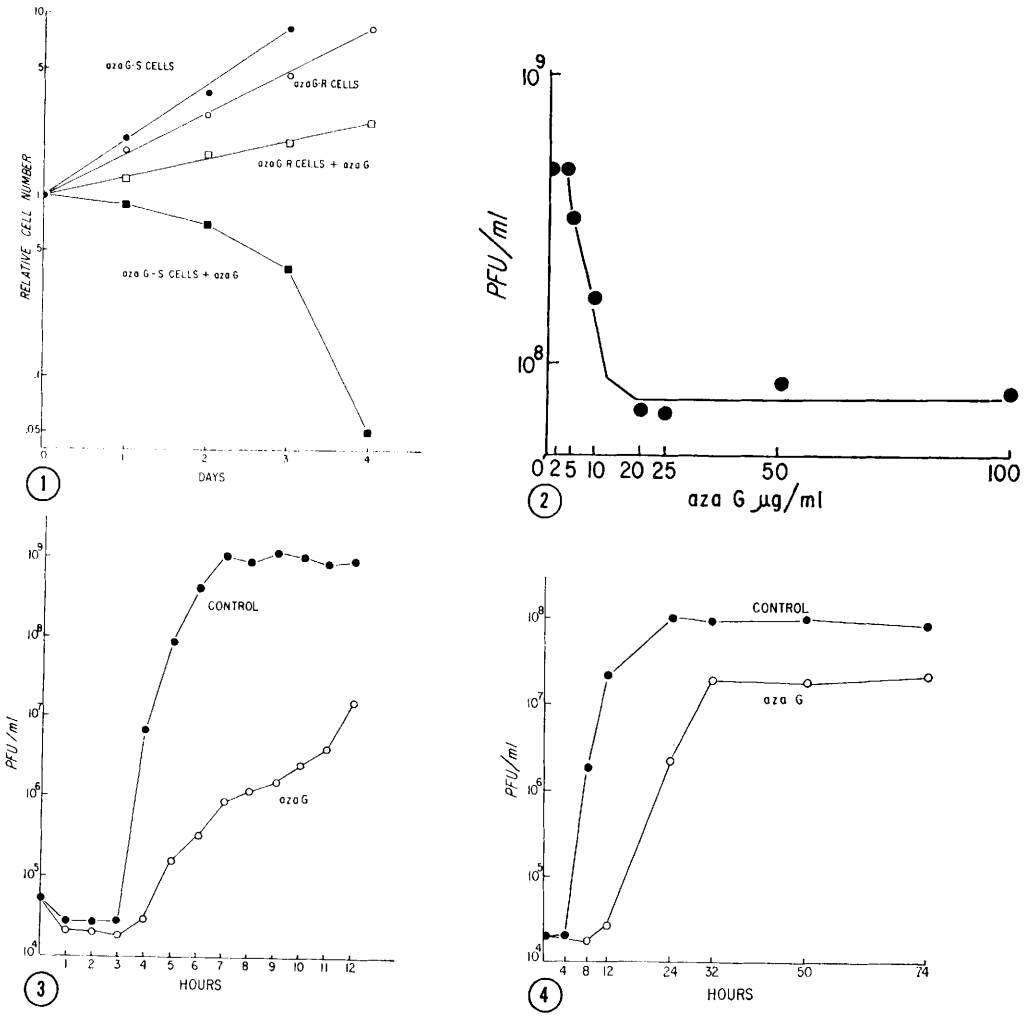


FIG. 1. Effect of azaG (100 μg/ml on day 0) on cell growth.
FIG. 2. Effect of azaG on EMC virus yields at 8 hours in azaG-S cells.
FIG. 3. Effect of azaG (20 μg/ml) on EMC virus growth in azaG-S cells, single cycle experiment.
FIG. 4. Effect of azaG (20 μg/ml) on EMC virus growth in azaG-S cells, multicycle experiment.

