SYNTHESIS AND SOME BIOLOGICAL PROPERTIES OF 4-AMINOPTEROYLASPARTIC ACID

By B. L. HUTCHINGS, J. H. MOWAT, J. J. OLESON, A. L. GAZZOLA, E. M. BOGGIANO, D. R. SEEGER,* J. H. BOOTHE, C. W. WALLER, R. B. ANGIER, J. SEMB, AND Y. SUBBAROW†

(From the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York)

(Received for publication, April 27, 1949)

In an earlier communication the synthesis and certain of the antipteroylglutamic acid properties of pteroylaspartic acid were described (1). The related compound, *N*-[4-{[(2,4-diamino-6-pteridyl)methyl]amino}benzoyl]aspartic acid, which will be designated as 4-aminopteroylaspartic acid, has been prepared and the inhibitory nature of the compound on the growth of bacteria, chicks, and rats has been determined. By comparing the relative activities of the two compounds it is possible to assess the effect of the introduction of an amino group in place of the hydroxyl group on the pteridine ring on the antipteroylglutamic acid properties of this class of compounds.

EXPERIMENTAL

Synthesis of 4-Aminopteroylaspartic Acid

p-Aminobenzoylaspartic Acid—A mixture of *p*-nitrobenzoylaspartic acid (1) (640 gm.) and water (8 liters) was adjusted to pH 3.0 and reduced with zinc dust (800 gm.) and concentrated hydrochloric acid (1350 ml.) as described by Boothe *et al.* (2). When the reduction was complete, the excess zinc was removed by filtration and the filtrate was used in the following reaction.

4-Aminopteroylaspartic Acid—In a procedure similar to that described by Seeger *et al.* (3) for the synthesis of 4-aminopteroylglutamic acid, 2,4,5,6-tetraaminopyrimidine sulfate (4) (770 gm.) and 1,3,3-tribromopropanone-2 (5) (430 ml.) were added to the well stirred filtrate containing the *p*-aminobenzoylaspartic acid. The mixture was then heated at 80° for 30 minutes and maintained at pH 2.0 by the addition of 2.5 N sodium hydroxide solution. The mixture was then adjusted to pH 3.5 to 4.0, cooled, and filtered. The filter cake was washed successively with 5 per cent sodium chloride solution, acetone, and ether.

* Present address, Calco Chemical Division, American Cyanamid Company, Bound Brook, New Jersey.

† Deceased, August 9, 1948.

This crude product was dissolved in 65 liters of 0.1 N sodium hydroxide solution at 35–40°, stirred for 30 minutes, and filtered. The filtrate was then heated to 70°, treated with 600 ml. of a 30 per cent calcium chloride solution, and again filtered. The filtrate was adjusted to pH 1.0 with hydrochloric acid, heated to 85–90°, and filtered while hot. On cooling, the product crystallized as small needles. Yield 131 gm. Chemical assay (6) indicated the product to be approximately 75 per cent pure. Further purification was effected by recrystallization from dilute hydrochloric acid.

$C_{18}H_{18}O_5N_8 \cdot HCl$.	Calculated.	C 46.71, H 4.14, N 24.21, Cl 7.66
	Found.	" 46.69, " 4.6, " 24.52, " 7.39

Inhibition of Bacterial Growth—The effect of 4-aminopteroylaspartic acid on the growth of certain bacteria was investigated. The medium used for each organism was as follows: *Streptococcus faecalis* R was grown on the medium of Teply and Elvehjem (7), *Escherichia coli* BG-22[1] was cultured on a similar medium adjusted to pH 6.0 from which the purines and pyrimidines were omitted, and *Lactobacillus arabinosus* was grown on a modified Teply and Elvehjem medium in which sodium acetate was used as the buffer and only 1 per cent glucose was added; *L. arabinosus* and *E. coli* were incubated 16 hours at 37° and *S. faecalis* R was incubated 16 hours at 32°.

When the inhibition index is reported, it is defined as the ratio of inhibitor to metabolite that will produce one-half maximum inhibition.

Rat and Chick Experiments—The experimental conditions were similar to those previously described (1).

