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THE ACTIVITIES OF SOME 2 : 4-DIAMINOPTERIDINES AND SULPHATHIAZOLE AGAINST *STREPTOCOCCUS FAECALIS* AND *STAPHYLOCOCCUS AUREUS*

BY

H. O. J. COLLIER AND PAMELA D. WATERHOUSE

From the Research Division, Allen and Hanburys Ltd., Ware, Herts

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The relationship between the biological activities of the nutrient 2-amino-4-hydroxypteridines, such as pteroylglutamic acid (PGA), and the 2:4-diaminopteridines possessing substituents in both the 6 and 7 positions has been investigated in *Streptococcus faecalis* and other organisms. Using a strain of *Str. faecalis* that required preformed PGA, Daniel, Norris, Scott, and Heuser (1947) found that some diaminopteridines actively inhibited growth, and that this inhibition was overcome by PGA. Using a similar strain of organism, we (Collier and Waterhouse, 1950b) have obtained similar results with a number of diaminopteridines, recently synthesized by Campbell, Dunsmuir, and Fitzgerald (1950, 1952). One of the objects of the present paper is to report the activities of further diaminopteridines against this strain of *Str. faecalis* and against three pathogenic strains of this species recently isolated from cases of bacterial endocarditis.

Since a number of the diaminopteridines examined showed high activities against the pathogenic strains, we thought it desirable to carry out therapeutic tests in infected animals. None of the strains of *Str. faecalis* used, however, proved to be pathogenic to mice. As Daniel and Norris (1947) had shown that diaminopteridines inhibited the growth *in vitro* of *Staphylococcus aureus* and as a strain of this organism causing a fatal bacteriaemia in mice was made available to us, we decided to carry out therapeutic tests in mice infected with *Staph. aureus*. We accordingly first examined *in vitro* the activity against *Staph. aureus* of the compounds more active against *Str. faecalis*. Although the two bacterial species showed considerable differences in reactions to the compounds, several pteridines exhibited comparable activities in both species. We chose one of these, 2 : 4-diamino-6 : 7-dibenzylpteridine (0/137 ; hereafter called dibenzylpteridine), for exploration of the therapeutic possibilities of compounds of this type in infections of mice with *Staph. aureus* ; these experiments are reported below.

Daniel and Norris (1947) showed that certain diaminopteridines act synergistically with sulphathiazole in inhibiting the growth *in vitro* of *Staph. aureus* and other organisms that do not require preformed PGA. We have therefore examined in a number of experiments described below whether dibenzylpteridine also acts synergistically with sulphathiazole in protecting mice against infections of *Staph. aureus* and in inhibiting the growth of this organism and of *Str. faecalis in vitro*.

Materials and Methods

Compounds.—The chemical names and serial numbers of the diaminopteridines employed are given in Table I. These compounds are for the most part the same as those previously screened against *Vibrio cholerae* (Collier and Waterhouse, 1950a). For work *in vitro* the free bases were used. The base, phosphate, and hydrochloride of dibenzylpteridine (0/137), the phosphate of the di-*sec*-butyl compound (0/142), and the bases of compounds 0/87, 0/104, and 0/128 were used for experiments in mice. For comparative purposes 4-aminopteroylglutamic acid, potassium penicillin G (1,575 units per mg.), chloramphenicol, and sulphathiazole were employed. In order to study antagonism of the antibacterial pteridines we used synthetic citrovorum factor (" Leucovorin ").

Strains.—In addition to the strain of *Str. faecalis* (M75) previously used by us (Collier and Waterhouse, 1950b), we employed three pathogenic strains, named Blandford, Kenwood, and Moat, which had recently been isolated by Cates, Christie, and Garrod (1951) from the blood in clinical cases of bacterial endocarditis. The pathogenic strains of *Str. faecalis* were maintained in blood-agar. For experiments with *Staph. aureus* we used the mouse pathogenic strain CN491. For experiments *in vitro* this was maintained on blood-agar. For work *in vivo* it was maintained by once-weekly passage through mice. *Lactobacillus casei* (ACTC 7469) was used for comparison with *Str. faecalis.*

Experiments in vitro.—All experiments with *Str. faecalis* and *L. casei* were carried out in the modified medium of Barton-Wright, Emery, and Robinson (1945) previously used by us (Collier and Waterhouse, 1950b); to this 2 mμg. PGA per ml. medium was added. For examination of antagonism by body fluids we also added to this medium 5 per cent (v/v) human urine before or 5 per cent (v/v) oxalated horse blood after autoclaving. The procedure for inoculation was that previously described, except that pathogenic strains were grown for 48 hr. before preparing the inoculum. The inoculum was adjusted by opacity tubes to give approximately 10^6 organisms per ml. test medium.