RNA and protein synthesis. The synthesis of RNA and protein was studied by a modification of the method described by Baltimore and Franklin(10). To investigate the effect of azaG on cellular RNA and protein synthesis, plates were seeded with 1.5-2.0 × 10^6 cells in a medium without azaG. After 24 hours, different doses of the analog were added to the plates. At hourly intervals, H^3 uridine‡ or H^3 leucine‡ was added to a final concentration of 2.5 μc/ml. When H^3 uridine was used, a 1000-fold excess of unlabeled thymidine§ was added to prevent any incorporation into DNA. Cells were pulse-labeled for 15 minutes. Further processing has been carried out according to Hare(11). To investigate the effect of azaG on RNA synthesis of infected cultures, 24-hour-old plates were infected at high multiplicity. After 30 minutes adsorption at 37°C, the inoculum was removed and replaced with medium, with or without the analog. The subsequent

‡ New England Nuclear.

§ Schwarz BioResearch.

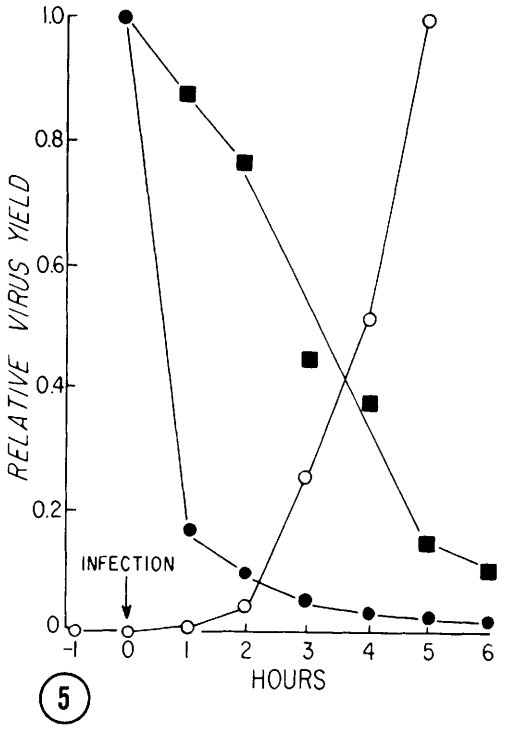
steps were carried out as outlined above.

Results. Effect of azaG on growth of azaG-S and azaG-R L cells. Several experiments were performed with both azaG-S and azaG-R cells to investigate the effect of the analog on cell growth. The pooled results of 2 experiments for azaG-S cells and of 3 experiments for azaG-R cells are shown in Fig. 1. It is apparent that in the presence of 100 μg/ml of azaG, normal cells fail to multiply, and detach from the plate within 4 days of exposure, while azaG-R cells continue to multiply although at a slower rate than unexposed controls. In these experiments, the growth rate of azaG-R cells in the absence of the analog was consistently lower than that of normal cells. Similar findings were reported by Littlefield(5). When the effect of azaG at 20 μg/ml was assayed on fully established monolayers, the number of surviving cells detached with trypsin after 3 cycles of washing indicated the rapid destruction of the azaG-S cells exposed to the analog (65% after 2 days).

Effect of azaG on virus yield. The effect of the analog on one cycle of virus multiplication was assayed by infecting azaG-S cells with EMC virus at a multiplicity of infection (m.o.i.) of about 4 in the presence of various amounts of the analog. Two or 3 replicate plates per dose of azaG were used in different experiments. At 8 hours, the plates were frozen and thawed 3 times and the fluids were later assayed. The results of Fig. 2, represent a pool of 2 experiments. Doses of azaG below 2 μg/ml, which inhibit normal L cell growth(4-6), have no significant effect upon the virus titer. Higher levels of the analog inhibit EMC replication, but no further decrease in titer occurs once a level of 20 μg/ml is attained. Even at the highest doses used here, azaG does not completely prevent viral replication, as an increase in titer of about 2 logs over the input level is observed at 8 hours.