Results

Bacterial Inhibition—The inhibition indices for a number of the pteroyl compounds as metabolites of *S. faecalis* R are tabulated in Table I. With *S. faecalis* R, 4-aminopteroylaspartic acid is a typical competitive antagonist for pteroylglutamic acid and related compounds. The action of the inhibitor is similar to that of pteroylaspartic acid but different from that of 4-aminopteroylglutamic acid. Oleson *et al.* (8) showed the latter compound to be a strong inhibitor of the growth of *S. faecalis* R. The inhibition could be reversed by the addition of excess pteroylglutamic acid, but it was noted that the reversal of the inhibition by the addition of graded amounts of pteroylglutamic acid was not a direct function of the ratios of the concentration of metabolite to inhibitor. The present data indicate that the replacement of the hydroxyl group of the pteridine ring by an amino group is not the sole determining factor for the abnormal behavior of 4-aminopteroylglutamic acid.

[1] This culture was originally isolated in these laboratories in 1917.

It is interesting to note that 4-aminopteroylaspartic acid is considerably more active than pteroylaspartic acid in inhibiting the growth induced by pteroic acid or pteroylglutamic acid. When the metabolites pteroyl-γ-glutamylglutamic acid and pteroyl-γ-glutamyl-γ-glutamylglutamic acid are used, the two antagonists show approximately the same activity. This is most probably due to the fact that the pteroyltriglutamic acid compound is utilized with difficulty by the organism. Hence, the association between the metabolite and enzyme is so weak that this, rather than the inhibitory nature of the antagonist, becomes the limiting

TABLE I

4-Aminopteroylaspartic Acid Inhibition of Metabolites for Streptococcus faecalis R

Metabolite	Concentration of metabolite	Inhibition index*
	γ per 10 ml.	
Pteroic acid	0.004	0.35
" "	0.04	0.39
" "	0.4	0.15
Pteroylglutamic acid	0.003	1.65
" "	0.03	0.50
" "	0.3	0.23
" "	3.0	0.73
Pteroyl-γ-glutamylglutamic acid	0.005	4.12
" "	0.05	2.5
" "	0.5	1.79
Pteroyl-γ-glutamyl-γ-glutamylglutamic acid	0.07	1.0
" "	0.7	0.28
" "	7.0	0.15

* Ratio of the amount of inhibitor to metabolite that will produce half maximum inhibition.

factor. The pteroyldiglutamic acid compound would also fall in this category.

The effect of 4-aminopteroylaspartic acid on the growth of *S. faecalis* R, *E. coli*, and *L. arabinosus* is outlined in Table II. *E. coli* and *L. arabinosus*, as examples of bacteria that do not require preformed pteroylglutamic acid, were completely inhibited by the addition of from 300 to 500 γ and 10 to 15 γ of 4-aminopteroylaspartic acid per 10 ml. of medium respectively. Data are presented on the results of the addition of compounds that have been shown to replace pteroylglutamic acid in the nutrition of various lactic acid bacteria (9, 10) or that have been shown to function in the synthesis of thymidine in certain microorganisms (11, 12).

TABLE II

Effect of Various Compounds on 4-Aminopteroylaspartic Acid Inhibition

When negative results are tabulated, the figure in parentheses represents the highest concentration used. When positive results are expressed, the effective concentration of the compound is indicated in parentheses. ± indicates half maximum reversal.

Organism	Compounds			
	Pteroylglutamic acid	Thymine	Vitamin B_{12}	Thymidine*
L. arabinosus	− (1 mg.)	− (4 mg.)	− (10 γ)	± (0.1 mg.)
E. coli	− (1 ")	± (0.5 mg.)	− (10 ")	± (0.1 ")
S. faecalis R	+	+ (7.5 γ)	− (10 ")	+ (0.1 ")

* Used as a concentrate.