Experiments with *Staph. aureus* were carried out in a slightly modified form of the medium of Daniel and Norris (1947). The inoculum was grown for 22 hr. in the medium described by the latter authors. The inoculum was adjusted to give approximately 10^4 organisms per ml. test medium.

Drugs were diluted serially as solutions or fine suspensions by twofold steps. Pteridines and sulphathiazole were autoclaved at 10 lb. for 10 min. in tubes of test medium after dilution. Antibiotics were diluted aseptically in previously autoclaved tubes of medium.

After incubation at 37° C. the extent of bacterial growth was read visually and in experiments with *Str. faecalis* (M75) also by nephelometer (Eel). Where blood was present in the medium, *p*H readings were made after incubation for 40 hr. in order to obtain evidence of bacterial growth. The end-point was assessed on a serial dilution scale as the least concentration in μg. per ml. required to inhibit growth for a specified time. The results were expressed as the geometric mean of the determinations.

Experiments in mice.—White mice weighing 18–26 g. were used in toxicity tests. In therapeutic tests, smaller mice (14–20 g.) were inoculated with approximately 10^7 organisms, suspended in 5 per cent mucin (Wilson's—type 1701-W). A washed standardized inoculum was prepared from an overnight culture in blood-broth. Drugs were administered in a single dose either by mouth or intraperitoneally between a quarter and one hour, according to the experiment, after infective inoculation. Both in toxicity and in therapeutic tests drugs were given in 10 per cent gum acacia in water.

RESULTS

Experiments with Str. faecalis

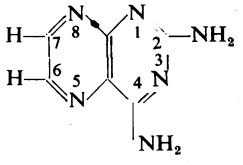
The results of screening tests of pteridine bases against *Str. faecalis* (M75) are included in Table I, which summarizes the responses of all four strains of this species

TABLE I

INHIBITION OF FOUR STRAINS OF *Str. faecalis* AND OF *Staph. aureus* BY 2 : 4-DIAMINOPTERIDINES

For *Str. faecalis* readings taken after 16–18 hours' incubation in medium containing 0.002 μg. PGA/ml.; for *Staph. aureus* after 24 hours' incubation.

Formula of 2 : 4-diaminopteridine



Serial No.	6 : 7-substituents in 2 : 4-diaminopteridine	Min. inhibitory concn. in μg. per ml.				
		Str. faecalis				*Staph. aureus*
		M75	Moat	Kenwood	Blandford	
0/154	None	>1.0				>100
0/58	dimethyl	>1.0				50
0/69	diethyl	0.075		1.0		25
0/71	di-*n*-propyl	0.0126		0.316		4.47
0/129	di*iso*propyl	0.0126	0.011	0.355	0.251	>50
0/128	di-*n*-butyl	0.0063	0.004	0.5	0.0316	0.20
0/113	di*iso*butyl	0.010		0.631		17.8
0/142	di-*sec*-butyl	0.0063	0.004	0.11	0.178	50
0/87	di-*n*-amyl	0.0063		0.355		0.20
0/150	di-*sec*-amyl	0.0063		0.255		4.47
0/138	(α-ethyl-*n*-propyl)-	0.125				17.8
0/103	di-*n*-hexyl	0.050		0.250		0.50
0/88	di-*n*-heptyl	0.158				>100
0/83	di*cyclo*hexyl	0.398				>100
0/104	di (*cyclo*hexylmethyl)-	0.031		1.0		0.215
0/137	dibenzyl	0.0126	0.003	0.166	0.266	0.210
0/97	camphano-(2′ : 3′–6 : 7 or 7 : 6)-	0.178				50
0/67	di (l-furyl)-	0.158				
0/171	indolo-(2′ : 3′-7 : 6)-	0.355				
0/120/I	1′-methylindolo-(2′ : 3′-7 : 6)-	0.355				
0/120/II	1′-methylindolo-(2′ : 3′-6 : 7)-	0.0133				
0/169	1′-ethylindolo-(2′ : 3′-7 : 6)-	0.20				
0/170	1′ *n*-propylindolo (2′ : 3′-7 : 6)-	0.079				
0/174	1′-benzylindolo-(2′ : 3′-7 : 6)-	0.045				
0/61	di-(*p*-methoxyphenyl)-	0.075				
0/114	di-(*o*-methoxyphenyl)-	0.50				
0/63	diphenyl	0.083		8.0		>100
4-aminopteroylglutamic acid		0.021	0.50	4.0	8.0	
Chloramphenicol		12.5	2.14	5.0	4.47	2.5†
Sulphathiazole						0.63†
Penicillin						0.039
Equal parts sulphathiazole and dibenzylpteridine (0/137)						0.050

to the drugs examined. Tests with strain M75 were carried out in duplicate. Agreement between duplicates was excellent and that between tests was fairly good. For example, the mean logarithm of 25 end-points obtained with aminopterin, which was generally put up for reference, was 1.32 mμg. per ml., and its standard error was ±0.098.

