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PTERIDINE DIURETICS AS BIOPTERIN ANTAGONISTS

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Abstract—A series of pteridines having varying degrees of diuretic activity were tested for their effects on the growth of the pteridine-requiring flagellate, *Crithidia fasciculata*. Inhibition of growth was correlated with diuretic activity insofar as compounds devoid of diuretic effects were uninhibitory and active inhibitors were also good to fair diuretics. Reversal of the growth inhibition was obtained with 2-amino-4-hydroxy-6-L-*erythro*-1',2',3'-trihydroxypropylpteridine in a competitive manner, while folate showed a logarithmic relationship to the drug concentration for 50 per cent inhibition.

THE TRYPANOSOMID flagellate, *Crithidia fasciculata*, has been shown to have a nutritional requirement which is best satisfied by biopterin, an unconjugated pteridine.[1] In this respect, this organism, as well as its close relatives, is unique.

After Kaufman[2] had demonstrated that tetrahydrobiopterin was a cofactor in the enzymatic conversion of phenylalanine to tyrosine, unconjugated pteridines were found to be involved as cofactors in a variety of reactions in which mixed function oxygenases were concerned.[3–7]

Because of our interest in the function of pteridines, we felt that additional information might be obtained by testing pteridine analogs such as those having pharmacological activity in mammals. Accordingly, a series of pteridines having various degrees of diuretic activity were tested for their effect on the growth of *C. fasciculata*. Most of the experiments were carried out using triamterene (2,4,7-triamino-6-phenylpteridine).

As early as 1958, it had been shown that triamterene (presumably an analog of pteroate) is an inhibitor of the conversion of folate to 5-formyltetrahydrofolate in chick liver homogenates.[8] In this system the 6-*o*-tolyl triaminopteridine was a better inhibitor than triamterene. For a more highly purified dihydrofolate reductase prepared from mouse Ehrlich ascites cells[9] a K_i of $1{\cdot}3 \times 10^{-8}$ was obtained for triamterene as compared to $<6 \times 10^{-10}$ for methotrexate. It is also of interest that for this enzyme biopterin was an exceedingly poor substrate, if utilized at all. Enzymes prepared from guinea pig liver and small intestine were also found to be sensitive with a concentration of $2{\cdot}6 \times 10^{-7}$ M giving 50 per cent inhibition.[10] For the dihydrofolate reductase prepared from L1210R murine lymphoma cells, a K_i of 1×10^{-8} was obtained.[11] It was also shown that the analog is much more tightly bound to the enzyme than is the natural substrate. The effect of triamterene on the reduction

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of folate to tetrahydrofolate by the enzyme from mouse leukemic cells was studied by Maass *et al.*[12] At a folate concentration of 10^{-4} M, 50 per cent inhibition by triamterene occurred at $8{\cdot}6 \times 10^{-7}$ M.

A study of the relatively crude preparation of the dihydrofolate reductase of *Trypanosoma equiperdum*, a rather distant relative of *C. fasciculata*, also showed half-maximal inhibition by triamterene at $2{\cdot}5 \times 10^{-6}$ M and that it was more active than the 6-tolyl compounds.[13] Folate was found not to be an effective substrate for the trypanosome enzyme. This may be the result of testing for activity at pH 7·0. With the guinea pig enzymes, for example, folate was reduced only at an acid pH,[10] and for the enzyme from chicken liver the optimal pH was 5·0.[14] Gutteridge *et al.*[15] studied the dihydrofolate reductases of *C. fasciculata* and *Crithidia oncopelti* and found that they resembled the enzyme of *Trypanosoma lewisi*[16] in their sensitivity to antifolates, but triameterene was not included in these studies.[15,16] The possibility also exists that in the trypanosomid flagellates there is a separate enzyme capable of reducing folate and pteridines at that oxidation level, which was not extracted in their enzyme preparation procedure. In any case, there must be a means for these flagellates to reduce dietary folate to the coenzyme form.

Both folate and unconjugated pteridines were tested for their ability to reverse the inhibition of the growth of the flagellate produced by certain of the diuretics.