TABLE III

Effect of 4-Aminopteroylaspartic Acid on Chicks

Group No.	Supplement	Average weights*			
		Initial	7 days	14 days	21 days
		gm.	*gm.*	*gm.*	*gm.*
1	None	39 (7)	76 (7)	105 (7)	110 (5)
2	0.25 mg. pteroylglutamic acid per kilo diet	38 (7)	83 (7)	147 (7)	212 (7)
3	As (2) + 0.25 mg. 4-aminopteroylaspartic acid per kilo diet	39 (7)	71 (7)	134 (6)	202 (6)
4	As (2) + 1.0 mg. 4-aminopteroylaspartic acid per kilo diet	39 (7)	76 (6)	134 (5)	196 (5)
5	As (2) + 2.0 mg. 4-aminopteroylaspartic acid per kilo diet	39 (7)	71 (7)	135 (7)	202 (7)
6	As (2) + 4.0 mg. 4-aminopteroylaspartic acid per kilo	39 (7)	74 (7)	130 (7)	185 (7)
7	None	39 (8)	60 (8)	72 (7)	
8	As (7) + 25 γ pteroylglutamic acid†	39 (8)	71 (8)	133 (8)	
9	" (7) + 0.5 mg. 4-aminopteroylaspartic acid†	39 (8)			
10	As (9) + 5 γ pteroylglutamic acid†	39 (8)	51 (5)	95 (3)	
11	" (9) + 10 γ " " †	39 (8)	51 (7)	106 (6)	
12	" (9) + 15 γ " " †	39 (8)	59 (7)	111 (7)	
13	" (9) + 20 γ " " †	38 (8)	64 (7)	114 (8)	
14	None	46 (8)	75 (8)	99 (7)	
15	As (14) + 50 γ pteroylglutamic acid†	47 (8)	84 (8)	140 (8)	
16	" (14) + 0.5 mg. 4-aminopteroylaspartic acid†	46 (8)	55 (6)	76 (1)	
17	As (16) + 20 γ pteroylglutamic acid†	46 (8)	73 (8)	128 (8)	
18	" (16) + 40 " " " †	47 (8)	79 (6)	127 (6)	
19	" (16) + 50 " " " †	47 (8)	87 (7)	139 (7)	

* The figures in parentheses indicate the number of survivors.

† Intramuscularly five times weekly.

With *S. faecalis* R the growth inhibition induced by 4-aminopteroylaspartic acid was completely reversed by the addition of pteroylglutamic acid, thymine, or a thymidine concentrate. However, the growth resulting from the addition of thymine or thymidine could not be inhibited by extremely high levels of 4-aminopteroylaspartic acid (2 mg. per 10 ml.).

Thymine and thymidine were able to reverse the inhibition partially when *E. coli* was used as the test organism. With *L. arabinosus* only the

TABLE IV

Effect of 4-Aminopteroylaspartic Acid on Rat

Group No.	Supplement	Average weights*				
		Initial	1 wk.	2 wks.	3 wks.	4 wks.
1	None	39 (7)	56 (7)	82 (7)	102 (7)	144 (7)
2	3.0 mg. 4-aminopteroylaspartic acid†	39 (10)	56 (10)	81 (10)	112 (10)	144 (10)
3	6.0 mg. 4-aminopteroylaspartic acid†	42 (9)	46 (9)	77 (5)	109 (5)	136 (5)
4	As (3) + 60 γ pteroylglutamic acid†	40 (4)	58 (4)	82 (4)	111 (4)	142 (4)
5	None	41 (7)	50 (7)	74 (7)	104 (7)	129 (7)
6	0.5 mg. 4-aminopteroylaspartic acid‡	43 (5)	51 (5)	71 (5)	92 (5)	116 (5)
7	1.0 mg. 4-aminopteroylaspartic acid‡	43 (5)	50 (5)	63 (5)	86 (5)	109 (5)
8	3.0 mg. 4-aminopteroylaspartic acid‡	41 (10)	46 (10)	60 (8)	80 (5)	101 (5)
9	None	44 (8)	63 (8)	91 (8)	120 (8)	146 (8)
10	0.5 mg. 4-aminopteroylaspartic acid‡	45 (8)	58 (8)	78 (6)	103 (6)	133 (5)
11	As (10) + 50 γ pteroylglutamic acid‡	45 (8)	59 (8)	72 (8)	105 (7)	132 (7)
12	As (10) + 100 γ pteroylglutamic acid‡	44 (6)	61 (6)	89 (6)	117 (6)	147 (6)

* The figures in parentheses indicate the number of survivors.

† Orally five times weekly.

‡ Intramuscularly five times weekly.

thymidine concentrate had a partial effect. The inhibition of this organism appears to be due in part to an interference in the synthesis of thymidine. These results are similar to those noted by Shive and coworkers (13) for *Leuconostoc mesenteroides.*

Various combinations of the compounds were tried, but no combination was more effective than the single compounds noted.

Chick—The results of the various chick experiments are tabulated in Table III. The data indicate that the route of administration is important. When administered orally, the compound is inactive at a ratio of metabolite to inhibitor of 1:16. On intramuscular injection the inhibitor is markedly more effective. At a ratio of 1:10 the growth effects of pteroylglutamic acid are neutralized. It is apparent with chicks and bacteria that 4-aminopteroylaspartic acid is approximately 50 times as active as pteroylaspartic acid in reversing the growth effects of pteroylglutamic acid.