Effect of azaG on virus growth. Growth curve experiments in presence of the analog were carried out to study the kinetics of virus multiplication. Monolayers of azaG-S cells were infected with an input m.o.i. of about 4. After 30 minutes adsorption, the inoculum was removed and medium containing 20 μg/ml of the analog was added to half of the plates. The remaining plates, as controls, were refed with medium without the analog. Duplicate plates of azaG-treated and control cells were frozen every hour over a 12-hour period, and saved for virus assay. The results of one such experiment are reported in Fig. 3. The growth of EMC virus in the absence of azaG begins between 3-4 hours, and is virtually complete by 6-7 hours. On the other hand, azaG delays the onset of virus growth until the fifth hour and strongly decreases the rate of growth. At the end of the experiment, the titer of the virus grown in presence of the analog has not reached a plateau and it is about 1% of that observed in control cells. To determine the virus production in a long-term experiment, a number of plates, with and without azaG, were infected at a lower m.o.i. and assayed at intervals until 74 hours. The results of this experiment are shown in Fig. 4. In azaG-treated cells the onset of growth is delayed about 8 hours over the control and the rate of viral growth is lower than in control cells. Maximum titers are obtained at 24 hours in controls, and at 32 hours in azaG-treated plates. The final yields show an inhibition of about 80% in the presence of the analog. In evaluating the results of this and other long-term experiments, the toxic effect of azaG on the cells has to be kept in mind. The cytocidal activity of the analog is likely to affect virus production independently from a direct effect on viral replication.

Effect of time of addition and time of removal of azaG on virus yields. In an attempt to relate the effect of azaG to some event in the virus replication cycle, a number of experiments were carried out in which the analog was added or removed at different times after infection. Controls without azaG were included and all plates were frozen for virus assay 8 hours after infection. In the "time of addition" experiments, azaG was added directly to the plates at different times after adsorption at a final concentration of 20 μg/ml. In the "time of removal" experiments, a number of plates were exposed to azaG at 20 μg/ml 1 hour prior to infection. The infection was carried out on bare mono-



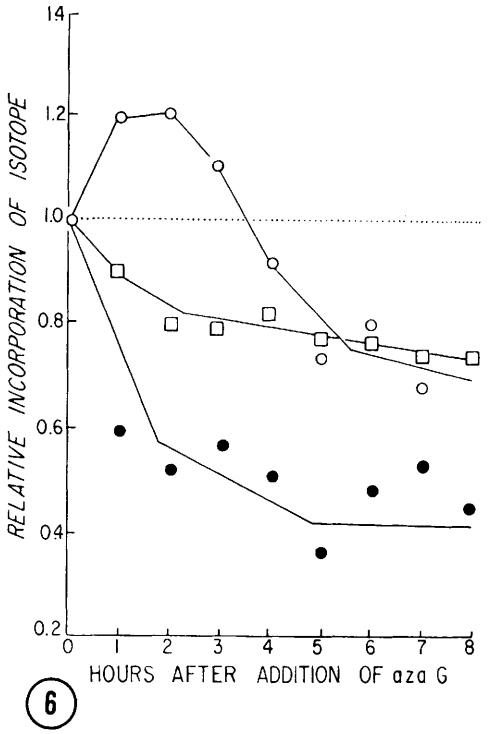


FIG. 5. Effect of addition or removal of azaG on EMC virus yields at 8 hours. azaG 20 μg/ml added to azaG-S cells at times shown (○———○) azaG 20 μg/ml added to azaG-S cells at —1 hour and removed at times shown (●———●); azaG 100 μg/ml added to azaG-R cells at —1 hour and removed at times shown (■———■).

FIG. 6. Effect of azaG on RNA and protein synthesis in azaG-S cells. H^3 uridine + azaG 10 μg/ml (○———○); H^3 uridine + azaG 100 μg/ml (□———□); H^3 leucine + azaG 10 μg/ml (●———●).

layers in the presence of the analog and, after adsorption, the plates were refed with azaG-containing media. At different times thereafter, the medium was removed, saved for assay and replaced with new medium. In order to minimize the effect of residual azaG, 500 μg/ml of adenine were incorporated in this medium. Adenine does not affect EMC virus replication in L cells(12), and in our hands, this base, in 25-fold excess, completely neutralizes the toxic effect of the analog on azaG-S cells. At the end of 8 hours the plates were frozen and prior to the virus assay the fluids from each plate were pooled with the azaG-containing fluids previously removed from the same plates. The results of a typical experiment, in which the titers at 8 hours are reported as a fraction of control titers, indicate that azaG-sensitive events take place over an extended period of time (Fig. 5). In fact, the removal of azaG at 1 hour following infection does not prevent a considerable inhibition of virus growth. On the other hand, addition of the analog at 3 and 4 hours still has an inhibitory effect. Addition of azaG at 5 hours has no effect on virus titer indicating that the analog does not affect virus release.

