FACTORS RELATED TO THE GROWTH OF PSITTACOSIS VIRUS (STRAIN 6BC)

I. Pteroylglutamic Acid, Vitamin B_{12}, and Citrovorum Factor*

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Previous studies with a strain of psittacosis virus (6BC) whose growth is inhibited by sulfonamide drugs (1) have demonstrated that this inhibitory effect is reversed competitively by *p*-aminobenzoic acid (PABA) and non-competitively by pteroylglutamic acid (PGA), giving evidence that PGA is essential for growth of the virus (2). Since vitamin B_{12} and citrovorum factor have certain biological relationships to PGA (3), it was of interest to study their influence on the inhibition of virus growth by the sulfonamides. Since present evidence for the role of PGA in growth of psittacosis virus (2) is of an indirect nature, the effect of PGA analogues on the multiplication of the virus in eggs was reinvestigated to secure more definitive evidence of the requirement of PGA for virus growth. Preliminary experiments (2) with a potent analogue of PGA, 4-aminopteroylglutamic acid, were unsuccessful, minute amounts of this substance being toxic for the chick embryo host, but the subsequent preparation of analogues of lower toxicity, such as 4-amino-N^{10}-methylpteroylglutamic acid (4) and 4-aminopteroylaspartic acid (5), suggested their trial in similar experiments. Moreover, since the toxic effects of PGA analogues were shown to be exerted primarily on the erythropoietic tissue of the chick embryo (6), experiments to determine the effects of these analogues on growth of the virus were carried out, using tissue cultures in which the viability of the host cells was not dependent on the functioning of intact blood-forming elements. Exploratory experiments (7) indicated that at least one PGA analogue would inhibit virus growth in the tissue cultures.

In the experiments presented here, it has been possible to obtain additional evidence of the importance of PGA for growth of psittacosis virus (6BC) and to investigate the possible relationships of vitamin B_{12} and citrovorum factor to virus multiplication.

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Materials and Methods

Viruses.—A strain (6BC) of psittacosis virus and one of meningopneumonitis virus, which had been repeatedly passed in eggs by the yolk sac route, were used. A pool of yolk sacs infected with each virus in nutrient broth was prepared with a Waring blendor. Aliquots were frozen in an alcohol and dry-ice mixture in sealed glass ampoules and stored in a dry-ice cabinet to provide a uniform inoculum for use in the experiments.

Compounds Tested.—Vitamin B_{12} was furnished in sterile solution by E. R. Squibb and Sons Citrovorum factor (CF) was provided by the Lederle Laboratories as a sterile aqueous solution containing 3 mg. per ml. with a biologic assay value of 20,000,000 units per ml. The folic acid analogues, 4-amino-PGA (aminopterin), 4-amino-N^{10}-methyl-PGA (amethopterin), and 4-aminopteroylaspartic (amino-an-fol) acid, and the sodium sulfadiazine (NaSD) were obtained from Lederle Laboratories as sterile preparations.

Experiments with Eggs.—The various materials were dissolved in sterile distilled water and injected into the yolk sacs of 7-day-old embryonated eggs from ½ to 1 hour before injection of the virus inoculum. Virus-infected controls and controls injected with each test compound alone to test its toxicity were included in each experiment. Eggs were incubated at 35°C., candled daily, and the deaths between 2 and 10 days were recorded. Those occurring within 48 hours were considered traumatic and disregarded. The average day of death of the eggs in each experimental series was calculated. As a check on the specificity of the deaths, yolk sacs of representative eggs were examined for the presence of psittacosis elementary bodies by examining smears stained by Macchiavello's method.

Experiments with Tissue Cultures.—A tissue culture method similar to that developed by Earle (8) was used. Perforated cellophane discs were fitted to cover the bottom of 10 ml. Erlenmeyer flasks which were then sterilized in the autoclave. Chick embryos 10 to 12 days old were minced with scissors in Hanks's balanced salt solution (9) without added $NaHCO_3$. This minced tissue was placed in the flasks under the cellophane disc. To each flask was added 2 ml. of a nutrient solution composed of Hanks's balanced salt solution (2 parts) and ox serum ultrafiltrate (1 part) which contained the virus inoculum. This fluid was removed 24 hours later and fresh nutrient fluid added. Thereafter, fluids bathing the cells were replaced at 4 day intervals and the various compounds under study were added to the nutrient fluid in the concentrations and at the intervals noted. Fluids removed from the cultures were diluted in broth, injected into eggs, and the virus titers calculated by the method described below.

At the conclusion of the experiment, representative cultures were treated with 10 per cent formalin in saline to fix the tissues and the cellophane discs carrying them were removed, stained with hematoxylin and eosin, and mounted on slides for microscopic examination of the adherent cells.