A few experiments were carried out using the ciliate, *Tetrahymena pyriformis*, which is capable of the biosynthesis of pteridines.[17]

MATERIALS AND METHODS

C. fasciculata was cultivated in a medium modified from medium 2 of Kidder and Dutta[18] by omission of the high concentration of folate and replacing it with 2-amino-4-hydroxy-6-L-*erythro*-1′,2′,3′-trihydroxypropylpteridine (THPP) kindly supplied by Dr. E. L. Patterson of the Lederle Laboratories, plus thymine (40 mg/ml) or THPP plus lower concentrations of folate, as noted below. After inoculation with suitably depleted organisms,[19] the cultures were incubated for 4 days at 25° in a slanted position for increased aeration. Growth was measured turbidimetrically using a Lumetron colorimeter with a red filter. The pteridine diuretics, obtained from Drs. A. R. Maass and J. Weinstock of Smith Kline & French, were dissolved by moistening the dry powder with 95% ethanol, adding a few drops of 1 N HCl, diluting, neutralizing with 2 N NaOH and bringing to the desired concentration. Assays were run in triplicate using 10 levels of the drug from one- to 10-fold for each concentration of THPP or folate tested.

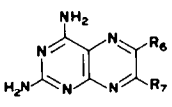
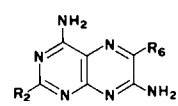
In other similar experiments, the effects of compounds capable of sparing the pteridine requirement of Crithidia[6,7] were investigated. Oleate was added to media from which Tween 80 was omitted at concentrations of 25, 50 and 100 μg/ml, linoleate at 25 and 50 μg/ml or cytidine at 50 μg/ml.

Three nonpteridine diuretics, Hydrodiuril (hydrochlorothiazide; 6-chloro-3,4-dihydro-7-sulfamoxl-2H-1,2,4-benzothiadiazine 1,1-dioxide), Orpidan (chlorozanil; 2-amino-4-(*p*-chloroanilo)-*s*-triazine) and Diamox (acetazolamide; 5-acetamido-1,3,4-thiadiazole-2-sulfonamide), kindly supplied by Dr. J. Weinstock, were also tested for their effects on growth.

RESULTS

In order to be able to control the pteridine concentration in the medium, it was necessary to reduce the folate content (or replace it by thymine) with the addition of THPP, because Crithidia is capable of converting folate to biopterin when the former is supplied at high concentrations (1–2 μg/ml).[20] In Table 1 are shown the amounts of the various pteridine diuretics required to give half-maximal inhibition of the growth of Crithidia in both the thymine–THPP medium and the folate–THPP medium.

TABLE 1. COMPARISON OF ACTIVITIES OF PTERIDINES AS INHIBITORS OF THE GROWTH OF *C. fasciculata* AND AS DIURETIC COMPOUNDS

	Substitutions		Amt. required for 50% inhibition (molarity × 10^5)		
Diaminopteridines	R_6	R_7	THPP + thymine†	THPP + folate‡	Duretic activity*
	C_2H_5	C_2H_5	3·7	2·2	1,0
	H	CH_3	7·2	4·4	2,1
	CH_3	CH_3	7·7	5·3	3,3
	CH_3	H	14·0	11·6	3,1
2,4-Diaminopteridines	C_4H_9	C_4H_9	n.i.§	n.i.§	0,–‖
		R_4			
	C_6H_5	NH_2	4·4	3·5	3,3
	2-$CH_3C_6H_4$	NH_2	5·1	6·7	2,1
	3-$CH_3C_6H_4$	NH_2	4·3		1,1
	4-$CH_3C_6H_4$	NH_2	14·8	17·9	2,1
	C_6H_5	$NHCH_3$	13·2		1,1
2,7-Diaminopteridines	4-$C_2H_5C_6H_4$	NH_2	n.i.¶	n.i.¶	0,–‖
		R_2			
	H_2NOC	C_6H_5	n.s.**	n.s.**	3,3
		C_6H_5	n.i.§	n.i.§	1,0
4,7-Diaminopteridines	C_6H_5		n.i.§	n.i.§	0, ‖
	C_6H_5	$(CH_3)_2N$	n.i.§	n.i.§	0,–‖

* From Weinstock *et al.*[21] on a scale of 0–3 of increasing activity. First figure = saline-loaded rat; second figure = sodium-deficient rat. Dose, 30 mg/kg.

† Medium containing THPP at 0·002 μg/ml and thymine at 40 μg/ml.

‡ Medium containing THPP at 0·002 μg/ml and folate at 0·01 μg/ml.

§ No inhibition at 100 μg/ml.

‖ Personal communication from Dr. Weinstock.

¶ No inhibition at 50 μg/ml.