In order to shed light upon the role of IMP-GMP pyrophosphorylases in mediating the effect seen in normal cells, the "time of removal" experiment was carried out with azaG-R cells. The following variations were introduced to increase the effect of the analog: (1) The level of azaG used in this experiment was raised to 100 μg/ml. (2) No adenine was used to antagonize the effect of azaG after removal. It is apparent that despite these conditions, no major inhibition occurs until after 3-4 hours of exposure to the analog (Fig. 5). The results of this experiment indicate

TABLE I. Effect of Actinomycin D* on Inhibitory Activity of azaG.†

Treatment	Exp 1			Exp 2		
	Titer	% Control	% Act. D	Titer	% Control	% Act. D
None	7.0×10^7	100.0	—	1.7×10^7	100.0	—
azaG	5.0×10^5	.7	—	2.1×10^5	1.2	—
Act. D	3.1×10^7	44.0	100.0	1.4×10^7	82.0	100.0
Act. D + azaG	1.8×10^5	.3	.6	2.3×10^5	1.4	1.6

* Actinomycin D, 2 μg/ml. † azaG, 20 μg/ml.

that the inhibitory effect of azaG is reduced in azaG-R cells as compared to normal cells; however, it is not completely suppressed.

Effect of Actinomycin D on azaG inhibitory action. To determine whether or not the effect of azaG is mediated by mechanisms which involve cellular RNA synthesis, experiments of EMC virus yield were carried out in cells pretreated with Actinomycin D at 2 μg/ml for 2 hours to inhibit cellular DNA-dependent RNA synthesis. The infection and the growth of the virus took place in the presence of the drug at the same concentration. In Table I are presented the titers of EMC virus at 8 hours after infection and the percentage of yield obtained in 2 experiments. The first experiment was carried out on fully grown cell monolayers; in the second experiment the cells were in a more dispersed condition and presumably in logarithmic phase of growth. Although it has been reported that multiplication of poliovirus(13), EMC virus and other members of the Columbia SK (14) group are not affected by the drug, in our hands the treatment with Actinomycin D lowered the titers of EMC virus about 50% in the first experiment and about 20% in the second one. The inhibition of virus production observed here is probably related to cellular functions, and it was more noticeable when cells in stationary phase were used. Similar effects of Actinomycin D, more evident with older cell cultures, have been recently observed also with poliovirus(15). In any case, azaG effectively inhibits viral replication with or without Actinomycin D indicating that the inhibitory effect of the analog is independent of cellular RNA synthesis.

Effect of azaG on cellular RNA and protein synthesis. These studies were carried out using the autoradiographic procedure described in *Materials and methods.*

Two different levels of the analog, 10 and 100 μg/ml, were used in RNA synthesis studies. The effect on protein synthesis was investigated at 10 μg/ml of azaG. The pooled results of several experiments are shown in Fig. 6. The synthesis of RNA decreases in 2 hours to a level of about 80% of control values when the cells are exposed to 100 μg/ml of azaG. At the 10 μg/ml level, there is an early transient stimulation, followed by an inhibition. Protein synthesis is inhibited by 10 μg/ml of azaG, and drops to lower percentage levels than those seen with respect to RNA. These results are in general agreement with those of Paul and Hagiwara(16) who observed inhibition of RNA, protein and DNA synthesis in L cells 4 hours after exposure to 10 μg/ml of azaG.

Effect of EMC infection on RNA synthesis. Several experiments were carried out to study the effect of viral infection on RNA synthesis. Pooled results of 4 experiments are presented in Fig. 7 (controls). EMC induces an immediate rapid decline in RNA synthesis followed by a sharp increase. The RNA synthesis reaches a maximum at 5 hours, then falls again. These results are in agreement with published data for EMC virus and are comparable to the results of RNA synthesis in Mengovirus-infected L cells(10), although the secondary rise observed in this last system achieved higher levels than in the experiments reported here. According to Baltimore and Franklin(10), the ascending limb of the curve represents viral RNA synthesis and it corresponds to the time course of significant viral RNA(17) and polymerase synthesis(18) in Krebs II ascites tumor cells.