To study further the possible toxic effects of the test compounds on chick embryo tissue, fragments of heart tissue from 12- to 14-day-old embryos were planted in plasma in Carrel flasks. Nutrient fluids containing the test compounds were added to these cultures which were incubated for 4 days at 35° C. Daily observations were made of the capacity of the fragments to beat and of the outgrowth of fibroblasts at the periphery of the explants.

Virus Titrations.—A single dilution of the virus-containing fluid from each tissue culture was prepared in broth and 0.25 ml. of the material was injected into the yolk sacs of 12 or more 7-day-old embryonated eggs. Eggs were observed daily as described in the previous section and the average day of death calculated. The virus titer was determined from this value by the method described by Golub (10).

EXPERIMENTAL

Effect of Vitamin B_{12} and Citrovorum Factor on the Sulfonamide Inhibition of Virus Growth.—Vitamin B_{12} in doses of 7.5 μg. was injected into the yolk sac of

eggs in admixture with 0.2 mg. of NaSD; ½ hour later, 10,000 LD_{50} of psittacosis virus (6BC) was injected into these eggs. After 10 days, all the infectious controls were dead, whereas 90 per cent of the eggs receiving NaSD alone were

TABLE I

Effect of Citrovorum Factor on the Growth Inhibition of Psittacosis Virus (6BC) by NaSD

NaSD	Inhibitor	No. of eggs	Survived 10 days
mg.			*per cent*
2.5	PGA—0.5 mg.	24	0
2.5	PGA—0.05 mg.	24	0
2.5	CF —10^6 units	24	0
2.5	CF —10^3 units	24	76
2.5	— 0	24	100
0	PGA—0.5 mg.	24	0
0	CF —10^6 units	24	0
	Drug controls‡		
2.5	— 0	24	100
0	CF —10^6 units	24	100

* 10,000 LD_{50} virus injected via yolk sac.

‡ Not infected.

TABLE II

Influence of 4-Aminopteroylaspartic Acid on the Growth of Psittacosis Virus (6BC)

4-Aminopteroylaspartic acid	PGA	No. of eggs	ADD*
mg.	*mg.*		
0.25	0.0	24	7.5
0.25	5.0	24	5.9
0.0	5.0	24	5.5
0.0	0.0	24	5.2
	Drug controls‡		
0.25	0.0	24	>10.0

* Average day of death following injection of 100,000 LD_{50} of virus.

‡ Not infected.

viable as were 80 per cent of those injected with the mixture of vitamin B_{12} and NaSD. Vitamin B_{12} in this dosage had no effect on the inhibiting action of minimal concentrations of NaSD.

In similar experiments, citrovorum factor (CF) was tested and found to reverse the inhibitory action of 2.5 mg. of NaSD on virus growth in doses of 1,000,000 units but not 1,000 units (Table I). In these experiments, 1,000,000

units of citrovorum factor was roughly equivalent to 0.05 mg. of PGA. These results indicate that CF plays a role in the intracellular growth of psittacosis virus and that its activity is related to that of NaSD, PABA, and PGA.

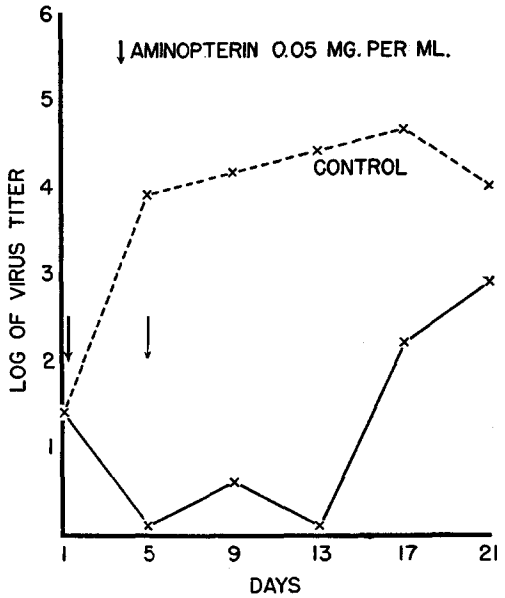

FIG. 1. The inhibition of growth of psittacosis virus (6BC) in tissue culture by aminopterin (4-amino-PGA).

TABLE III

Inhibition of Growth of Meningopneumonitis Virus by 4-Aminopteroylaspartic Acid

4-Aminopteroylaspartic acid	PGA	No. of eggs	ADD*
mg.	*mg.*		
0.25	0.0	24	6.5
0.25	5.0	24	5.2
0.0	0.0	24	4.9
	Drug controls‡		
0.0	0.0	24	>10.0

* Average day of death following injection of 100,000 LD_{50} of virus.

‡ Not infected.