** No inhibition within limit of solubility.

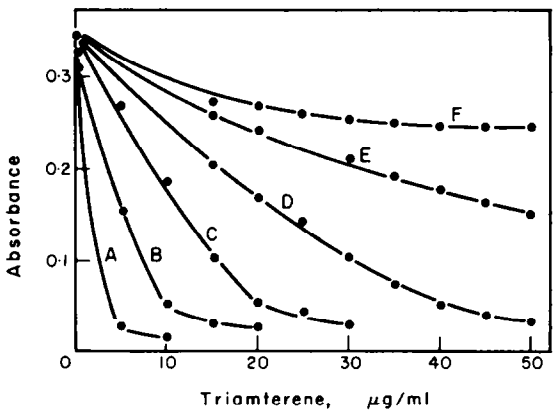


FIG. 1. Response of *C. fasciculata* to triamterene in the presence of varying concentrations of trihydroxypropylpteridine (THPP). Medium minus folate, plus thymine (40 μg/ml). Curve A, THPP 0·5 ng/ml; B, THPP 1·0 ng/ml; C, THPP 2·0 ng/ml; D, THPP 4·0 ng/ml; E, THPP 8·0 ng/ml; F, THPP 16·0 ng/ml.

Also shown in Table 1 are the results of tests of diuretic potency of these compounds (Weinstock *et al.*[21]).

As may be seen from Fig. 1, almost complete reversal of the inhibition produced by triamterene can be brought about by THPP. The inhibition is of the competitive type (Fig. 2). Several of the other analogs, the first four compounds listed in Table 1, were tested and found to be reversed by THPP.

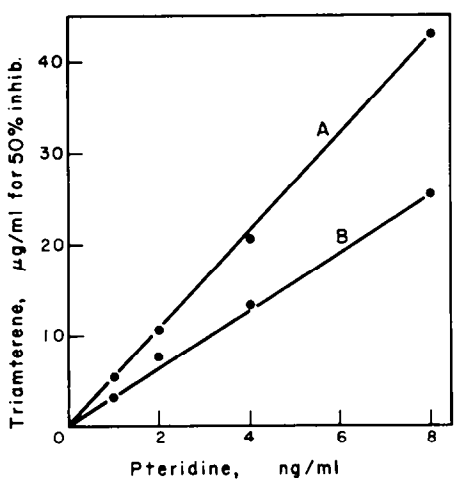


FIG. 2. Comparison of the inhibition of the growth of *C. fasciculata* by triamterene in the presence of increasing concentrations of trihydroxypropylpteridine with the addition of either thymine (40 μg/ml), curve A, or folate (0·01 μg/ml), curve B.

When folate was used in attempts at reversal of inhibition by triamterene, it was found that the relationship of the amount of folate to the amount of inhibitor to give half-maximal inhibition was exponential (Fig. 3).

Of the compounds most active in sparing the pteridine requirement of *C. fasciculata*, three, cytidine, oleate and linoleate, were selected for their effects on inhibition by

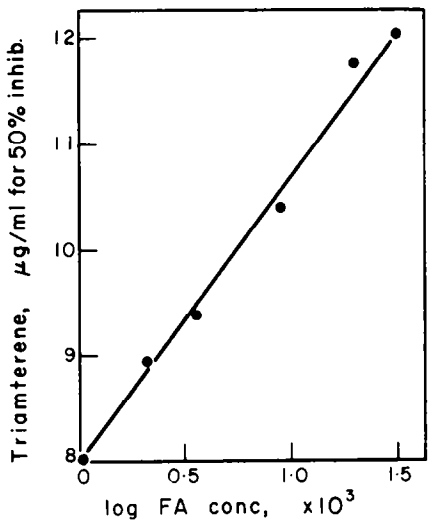


FIG. 3. Effect of increasing folate concentration on inhibition of the growth of *C. fasciculata* by triamterene; trihydroxypropylpteridine concentration 4·0 ng/ml.

triamterene. Cytidine had little effect, while either oleate or linoleate gave almost complete reversal of the noncompetitive type.

The nonpteridine diuretics, hydrochlorothiazide, chlorozanil and acetazolamide, at concentrations of 100 μg/ml were not inhibitory to the growth of Crithidia. Nor was *T. pyriformis* inhibited by triamterene at 50 μg/ml. The ciliate, like the vertebrate,[1,22] is capable of biosynthesis of unconjugated pteridines from a derivative of guanine.[23]

DISCUSSION

Triamterene has been stated to be a folate antagonist using growth of *Streptococcus faecalis* as the test organism.[24] Since the published curves do not show that an increased concentration of the drug is necessary for a given degree of inhibition as the folate supply was increased four-fold, this statement is unconvincing. However, experiments with dihydrofolate reductase[8,13] have shown that both activities are sensitive to inhibition by triamterene, although the inhibitor of dihydrofolate reductase is 100-fold less sensitive to triamterene than it is to methotrexate.