Effect of azaG on RNA and protein synthesis of EMC infected cells. Experiments similar to the ones mentioned above were carried out with virus-infected cells in the

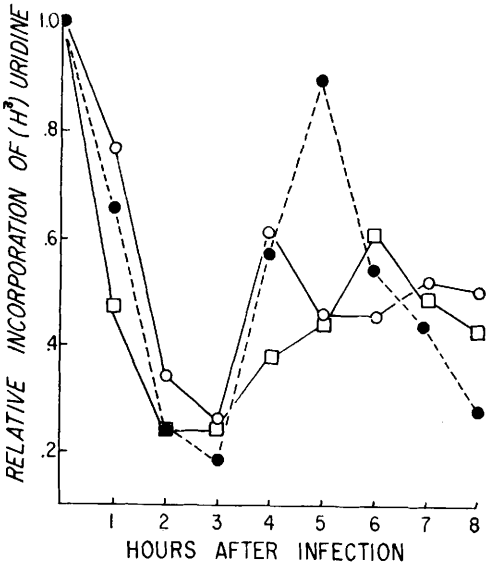


FIG. 7. Effect of azaG on RNA synthesis of infected azaG-S cells. Controls (● - - - - ●); azaG 20 μg/ml (○——○); azaG 100 μg/ml (□——□).

presence of azaG as described in *Materials and methods*. The results of 2 experiments in which 20 and 100 μg/ml of azaG were used are reported in Fig. 7.

The early drop in RNA synthesis at both concentrations of azaG does not differ essentially from that of infected controls. The subsequent increase in cultures exposed to 20 μg/ml of azaG is similar to that of control cultures but it stops at 4 hours. The analog at 100 μg/ml decreases the slope of the secondary rise and a maximum is reached only at 6 hours when the synthesis of RNA is about 60% of control values. While the experiments were not extended beyond 8 hours, the available data suggest that the overall amounts of RNA synthesized in cultures treated with 20 and 100 μg/ml of azaG are rather comparable. This observation is in agreement with the results (Fig. 2) on virus yield at 8 hours when virus growth took place in the presence of these 2 levels of azaG. Preliminary studies of the effect of azaG on protein synthesis in virus-infected cells show an early decline similar to that of controls. A minimum value is observed between 2-4 hours. The analog alters the kinetics of the secondary rise in synthesis in a manner grossly similar to its effect on the synthesis of RNA.

Discussion. The results presented here indicate that the inhibitory effect of azaG on EMC replication is only partial and manifests itself with a delay in both onset and rate of growth. The partial inhibition of viral growth in these experiments seems consistent with the appearance of very small plaques, after a 24-hour delay, under solid media(7). Analog-sensitive events take place over a period of several hours because late events are certainly affected as shown by the "time of addition" experiment, and early events seem also to be modified as the removal of azaG at 1 hour does not prevent a considerable inhibition.

The results of experiments, in which Actinomycin D was used, indicate that in both azaG-S and azaG-R cells the effect of azaG is not prevented by the drug, and, therefore, seems independent of incorporation of azaG products into cellular messenger RNA. Consequently, it seems also independent of cellular protein synthesis. This consideration brings attention upon virus-induced processes. The ability of azaG to affect viral RNA synthesis is shown by the studies with H^3 uridine, and preliminary work suggests a comparable effect on protein synthesis. As mentioned above, the period of the replication cycle sensitive to the analog covers the time course of virus RNA and polymerase synthesis(17, 18).

The question whether azaG is incorporated into viral RNA and only thereby affects protein synthesis and growth may be considered. The "time of removal" experiment does not support this hypothesis. The evidence so far accumulated indicates that while virus-specific RNA synthesis may initiate shortly after infection, no significant amounts are produced until 3-4 hours. When azaG is removed at 1 hour and a 500-fold amount of adenine, an antagonist of azaG, is added, it should be expected that by that time little azaG should be available. Nevertheless, a considerable inhibition is observed in these conditions. It is difficult to see how this inhibition could be mediated by significant incorporation of azaG into the RNA of the virus. Moreover, the RNA-mediated inhibition demands that the messenger function

of the viral RNA be substantially altered. In the experiments of Grünberger *et al*(19), 100% azaG-substituted trinucleoside diphosphates still promote the binding of L-(C^{14}) valine with at least 50% efficiency. It has also been shown that azaG messenger RNA stimulates polypeptide synthesis in *B. cereus* (20).