Rat—As an inhibitor of the growth of rats 4-aminopteroylaspartic acid is markedly inferior to the corresponding glutamic acid analogue. The oral feeding of 6 mg. of the inhibitor per day produces approximately 50 per cent mortality and a partial inhibition of growth in the survivors. When 3 mg. per day are injected intramuscularly, a more pronounced growth inhibition is evident. With 0.5 mg. intramuscularly some growth retardation occurs. This effect is counteracted by the injection of 100 γ of pteroylglutamic acid. The inhibitor produced no gross symptoms of pteroylglutamic acid deficiency other than that of a general retardation in growth.

DISCUSSION

Under the conditions of the present investigation, 4-aminopteroylaspartic acid behaves as a competitive metabolite antagonist in that the degree of inhibition is a function of the concentration of the metabolite.

The deficiency induced in the chick and the rat is not of a precipitous nature but is characterized by a gradual onset of the deficiency symptoms more akin to those of a simple dietary depletion of the vitamin. These results are in contrast to those obtained previously with 4-aminopteroylglutamic acid. It is of interest to speculate whether the 4-amino group is responsible for the strong inhibitory action of the glutamic acid analogue or whether the activity of the compound can be presumed to be due to its very close structural similarity to pteroylglutamic acid. If the amino group is responsible, other 4-amino analogues of the pteroyl compounds should also show to a certain extent the biological properties associated with 4-aminopteroylglutamic acid. The present results would indicate that the 4-amino group increases the inhibitory power of the compound but that the competitive nature of the antagonist is retained.

The enhanced activity of 4-aminopteroylaspartic acid is reflected in the increased inhibitory activity of the compound for bacteria and the chick but more noticeably in the ability of the 4-amino derivative to inhibit the growth of bacteria that do not require preformed pteroyl-

glutamic acid and to produce a growth retardation in the rat. Pteroylaspartic acid was inactive under these latter conditions.

SUMMARY

The synthesis of 4-aminopteroylaspartic acid from *p*-aminobenzoylaspartic acid, 1,3,3-tribromopropanone-2, and 2,4,5,6-tetraaminopyrimidine is described. The compound is a competitive antagonist of pteroylglutamic acid in the chick, the rat, and in certain bacteria that require preformed pteroylglutamic acid as a metabolite.

It is a pleasure to express our appreciation to Mr. L. Brancone and coworkers for the microanalyses.

BIBLIOGRAPHY

1. Hutchings, B. L., Mowat, J. H., Oleson, J. J., Stokstad, E. L. R., Boothe, J. H., Waller, C. W., Angier, R. B., Semb, J., and SubbaRow, Y., *J. Biol. Chem.*, **170,** 323 (1947).
2. Boothe, J. H., Semb, J., Waller, C. W., Angier, R. B., Mowat, J. H., Hutchings, B. L., Stokstad, E. L. R., and SubbaRow, Y., *J. Am. Chem. Soc.*, **71,** 2304 (1949).
3. Seeger, D. R., Cosulich, D. B., Smith, J. M., Jr., and Hultquist, M. E., *J. Am. Chem. Soc.*, **71,** 1753 (1949).
4. Traube, W., *Ber. chem. Ges.*, **37,** 4545 (1904).
5. Watson, H. B., and Yates, E. D., *J. Chem. Soc.*, 1207 (1932).
6. Hutchings, B. L., Stokstad, E. L. R., Boothe, J. H., Mowat, J. H., Waller, C. W., Angier, R. B., Semb, J., and SubbaRow, Y., *J. Biol. Chem.*, **168,** 705 (1947).
7. Teply, L. J., and Elvehjem, C. A., *J. Biol. Chem.*, **157,** 303 (1945).
8. Oleson, J. J., Hutchings, B. L., and SubbaRow, Y., *J. Biol. Chem.*, **175,** 359 (1948).
9. Stokstad, E. L. R., *J. Biol. Chem.*, **139,** 475 (1941).
10. Stokes, J. L., *J. Bact.*, **48,** 201 (1944).
11. Shive, W., Ravel, J. M., and Eakin, R. E., *J. Am. Chem. Soc.*, **70,** 2614 (1948).
12. Skeggs, H. R., Huff, J. W., Wright, L. D., and Bosshardt, D. K., *J. Biol. Chem.*, **176,** 1459 (1948).
13. Shive, W., Eakin, R. E., Harding, W. M., Ravel, J. M., and Sutherland, J. E., *J. Am. Chem. Soc.*, **70,** 2299 (1948).