Interference with dihydrofolate reductase may account for the fact that triamterene and some of the other diuretics are somewhat more inhibitory to Crithidia in the folate–THPP medium than in the thymine–THPP medium (Fig. 2). Inasmuch as Crithidia can be maintained indefinitely[19] in media devoid of folate when thymine is added, inhibition of dihydrofolate would not account for the effects of triamterene. In the presence of thymine, there should be noncompetitive reversal if dihydrofolate reductase were the only site of inhibition. The data of Dewey *et al.*[25] for the inhibition of the growth of *T. pyriformis* by amethopterin (methotrexate) show a logarithmic relationship of folate concentration to the amount of inhibitor required for 50 per cent inhibition. A similar relationship is shown by the pteridine inhibitor, 2,4-diamino-5,6,7,8-tetrahydroquinazoline, to the pteridine concentration required for reversal of the inhibition of growth of Crithidia.[26]

The only other compounds which have been reported to act as antagonists of biopterin in the growth of Crithidia are a series of 4-alkoxy- and 4-aryloxy-2,6-diaminopyridines.[27,28] No information regarding the pharmacological activity of these compounds* or of the quinazoline has been reported.

The fact that two sequential enzymatic reactions, folate reduction[8,12] and dihydrofolate reduction,[9–11] are both inhibited may account for the relative inefficacy of folate in reversing growth inhibition. In an organism such as *T. pyriformis* which is independent of exogenous unconjugated pteridine, the effects of the drug on folate metabolism appear to be negligible, since there is no inhibition at high concentrations of triamterene. However, the dihydrofolate reductase of the ciliate must be unusual in that growth is not inhibited by aminopterin, although it is by methotrexate.[25,30]

That the dihydrofolate reductases of trypanosomids may differ in their properties from those previously studied may be indicated by their high molecular weight.[31]

Crithidia differs from all but its close relatives in its requirement for simple pteridines. This requirement implies a capacity for the reduction of pteridines at the oxidation level of folate to the tetrahydro form which is active enzymatically.[2] Such an enzyme might be termed a "pteridine reductase." In bacteria capable of folate synthesis,[23] the first pteridine formed is D-*erythro*-7,8-dihydroneopterin, which is not further reduced until after conversion to dihydrofolate in several enzymatic steps.[32]

Evidence for a dihydropteridine reductase as opposed to dihydrofolate reductase was obtained by Kaufman[33] in his studies of phenylalanine hydroxylation. The substrate for this enzyme is an unstable quinonoid dihydrobiopterin[34] which is converted to 7,8-dihydrobiopterin in the presence of phosphate ions.[33] This compound is a substrate for dihydrofolate reductase, but not for dihydropteridine reductase.[33] Although sensitivity of phenylalanine hydroxylation to aminopterin could be demonstrated when crude preparations of the sheep liver enzymes were used[35] as a source of enzymes to reduce the dihydropteridine produced in the hydroxylation reaction, its effect was probably on the dihydrofolate reductase necessitated by the use of phosphate buffer in the system studied.[35] Later work by Musacchio[36] has shown that the dihydropteridine reductase from adrenal gland is insensitive to aminopterin and methotrexate at 0·1 M.

In view of these facts, it appears unlikely that dihydropteridine reductase is inhibited by triamterene, and an additional site of action must exist in Crithidia to account for inhibition in the presence of thymine. Inhibition of the growth of Crithidia by aminopterin is completely reversed by thymine (unpublished results). The postulated "pteridine reductase" could be the enzyme inhibited by triamterene.

While there is not good correspondence in the activity of the various compounds as diuretics and as inhibitors of the growth of Crithidia, all which are good diuretics also inhibit growth (excepting one which was relatively insoluble) and those which were not diuretic were uninhibitory. Further evidence is needed before it can be concluded that diuretic activity can be attributed to antagonism of the cofactor action of pteridines.

* Since submission of this paper for publication, it has been brought to our attention that a patent, 3,329,569, issued to R. E. Tedeschi and J. Weinstock on July 4, 1967 ascribes hypotensive activity to 2,6-diamino-4-substituted-pyridines. Presumably their activity is similar to that of a naturally occurring hypotensive agent which acts as a competitive inhibitor of tetrahydropteridine in the tyrosine hydroxylase reaction.[29]

REFERENCES

1. E. L. Patterson, H. P. Broquist, A. M. Albrecht, M. H. von Salza and E. L. R. Stokstad, *J. Am. chem. Soc.* **77,** 3167 (1955).
2. S. Kaufman, *Proc. natn Acad. Sci. U.S.A.* **50,** 1085 (1963).
3. T. Nagatsu, M. Levitt and S. Udenfriend, *Fedn Proc.* **23,** 480 (1964).
4. F. D. Marshall, Jr., S. K. Chatterjee and E. M. Gal, *Fedn Proc.* **23,** 529 (1964).
5. D. D. Hagerman, *Fedn Proc.* **23,** 480 (1964).
6. G. W. Kidder and V. C. Dewey, *Biochem. biophys. Res. Commun.* **12,** 280 (1963).
7. V. C. Dewey and G. W. Kidder, *Fedn Proc.* **23,** 376 (1964).
8. V. M. Doctor, *J. biol. Chem.* **232,** 617 (1958).
9. J. R. Bertino, J. P. Perkins and D. G. Johns, *Biochemistry, N.Y.* **4,** 839 (1965).
10. J. R. Bertino, A. T. Iannotti, J. P. Perkins and D. G. Johns, *Biochem. Pharmac.* **15,** 563 (1966).
11. J. P. Perkins and J. R. Bertino, *Biochemistry, N.Y.* **5,** 1005 (1966).
12. A. R. Maass, V. D. Wiebelhaus, G. Sosnowski, B. Jenkins and G. Gessner, *Toxic appl. Pharmac.* **10,** 413 (1967).
13. J. J. Jaffe and J. J. McCormack, Jr., *Molec. Pharmac.* **3,** 359 (1967).
14. S. Futterman, *J. biol. Chem.* **228,** 1031 (1957).
15. W. E. Gutteridge, J. J. McCormack and J. J. Jaffe, *Biochim. biophys. Acta* **178,** 453 (1969).
16. J. J. Jaffe, J. J. McCormack and W. E. Gutteridge, *Expl. Parasit.* **25,** 311 (1969).
17. G. W. Kidder and V. C. Dewey, *J. biol. Chem.* **243,** 826 (1968).
18. G. W. Kidder and B. N. Dutta, *J. gen. Microbiol.* **18,** 621 (1958).
19. V. C. Dewey and G. W. Kidder, in *Methods in Enzymology* (Eds. D. B. McCormick and L. D. Wright), Vol. 18 B, p. 618. Academic Press, New York (1971).
20. G. W. Kidder, V. C. Dewey and H. Rembold, *Arch. Mikrobiol.* **59,** 180 (1967).
21. J. Weinstock, J. W. Wilson, V. D. Wiebelhaus, A. R. Maass, F. T. Brennan and G. Sosnowski, *J. med. chem.* **11,** 573 (1968).
22. C. C. Levy, *J. biol. Chem.* **239,** 560 (1964).
23. G. M. Brown, *Adv. Enzymol.* **35,** 35 (1971).
24. M. E. Rosenthale and C. G. Van Arman, *J. Pharmac. exp. Ther.* **142,** 111 (1963).
25. V. C. Dewey, G. W. Kidder and R. E. Parks, Jr., *Proc. Soc. exp. Biol. Med.* **78,** 91 (1951).
26. V. C. Dewey and G. W. Kidder, *Biochem. biophys. Res. Commun.* **34,** 495 (1969).
27. D. G. Markees, V. C. Dewey and G. W. Kidder, *Archs Biochem. Biophys.* **86,** 179 (1960).
28. D. G. Markees, V. C. Dewey and G. W. Kidder, *J. med. chem.* **11,** 126 (1968).
29. H. Umezawa, T. Takeuchi, H. Iinuma, K. Suzuki, M. Ito, M. Matsuzaki, T. Nagatsu and O. Tanabe, *J. Antibiot.* **23,** 514 (1970).
30. V. C. Dewey and G. W. Kidder, *Proc. Soc. exp. Biol. Med.* **83,** 586 (1953).
31. W. E. Gutteridge, J. J. Jaffe and J. J. McCormack, Jr., *Biochim. biophys. Acta* **191,** 753 (1969).
32. T. Shiota, C. M. Baugh and R. Dillard, *Biochemistry, N.Y.* **8,** 5022 (1969).
33. S. Kaufman, in *Pteridine Chemistry* (Eds. W. Pfleiderer and E. C. Taylor), p. 307. Macmillan, New York (1964).
34. S. Kaufman, *J. biol. Chem.* **239,** 332 (1964).
35. S. Kaufman and B. Levenberg, *J. biol. Chem.* **234,** 2683 (1959).
36. J. M. Musacchio, *Biochim. biophys. Acta* **191,** 485 (1969).