A primary effect upon the synthesis of protein independent of incorporation into viral RNA may be considered. The altered kinetics of RNA synthesis and the decreased rate of growth induced by the analog can be explained by an effect upon the synthesis of viral RNA-RNA polymerase and coat proteins. There is no reason at present to suggest that azaG inhibition would affect specifically one or the other of these proteins. Actually, there are indications that protein synthesis in general can be inhibited by the analog. The sensitivity of mammalian protein synthesis to the deleterious effects of the analog has already been reported(16). In our hands, azaG induces a severe inhibition of L cell protein synthesis at a concentration which causes transient stimulation of RNA synthesis (Fig. 6). If, however, a general inhibition of protein synthesis plays a major role in the viral inhibition of azaG, the lack of an effect on the early drop of host cell RNA synthesis induced by virus-directed proteins has to be explained. It is conceivable that at the time when early proteins are synthesized, an insufficient amount of azaG products was present in the cells. Alternatively, although the mechanism of action of the early proteins responsible for inhibition of cellular RNA and protein synthesis is not clear(21), it is possible that a relatively low number of molecules is sufficient for effective inhibition of cellular RNA and protein synthesis.

It seems possible that the effect of azaG is mediated by an azaG-substituted small molecule. GTP has been shown to play an important role in the transfer of amino acids from s-RNA into protein(22). A good deal of evidence indicates that it acts as a cofactor (23-25).

If an azaG-cofactor is responsible for the inhibitory effect of azaG, the activity of the analog in azaG-R cells, which have a low level of IMP-GMP pyrophosphorylases, could be explained. While azaG-R cells cannot convert large amounts of azaG to its nucleotide *via* pyrophosphorylation, a secondary mechanism exists which can use azaG as a substrate. This mechanism involves formation of the nucleoside(26) which is followed by the synthesis of the mono-(27), di-, and triphosphate(28).

The analog could inhibit EMC virus multiplication in azaG-R cells *via* this secondary mechanism which, however, is less efficient than pyrophosphorylation in providing the azaG toxic metabolites. The results of the "time of removal" experiments in azaG-S and azaG-R cells show that more azaG had to be added to the azaG-R cells in order to achieve after a 5-hour exposure an inhibition comparable to that seen after 1-hour exposure in azaG-S cells (Fig. 5).

The possibilities of an azaG-cofactor could also account for the reduced rate of growth of azaG-R cells in the presence of the analog.

Summary. The effect of azaG on EMC virus multiplication was investigated. It was found that the analog induces a delay in the onset of virus growth and slows the growth rate. This effect seems to be independent of host cells macromolecular synthesis, and indirect evidence suggests that it is also independent of incorporation into viral RNA. There is evidence that the synthesis of various virus-directed proteins may be primarily affected. It is suggested that an azaG-substituted GTP cofactor may interfere in the transfer capacity of s-RNA. This hypothesis could explain the ability of azaG to inhibit EMC replication at low intracellular concentration of azaG products as it would be expected in azaG-R cells which lack IMP-GMP pyrophosphorylases. A known secondary mechanism for the formation of azaG nucleotides could provide sufficient amounts of an azaG-cofactor to inhibit viral multiplication in this system.

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A Simplified Method for Separation of Urine and Feces in the Immature Fowl.*† (31501)

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The urine of the fowl flows from the ureters into the cloaca where it becomes mixed with feces; consequently, the collection of uncontaminated samples of urine or feces presents considerable practical problems. Non-surgical methods such as collecting urine by means of a funnel inserted into the urodeum were employed by Davis(1) and Coulson and Hughes(2), and the cannulation of the ureters has been employed to measure urine flow(3). The catheter (funnel) and cannula techniques facilitate only the collection of urine and, at best, for only limited periods (usually one-half to 24 hours). Exteriorization of the ureteral openings has been described by Hart and Essex(4) and more recently by Ainsworth(5). The latter method requires several incisions and sutures and usually requires that the bird wear a harness to facilitate urine and/or feces collection. A method has also been described for constructing an artificial anus(4), but the authors reported that birds remained suitable for experimental purposes for only 2 or 3 weeks. The method described here does not suffer from many of these disadvantages.

Anesthesia. White-Leghorn cockerels of approximately 9 weeks of age were selected and fasted 8 hours to insure that all feces were voided from the lower portion of the gut. The birds were given a general anesthetic by administering 20 mg/kg body weight of pentobarbital sodium, (Abbott Laboratories, North

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