Influence of Analogues of PGA on the Growth of Virus in Embryonated Eggs.— As indicated by the data in Table II, it was possible to inhibit partially the growth of psittacosis virus (6BC) in eggs by means of quantities of 4-aminopteroylaspartic acid that were just below the toxic dose of 0.5 mg. for the chick

embryo. This degree of growth inhibition was significant, however, since a prolongation of 2.3 days in the average day of death of the treated eggs indicates a definite decrease in the rate of multiplication of virus in the eggs injected with the PGA analogue. The inhibitory action of 4-aminopteroylaspartic acid is readily reversed by 20 times the amount of PGA. Another analogue, 9-methyl-PGA, had similar though less striking effects on the growth of the virus in eggs.

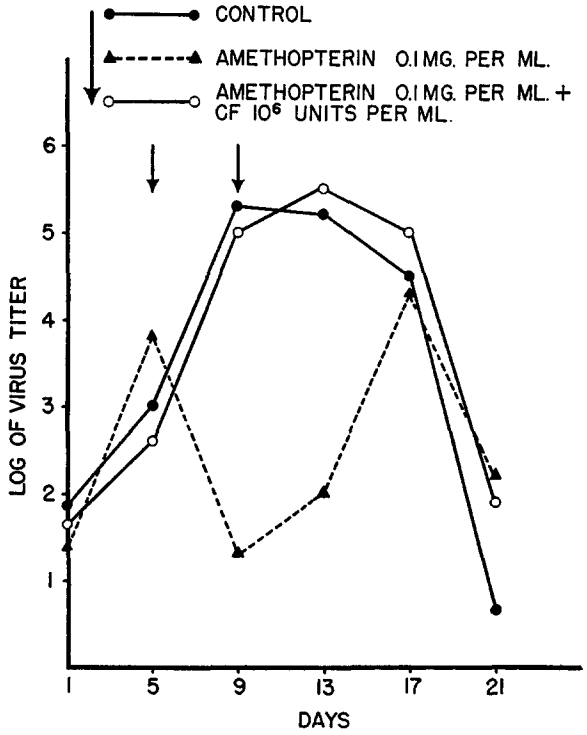

FIG. 2. Inhibition of psittacosis virus (6BC) growth in tissue culture by amethopterin (4-amino-N^{10}-methyl-PGA) and its reversal by citrovorum factor (CF).

Since meningopneumonitis virus is not susceptible to the action of sulfonamides (11), it was of interest to determine if analogues of PGA would interfere with its growth. Data summarized in Table III show that 4-aminopteroylaspartic acid will suppress the growth of meningopneumonitis virus in eggs. This effect is reversed by PGA.

Experiments in Tissue Cultures.—Three PGA analogues were tested: 4-amino-PGA, 4-amino-N^{10}-methyl-PGA and 4-aminopteroylaspartic acid. The analogue, 4-amino-PGA, produced marked inhibition of psittacosis virus (6BC) growth at 0.05 mg./ml. levels (Fig. 1). 4-Amino-N^{10}-methyl-PGA and 4-aminopteroylaspartic acids gave some evidence of inhibitory activity at 0.05 mg./ml.,

and at 0.1 mg./ml. exhibited striking effects (Figs. 2 and 3). 4-Aminopteroylaspartic acid had similar effects on meningopneumonitis virus in tissue culture. At these concentrations, the PGA analogues produced no obvious toxic effects on cultures of chick embryo heart tissues, in accord with previously reported observations (12).

The virus inhibitory effect of 4-aminopteroylaspartic acid at 0.1 mg./ml. was partially reversed by addition of 10 times the amount of PGA, but reversal of

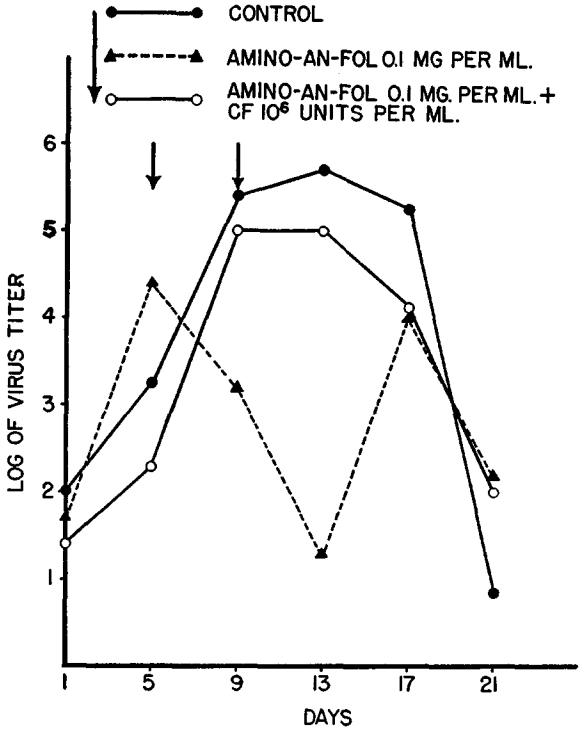

FIG. 3. Inhibition of multiplication of psittacosis virus (6BC) by amino-an-fol (4-aminopteroylaspartic acid) with reversal of its effects by citrovorum factor (CF).

the inhibitory action of 4-amino-N^{10}-methyl-PGA (0.1 mg./ml.) and 4-amino-PGA (0.05 mg./ml.) was not readily achieved with similar amounts of PGA. However, with citrovorum factor (CF), the inhibitory effects of 0.1 mg./ml. of 4-amino-N^{10}-methyl-PGA or 4-aminopteroylaspartic acid were completely reversed with 1,000,000 units/ml. of CF (Figs. 2 and 3). These observations confirm the importance of CF for the growth of psittacosis virus (6BC).

DISCUSSION

Experiments presented here on the effects of PGA analogues on the growth of psittacosis (6BC) and meningopneumonitis virus provide evidence of the

importance of PGA for the growth of these viruses. In the studies with psittacosis virus (6BC), data have been obtained which indicate that inhibition of the growth of this virus by NaSD involves the metabolic functions of PGA and CF within the cell and that vitamin B_{12} is not an important factor in such metabolic relationships. These findings raise the question furthermore of the possible functions of PGA and CF in the growth of psittacosis virus (6BC). Since both of these substances have been linked to the intracellular synthesis of desoxyribonucleic acid in bacteria (3), the possibility that they have a similar function in viral synthesis is suggested. This hypothesis receives further support from the finding that psittacosis virus contains considerable quantities of desoxyribonucleic acid (13, 14) indicating that virus growth would involve the synthesis of this nucleic acid. A subsequent paper in this series examines further the relationship of the synthesis of desoxyribonucleic acid to virus multiplication.

The inhibition of growth of meningopneumonitis virus in embryonated eggs and tissue cultures by 4-aminopteroylaspartic acid suggests the importance of PGA for the growth of this virus, which is not susceptible to the inhibitory action of the sulfonamides. PGA, therefore, may be an important growth factor for viruses of the psittacosis-lymphogranuloma venereum group irrespective of their sulfonamide sensitivity.

In interpreting the inhibition of virus growth by any material, the question of its possible toxic effects on the host cells must be considered, since death of these cells would halt virus proliferation. Toxicity tests of the test compounds on chick embryo tissues indicate that death of host cells cannot be the cause for the observed effects of PGA analogues on viral growth. Chick embryo heart in tissue cultures continued to beat, produce acid, and show fibroblastic proliferation when cultivated in the presence of concentrations of the PGA analogues identical with those of the experiments. Also, the prompt reappearance of virus after the removal of the analogues from the cultures indicates that these cells are still capable of supporting virus growth.

The tissue culture technic offers certain advantages for the study of the action of substances on the growth of viruses: (*a*) the action of the compound on virus growth can be studied without the limitation that would be imposed if it possessed special toxic properties for certain tissues of the host (*e.g.*, PGA analogues for hematopoietic tissue); (*b*) known concentrations of the test compound can be placed in contact with the infected host cell; (*c*) the cells are assuredly infected and intracellular synthesis of virus is underway when the compound is added; (*d*) the effect of any compound on virus growth can be roughly quantitated by serial determinations of virus released from the cells before and after its addition; (*e*) the absence of detrimental effects of the test substance on the host cells is indicated by the reappearance of virus when the compound is removed; (*f*) information as to the nature of the action of the inhibitory compound can be obtained by the simultaneous addition of various substances to deter-

mine their possible reversal of its suppression of virus growth; and (*g*) the tissues can be examined microscopically at the end of the experiment.

The various advantages of this tissue culture technic suggest its wider application for the study of virus growth and its inhibition or stimulation by various substances.

SUMMARY

The inhibitory action of sodium sulfadiazine on the growth of psittacosis virus (6BC) in embryonated eggs is readily reversed by citrovorum factor but not by small amounts of vitamin B_{12}.

In embryonated eggs, the pteroylglutamic acid analogues, 9-methylpteroylglutamic acid and 4-aminopteroylaspartic acid, produced some suppression of the growth of psittacosis virus (6BC). 4-Aminopteroylglutamic acid, 4-amino-N^{10}-methylpteroylglutamic acid, and 4-aminopteroylaspartic acid inhibited the growth of this virus in tissue cultures at concentrations which were not toxic for the host tissue. The inhibitory action of 4-amino-N^{10}-methylpteroylglutamic acid and 4-aminopteroylaspartic acid was readily overcome by addition of citrovorum factor.

Growth of meningopneumonitis virus in embryonated eggs or tissue culture is suppressed by 4-aminopteroylaspartic acid.

The advantages of the tissue culture technic for studies on the growth of viruses are discussed.

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