It will be seen from Table I that while all strains of *Str. faecalis* are highly sensitive to certain diaminopteridines, the Kenwood and Blandford strains are considerably less sensitive than the other two. Experiments showed that these two strains were able to grow without addition of PGA to the medium, while, on the contrary, strains M75 and Moat required PGA or its equivalent for growth.

This difference between the strains was also reflected in their reaction to a mixture of dibenzylpteridine and sulphathiazole. Table II gives an example of

TABLE II

EFFECT OF SULPHATHIAZOLE ON THE ACTIVITY OF DIBENZYLPTERIDINE (0/137) AGAINST FOUR STRAINS OF *Str. faecalis*

Medium contained 0.002 μg. added PGA per ml. Readings taken after incubation for 40 hours.

Strain	Requirement for PGA	Drug	Min. inhib. concn. in μg./ml.
M75	Required	Sulphathiazole Dibenzylpteridine S'thi: + dibenzylpteridine	>500 0.0078 3.9 S'thi: + 0.00195 pteridine
Moat	Required	Sulphathiazole Dibenzylpteridine S'thi: + dibenzylpteridine	>500 0.0156 15.6 S'thi: +0.0078 pteridine
Kenwood	Not required	Sulphathiazole Dibenzylpteridine S'thi: + dibenzylpteridine	>500 0.25 3.9 S'thi: + 0.00195 pteridine
Blandford	Not required	Sulphathiazole Dibenzylpteridine S'thi: + dibenzylpteridine	>500 0.25 7.8 S'thi: + 0.0039 pteridine

experiments in which the inhibitory activities of sulphathiazole and of the pteridine were compared with that of a mixture containing 2,000 parts of sulphathiazole to 1 part of pteridine. In the presence of sulphathiazole, the activity of the pteridine against the two strains that do not need PGA for growth is considerably increased. Although sulphathiazole alone is inactive at 500 μg. per ml., it appears to convert the strains not requiring PGA to the same degree of sensitiveness to diaminopteridines as those requiring this nutrient.

It will be seen in Table I that some of the new pteridines studied are more effective than aminopterin in inhibiting the growth of *Str. faecalis* in a medium containing a defined concentration of PGA. It is of interest to report that in similar conditions these pteridines are very much less effective than aminopterin in inhibiting the growth of *L. casei*. In a medium containing 0.1 mμg. PGA per ml., one part by weight of aminopterin proved as effective as about 750 parts of pteridine 0/120/II, 3,000 of 0/137, 10,000 of 0/129, and 70,000 of 0/128.

As we were interested in the possible therapeutic value of diaminopteridines in human infections with *Str. faecalis,* we examined whether these substances were antagonized by blood or urine. In our experiments 5 per cent human urine did not antagonize the dibenzyl- or the di-*sec*-butyl-pteridines. Oxalated horse blood possibly exerted a slight antagonism.

We have previously reported (1950b) that pteridines of the type under investigation are antagonized by PGA. We find, using *Str. faecalis* (M75), that synthetic citrovorum factor antagonizes pteridines 0/69, 0/120/II, and 0/129 in a similar manner to PGA, but with considerably greater effectiveness.

Experiments with Staph. aureus in vitro

Table I summarizes the results of experiments with *Staph. aureus in vitro.* In these experiments the diaminopteridines more active against *Str. faecalis* were examined; sulphathiazole was used for reference in most tests. There was good agreement between tests. For example, the mean logarithm of seven end-points for sulphathiazole was 2.8 mμg. per ml. and its standard error ± 0.113.

It will be seen (Table I) that the most active diaminopteridines were rather more effective than sulphathiazole, though less effective than penicillin, in suppressing growth of this strain of *Staph. aureus* on the medium used. It will also be seen that a mixture of equal parts of sulphathiazole and dibenzylpteridine was more effective than either substance alone. Analysis showed that this difference was significant.

Experiments on toxicity

The four pteridine bases most active against *Staph. aureus,* 0/87, 0/104, 0/128, and 0/137, were administered in suspension in gum acacia intraperitoneally to mice at 4, 2, and 1 g. per kg. This preliminary experiment, involving 55 mice, showed that the order of toxicities was as follows: di-*n*-butyl (0/128)>di-*n*-amyl (0/87) >di(*cyclo*hexylmethyl) (0/104)>dibenzyl (0/137). Whereas a dose of 2 g. 0/128 per kg. killed all of five mice treated, a dose of 4 g. 0/137 per kg. killed no animals. Since dibenzylpteridine was the least toxic of the pteridines administered to mice and among the most active of those tested against *Staph. aureus in vitro,* and since it showed comparable activity against *Str. faecalis,* it was chosen for exploration of the therapeutic possibilities of compounds of this type in mice infected with *Staph. aureus.*

Toxicity tests were somewhat limited by the quantities of pteridine salts available. The results of experiments with dibenzylpteridine base and with the phosphate and hydrochloride, with sulphathiazole, and with a mixture of the latter two drugs may be summarized by the statement that the toxicity of the pteridine salts appears to be of the same order as that of sulphathiazole. The toxicity of the mixure of the two drugs may be a little higher, but if so the difference is slight.

Eight or more days after administration of the drugs, some surviving mice were sacrificed and examined *post mortem.* In animals receiving the pteridine, deposits of drug were found in the connective tissue of the peritoneal cavity and this tissue had evidently increased in bulk. All animals surviving the drug and not sacrificed for examination *post mortem* remained healthy for at least 28 days after administration of the pteridine.

Doses of 1.0 and 2.0 g. of the dibenzylpteridine phosphate were administered to mice by mouth without any apparent toxic effect. The di-*sec*-butylpteridine phosphate administered intraperitoneally to mice for comparison with the dibenzyl compound killed all mice at a dose of 100 mg. per kg. and none at 50 mg. per kg.

Protective experiments in mice

In an initial series of experiments, involving 170 mice, the activities of the dibenzylpteridine, sulphathiazole, and chloramphenicol were compared when each drug was administered by mouth. The results of these tests, summarized in Table III, show that single doses of dibenzylpteridine phosphate exhibit definite protective

TABLE III

THERAPEUTIC ACTIVITY IN MICE INFECTED WITH *Staph. aureus* OF DIBENZYLPTERIDINE, SULPHATHIAZOLE, AND CHLORAMPHENICOL

Summary of tests where each drug was given by mouth in one dose between a quarter and one hour after infective inoculation.

Drug	Oral dose in mg./kg.	Survivors to total No. of mice at			
		2 days	3 days	5 days	6 days
Sulphathiazole	500	18/20	14/20	4/10	1/10
	250	9/10	8/10	6/10	3/10
	125	5/5	4/5	0/5	0/5
	62.5	8/10	7/10	2/10	1/10
Dibenzylpteridine base	500	3/10	2/10	1/10	0/10
Dibenzylpteridine phosphate ..	1,000	14/15	13/15	11/15	8/15
	500	15/25	13/25	6/25	5/25
	125	0/5	0/5	0/5	0/5
Chloramphenicol	500	5/5	5/5	5/5	5/5
	125	14/15	14/15	13/15	11/15
All untreated controls		8/60	5/60	4/60	2/60

activity. When given by mouth, a dose of 1 g. of this compound per kg. exerts a more prolonged effect than 0.5 g. sulphathiazole, but less effect than 0.125 g. chloramphenicol.

Since we thought it possible that the pteridine was not readily absorbed from the alimentary canal, we compared the activities of the phosphate of dibenzylpteridine with those of sulphathiazole and penicillin G, when each drug was administered by the intraperitoneal route. This experiment, illustrated in Table IV, shows that, given by this route, the pteridine prolongs life more effectively than the sulphonamide, though less than the antibiotic.

For reasons already stated, we also examined the therapeutic activity of mixtures of sulphathiazole and dibenzylpteridine phosphate. Synergism between the two drugs is shown in Table IV. It was confirmed in other experiments. In one of these both drugs were administered by mouth half an hour after intraperitoneal

TABLE IV

THERAPEUTIC ACTIVITY IN MICE INFECTED WITH *Staph. aureus* OF DIBENZYLPTERIDINE, SULPHATHIAZOLE, AND PENICILLIN G

One intraperitoneal dose of drug given half an hour after infective inoculation.

Drug	Dose in mg. per kg.	No. survivors out of 10 animals at			
		2 days	3 days	5 days	7 days
Sulphathiazole	125	6	4	1	1
	31.25	3	1	0	0
Dibenzylpteridine phosphate ..	250	10	8	3	1
	62.5	10	10	5	1
Mixture equal parts sulphathiazole and pteridine	31.25	10	10	9	5
	7.8	10	10	6	3
Penicillin G	15.6	10	10	9	7
	3.9	10	8	7	4
	0.975	9	9	6	5
Untreated controls		1	0	0	0

inoculation with *Staph. aureus*. In another experiment, illustrated in Table V, sulphathiazole was given by mouth and the pteridine intraperitoneally. In this experiment, judged by survivals at seven days, a combination of the two drugs appeared to exert more protective effect than 16 times the dose of either drug alone.

DISCUSSION AND CONCLUSIONS

In experiments described above, both *Str. faecalis* and *Staph. aureus* show themselves to be sensitive to the structure of substituents in the 6 and 7 positions of the pteridine molecule. Their reactions show interesting similarities and differences. Both species are readily inhibited by the dibenzyl and di*cyclo*hexylmethyl compounds

TABLE V

SYNERGISM *in vivo* BETWEEN SULPHATHIAZOLE AND DIBENZYLPTERIDINE

Drugs administered half an hour after inoculation with *Staph. aureus*.

Dose of drug in mg./kg.		No. survivors out of 10 animals at			
Sulphathiazole orally	Dibenzylpteridine phosphate I.P.	2 days	3 days	5 days	7 days
250	0	9	8	6	1
62.5	0	8	7	2	0
0	250	10	9	3	0
0	62.5	10	6	3	0
31.25	31.25	10	10	6	2
7.8	7.8	10	9	8	6
	Untreated controls	2	0	0	0

and by those possessing straight alkyl chains of three to six carbon atoms. *Staph. aureus*, however, is much less readily inhibited by the corresponding branched chain dialkyl compounds, although *Str. faecalis* is as sensitive to these as to the straight-chain pteridines.

Three of the compounds (0/154, 0/58, and 0/63) tested by us were among those examined by Daniel *et al.* (1947). Two of them (0/58 and 0/63) were also studied by Swendseid *et al.* (1949), using *Str. faecalis*. In agreement with these authors we found 0/154 and 0/58 relatively less active than 0/63. We did not find 0/63, however, as active as might have been expected from previously published results. A sample of this pteridine, kindly supplied by Dr. Cain, gave similar results in our hands to that prepared by Campbell, Dunsmuir, and Fitzgerald. When compared directly against *Str. faecalis*, 0/63 appears to possess about the same activity as 0/69, although we previously thought 0/63 to be the more active, on the basis of an indirect comparison (see Collier and Waterhouse, 1950b).

In some of the experiments performed, we found that strains of *Str. faecalis* requiring preformed PGA, or its equivalent, were more readily inhibited by diaminopteridines than those not requiring PGA. The addition of sulphathiazole to the medium greatly increases the sensitiveness to diaminopteridines of the strains capable of synthesizing PGA. This observation agrees with that of Daniel and Norris (1947). The effect of sulphathiazole might be attributed to its blocking 4-hydroxypteridine synthesis, which has been described by Lampen and Jones (1947) and by Nimmo-Smith, Lascelles, and Woods (1948) in various organisms.

Experiments showed that a quarter of an hour after intraperitoneal inoculation of suspensions of *Staph. aureus* into mice, bacteria could be recovered from the heart blood. We can therefore regard those experiments described above, in which drugs were administered intraperitoneally to mice half an hour after infective inoculation, as tests of therapeutic activity. In these experiments the therapeutic index of the dibenzyl compound, calculated as the ratio of the maximum tolerated dose to the minimum dose effective in prolonging life, exceeds 20, while the index of the pteridine-sulphonamide mixture exceeds 60. From the therapeutic point of view the marked synergism between this pteridine and sulphathiazole *in vivo* may be important. Since sulphonamides are established chemotherapeutic remedies, it seems practical to regard the antibacterial pteridines as potentiators of sulphonamides. In this role the pteridines may be able to bring within the scope of sulphonamide therapy infections that have responded poorly or not at all to sulphonamides alone.

From the clinical point of view, the above results suggest the possibility of using appropriate 2 : 4-diaminopteridines for the treatment of infections of man with bacteria such as *Str. faecalis* (Moat) that are not able to synthesize PGA. They also suggest that a mixture of pteridine and sulphonamide might be valuable for treating infections with bacteria that can synthesize PGA and that normally do not respond to sulphonamides, such as *Str. faecalis* (Blandford and Kenwood) and *Staph. aureus*.

SUMMARY

1. The activities *in vitro* of twenty-seven 2 : 4-diaminopteridines against four strains of *Str. faecalis* are reported. