STUDIES ON THE RELATIONSHIP OF PTEROYLGLUTAMIC ACID TO THE GROWTH OF PSITTACOSIS VIRUS (STRAIN 6BC)*

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The studies by Woods (1) on the relationship of *p*-aminobenzoic acid (PABA) to the growth inhibitory action of the sulfonamides for bacteria have provided a useful tool for metabolic studies with PABA and related compounds such as pteroic acid and pteroylglutamic (synthetic folic) acid (PGA) which contain the PABA moiety (2, 3). Demonstration of the growth inhibitory effect of sulfadiazine on psittacosis virus (strain 6BC) (4) and the subsequent studies showing that this inhibitory action was antagonized by PABA and PGA (5) suggested the usefulness of this system for a study of certain factors concerned in the growth of this virus. The present communication reports the results of experiments on the effect of PABA, pteroic acid, PGA, and related compounds on sulfonamide inhibition of the growth of psittacosis virus and attempts to use this phenomenon to study certain possible synthetic activities of this virus.

Since certain analogues of PGA[1] have recently become available, it was of interest to test them for their effect on growth of the virus and for their influence on the inhibitory action of the sulfonamides since, in other systems, some of them behave like PGA (2) and others act as PGA antagonists (6, 7).

Materials and Methods

Psittacosis virus (strain 6BC) which had been repeatedly passed in eggs by the yolk sac route was used. A pool of infected yolk sacs was prepared in nutrient broth with a Waring blendor. Aliquots were placed in sealed glass ampoules and stored in the dry ice cabinet to provide a uniform inoculum for use in the experiments. This yolk sac suspension was titrated by injecting 0.25 ml. amounts of serial tenfold dilutions into the yolk sac of 6 to 7 day old embryonated eggs. The infected eggs were incubated at 35°C. for 10 days during which time deaths were recorded and the LD_{50} titer calculated (8). Deaths occurring during the first 48 hours were considered traumatic and disregarded. The day of death of the embryos showed an inverse correlation with the size of the infectious inoculum. For subsequent experiments, a dilution of the seed virus containing 10,000 LD_{50} doses in 0.25 ml. was used.

The various compounds to be tested were dissolved in sterile distilled water for injection. The sulfadiazine was obtained as a sterile solution of the sodium salt (NaSD) and dosage was

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[1] Obtained through the courtesy of Lederle Laboratories.

recorded in terms of NaSD. Weighed amounts of crystalline PABA, *p*-aminohippuric acid (PAHA), pteroic acid, glutamic acid, and PGA were placed in volumetric flasks with distilled water and a few drops of phenol red. Sodium hydroxide (1 N) was slowly added until the acids dissolved. The solutions were then sterilized in the autoclave at 10 pounds for 10 minutes and adjusted to volume with sterile distilled water. Other PGA derivatives were furnished as sterile salts and dissolved in sterile distilled water. The dosages of these materials are listed in terms of the free acid.

In the tests for sulfonamide antagonism, the solutions of the antagonist and the NaSD were mixed just before their injection unless otherwise noted. These various materials were injected into the yolk sac in 0.25 ml. volumes, and, after an interval of from one-half to 1 hour, the virus inoculum was injected by the same route. Infectious controls and uninfected eggs injected with the various compounds as a check on their toxicity were included in each experiment.

Eggs were candled daily and deaths recorded. Yolk sacs of representative embryos dying during the observation period were examined for the presence of psittacosis elementary bodies by examining smears stained with Macchiavello stain. Some yolk sacs were cultured on blood agar and in thioglycollate broth to exclude the presence of bacterial contaminants.

PABA determinations were done, as for sulfanilamide, by the method of Bratton and Marshall (9). The method described for determinations on blood was used with yolk samples and that suggested for urine specimens with the allantoic fluid levels.

Determination of the Minimal Experimental Inhibiting Dose of NaSD

In order to determine the efficacy of the action of the various sulfonamide antagonists, it was necessary to determine the effective dose of NaSD. Groups of eggs were injected with from 0.001 to 5.0 mg. of NaSD prior to infection with 10,000 LD_{50} of the virus. The results are presented in Table I. These data show that for an infectious inoculum of 10,000 LD_{50}, the minimal effective inhibiting dose of NaSD was between 0.1 and 0.5 mg. To insure that an adequate amount of NaSD was present, 2.5 mg. were used in the subsequent experiments on antagonism unless a range of test dosages was employed.

Effect of PABA and PGA on the Growth of Psittacosis Virus and Their Antagonistic Effect on Inhibition of Growth by NaSD

Experiments were carried out to determine the range of activity of the antagonistic action of PABA and PGA on the growth inhibition of psittacosis virus by NaSD. These data are summarized in Table II.

It is apparent that on a weight basis, PABA was more than 10 times as active as PGA. However, if the molecular weights are taken into account, PABA is only slightly more than 3 times as potent as PGA. This difference in magnitude approaches the range of experimental error for this test system. PABA alone in doses up to 5 mg. and PGA to 10 mg. had no effect on the growth of the virus and no serious degree of toxicity for the chick embryos.

A question that immediately arises is whether the activity of the PGA might be due to the fact that it was being broken down by the chick embryo with the release of PABA. When PABA was injected into eggs *via* the yolk sac route in doses as low as 0.5 mg., detectable levels could be measured in the yolk and

allantoic fluids after 2, 4, 6, and 9 days. With doses of PGA as large as 10 mg. per egg, the yolk and allantoic fluids contained no free PABA after the same

TABLE I

Determination of the Minimal Inhibiting Dose of NaSD on the Growth of Psittacosis Virus (Strain 6BC) in Eggs

NaSD	No. of eggs	Survived 10 days*
mg.		*per cent*
5.0	24	96
2.5	29	97
1.25	29	90
0.5	16	82
0.1	25	0
0.05	12	0
0.01	22	0
0	8	0

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

TABLE II

Effect of PABA and PGA on the Growth Inhibition of Psittacosis Virus (Strain 6BC) by NaSD

NaSD	Inhibitor	No. of eggs	Survived 10 days*
mg.	*mg.*		*per cent*
2.5	PABA 0.5	12	0
"	" 0.05	11	0
"	" 0.005	12	0
"	" 0.001	9	55
"	" 0.0005	10	100
"	PGA 5.0	12	0
"	" 0.5	12	0
"	" 0.25	11	66
"	" 0.01	10	100
"	" 0.005	9	100
"	— 0	12	92
0	PABA 5.0	8	0
0	PGA 10.0	10	0
	Drug Controls‡		
0	PABA 5.0	20	95
0	PGA 10.0	19	95

* 10,000 LD_{50} virus injected *via* yolk sac.
‡ Not infected.

intervals of time. This evidence strongly suggests that PGA is not broken down with release of PABA to any appreciable extent and, therefore, that PGA

is active *per se* as a sulfonamide antagonist. PAHA was also tested and no sulfonamide antagonism was demonstrated.

TABLE III

Comparison of the Antagonism of Sulfonamide Inhibition of Psittacosis Virus (Strain 6BC) by PABA and PGA

NaSD	Inhibitor	No. of eggs	Survived 10 days*
mg.	*mg.*		*per cent*
0.5	PABA 0.01	7	0
2.0	" "	8	0
5.0	" "	6	0
10.0	" "	19	47
25.0	" "	13	85
50.0	" "	12	100
0.5	PABA 0.05	5	0
2.0	" "	8	0
5.0	" "	8	0
10.0	" "	18	0
25.0	" "	11	8
50.0	" "	4	100
0.5	PGA 0.1	7	0
2.0	" "	8	0
5.0	" "	6	0
10.0	" "	16	0
25.0	" "	11	0
50.0	" "	18	0
0.5	PGA 0.5	8	0
2.0	" "	7	0
5.0	" "	7	0
10.0	" "	20	0
25.0	" "	10	0
50.0	" "	7	0
0.5	— 0	16	82
5.0	— 0	24	96
0	PABA 5.0	8	0
0	PGA 10.0	10	0
0	— 0	16	0
	Drug Controls‡		
50.0	— 0	8	85
0	PABA 5.0	26	95
0	PGA 10.0	19	95

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

‡ Not infected.

Since penicillin has been shown to inhibit the growth of psittacosis virus (10), the effect of PABA and PGA on its action was tested. In doses of 5 mg. of

PABA and 5 mg. of PGA these compounds failed to show any effect on the protective action of 500 units of penicillin which gave an 80 per cent survival rate in embryos infected with 10,000 LD_{50} of psittacosis virus. This provides additional evidence for the specific nature of the metabolic interrelationships of NaSD, PABA, and PGA in the growth of psittacosis virus.

Since liver extract as well as PGA has an important relationship to pernicious anemia, liver extract was tested in doses of 4.2 units (purified, Lilly) for possible antagonism of 2.5 mg. NaSD and was shown to be without any effect on the action of the drug.

Type of Sulfonamide Antagonism Produced by PABA and PGA

The previous studies of sulfonamide antagonism with bacteria had shown that PABA exerted a competitive antagonism on the sulfonamides, *i.e.* the amount of antagonist required bore a direct relationship to the dose of sulfonamide used, while PGA exerted its effect without regard to the amount of sulfonamide present (2, 3). It was of interest, therefore, to determine the nature of the action of these antagonists in the system being studied. Two doses of PABA and PGA, representing 2 and 10 times their minimal effective amounts for 2.5 mg. of NaSD, were therefore tested against increasing amounts of NaSD. These data are presented in Table III.

There is a direct relationship between the amount of SD used and the amount of PABA required to antagonize its inhibitory effect which demonstrates a competitive type of inhibition for PABA. On the other hand, once an effective dose of PGA is given, the dose of NaSD may be increased as much as 50 times without overcoming this antagonistic effect. Therefore, PGA exerts a non-competitive type of antagonism.

Effect of Other Components of PGA on Sulfonamide Action

Since PGA is composed of glutamic acid, PABA, and the pteridine nucleus, it was of interest to test components other than PABA for sulfonamide antagonism. Glutamic acid and pteroic acid were available for testing. The pteroic acid had been purified so that it contained only 0.026 per cent of free PABA and 7 per cent of other pterin materials as contaminants.[2] Table IV presents the data obtained in these experiments.

Results of these experiments show that glutamic acid in doses up to 10 mg. exerts no effect on the sulfonamide inhibition of virus growth while pteroic acid in 0.05 and 0.25 mg. amounts shows a competitive antagonistic effect.

Effects of Certain Analogues of PGA on the Growth of Psittacosis Virus and Its Inhibition by NaSD

The following analogues of PGA were tested: pteroyldiglutamic acid, pteroyl γ-diglutamic acid, pteroyltriglutamic acid, pteroylaspartic acid, and 4-amino-

[2] Data furnished by Dr. C. W. Waller of the Lederle Laboratories.

pteroylglutamic acid. Another folic acid antagonist, *N*-methylpteroic acid, was also tested (6). The data are presented in Table V.

In doses up to 2.5 mg. per egg, pteroyldiglutamic acid, pteroyl-γ-diglutamic acid and pteroyltriglutamic acid had no effect on the growth of psittacosis virus. These compounds produced sulfonamide antagonism for 2.5 mg. NaSD in amounts of 0.1 mg. Therefore, they appeared to act in the same manner as PGA in sulfonamide antagonism. However, pteroyl-γ-diglutamic acid when

TABLE IV

Effect of Components of PGA on the Sulfonamide Inhibition of Psittacosis Virus

SD	Inhibitor		No. of eggs	Survived 10 days*
mg.	*mg.*			*per cent*
2.5	Glutamic acid	1.0	9	89
"	" "	10.0	9	78
"	" "	20.0	6	83
1.0	Pteroic acid	0.05	12	0
2.5	" "	"	11	0
5.0	" "	"	11	36
10.0	" "	"	11	73
20.0	" "	"	12	100
2.5	Pteroic acid	0.25	12	0
5.0	" "	"	12	0
10.0	" "	"	11	0
20.0	" "	"	11	36
1.0	—	0	8	100
20.0	—	0	8	85
0	—	0	6	0
	Drug controls‡			
0	Glutamic acid	10.0	11	82
20.0	—	0	6	83
0	Pteroic acid	5.0	6	100

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

‡ Not infected.

tested for sulfonamide antagonism produced deaths of the embryos from 2 to 3 days later than those occurring with the other compounds. This delay indicates that this compound was somewhat less active than the others as an antagonist for NaSD.

Pteroylaspartic acid failed to produce sulfonamide antagonism in doses of 0.1 mg. but did so in 0.5 mg. amounts. In doses of 10 mg. it had no effect on the growth of the virus.

The compound *N*-methylpteroic acid produced definite sulfonamide antagonism only with doses of 5 mg. This effect was probably due to the small

amounts of PABA present in this preparation. It had no effect on the growth of the virus in 10 mg. amounts.

Attempts to study the effect of 4-aminopteroylglutamic acid on the growth of the virus were difficult since this compound produced deaths in from 5 to 10

TABLE V

Effect of Analogues of Folic Acid on the Growth of Psittacosis Virus and Its Inhibition by NaSD

NaSD	Inhibitor		No. of eggs	Survived 10 days*
mg.	*mg.*			*per cent*
2.5	Pteroylglutamic acid	0.1	8	0
"	" "	0.5	10	0
"	Pteroyldiglutamic acid	0.1	10	10
"	" "	0.5	9	0
"	Pteroyl-γ-diglutamic acid	0.1	10	0
"	" "	0.5	10	0
"	Pteroyltriglutamic acid	0.1	8	0
"	" "	0.5	10	0
"	Pteroylaspartic acid	0.1	10	50
"	" "	0.5	10	0
"	*N*-methylpteroic acid	0.5	6	66
"	" "	5.0	8	0
0	Pteroyldiglutamic acid	2.5	8	0
0	Pteroyl-γ-diglutamic acid	2.5	8	0
0	Pteroyltriglutamic acid	2.5	8	0
0	Pteroylaspartic acid	10.0	6	0
0	*N*-methylpteroic acid	10.0	7	0
2.5	—	0	12	100
0	—	0	12	0
	Drug controls‡			
0	Pteroyldiglutamic acid	2.5	10	100
0	Pteroyl-γ-diglutamic acid	2.5	10	100
0	Pteroyltriglutamic acid	2.5	10	90
0	Pteroylaspartic acid	10.0	9	75
0	*N*-methylpteroic acid	10.0	10	100

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

‡ Not infected.

days in chick embryos when given in doses of 0.001 to 0.005 mg. When virus-infected eggs were given 3 injections of 0.002 mg. over a period of 7 days and the yolk sacs were harvested and titrated, the content of virus was about the same as in untreated and infected controls. Small doses of 4-aminopteroyl-glutamic acid (0.002 mg.) combined with 0.25 to 0.01 mg. of NaSD gave no evidence of potentiating nor antagonizing the virus-inhibiting action of the

sulfonamide. Within the limitations of these experimental techniques no effect of 4-aminopteroylglutamic acid on virus growth could be demonstrated.

DISCUSSION

Among the members of the psittacosis-lymphogranuloma group, the viruses of lymphogranuloma venereum (11), mouse pneumonitis (11), and at least two strains of psittacosis virus, *i.e.* 6BC (4) and Gleason (12), have been shown to be susceptible to the chemotherapeutic action of sulfonamide drugs. Studies on the antagonistic action of PABA on the sulfonamide inhibition of growth of lymphogranuloma venereum virus have yielded conflicting results. Findlay (13) reported that PABA antagonized the chemotherapeutic action of sulfanilamide in mice infected with this virus that received 10 mg. of each compound daily. In direct contrast, Seeler *et al.* (14) using a diet containing 0.3 per cent PABA and 0.3 per cent sulfanilamide were unable to demonstrate any effect of PABA. Rodaniche (15) feeding a diet containing 0.5 per cent PABA and 2 per cent sulfathiazole found no effect, but noted some evidence of sulfonamide antagonism when the amount of PABA was increased to 2 per cent.

The discrepancies in the results previously reported may be due to the fact that PABA is rapidly excreted by the mouse (16) so that it may not have been present in the body in adequate amounts.

The data presented here show clearly that PABA will antagonize the inhibition of psittacosis virus growth by the sulfonamides. The further demonstration that PGA has a similar action has provided a useful tool for the investigation of possible metabolic activities and growth requirements of the psittacosis virus.

Demonstration of the competitive nature of the sulfonamide antagonism by PABA, and, the finding that once an adequate dose of PGA is given, the sulfonamide dosage may be increased without any effect have certain interesting implications. If the systems which have been worked out in the case of several bacteria (2, 3) can be applied here, then it can be reasoned that the sulfonamide prevents the growth of psittacosis virus by interfering with its use of PABA in the synthesis of PGA which it requires for growth. This reasoning is based on the fact that PABA can competitively reverse the growth inhibitory action of the sulfonamide. Furthermore, once PGA is supplied, the virus can grow in the presence of large amounts of NaSD since this essential metabolite is furnished. Further evidence for this hypothesis is presented in the data obtained with the experiments using glutamic and pteroic acids. Glutamic acid, as expected, was found to be without effect on sulfonamide action. On the other hand, pteroic acid antagonized sulfonamide inhibition of virus growth in a competitive manner. This finding suggests that pteroic acid is an intermediate step in the synthesis of PGA by psittacosis virus.

Of course, it could be pointed out that these effects might be operating on the

host rather than the virus, but there is evidence that chicks do not synthesize PGA since it is a dietary requirement for them (17).

Of the analogues tested, all but pteroylaspartic acid, 4-aminopteroylglutamic acid, and *N*-methylpteroic acid have previously been shown to have properties similar to those of PGA. Similar data were obtained in these experiments. The compounds 4-aminopteroylglutamic acid and *N*-methylpteroic acid are known to be active antagonists for PGA (6). In this study, they differed from PGA in that they showed no activity as sulfonamide antagonists. Within the limitations of this experimental technique, they showed no inhibitory effect on psittacosis virus growth. However, they did kill the chick embryos and histological examination of their erythropoietic tissue showed a marked maturation arrest which could be reversed by the administration of PGA (18). These findings suggest that these compounds were active as folic acid antagonists in the chick embryo tissues. Therefore, the fact that, in the small doses that could be tolerated, they did not affect growth of the psittacosis in these tissues may provide additional evidence that the virus was synthesizing its own PGA since it would not then be affected by their action. Obviously, if the virus is capable of synthesizing PGA, it should be present in higher concentrations in infected chick tissues and fluids in comparison with the same materials from normal embryos. Experiments are now underway to elucidate this point.

It is of special interest that these experiments give evidence of the relationship of PGA to growth of this virus since recent studies (19) have suggested that this compound has an important rôle in the synthesis of nucleic acids and certain of their derivatives which are important factors in the chemical constitution of viruses.

SUMMARY AND CONCLUSIONS

p-Aminobenzoic and pteroic acids antagonize the inhibition of the growth of psittacosis virus (strain 6BC) by sulfadiazine in a competitive manner. Pteroylglutamic acid exerts a non-competitive type sulfonamide antagonism.

The effects of certain analogues of pteroylglutamic acid and of pteroic acid on the growth of psittacosis virus and its inhibition by sulfadiazine are reported.

The implications of these findings with regard to the possible synthesis of pteroylglutamic acid by psittacosis virus and its suggested rôle as an essential factor for growth of this virus are discussed.

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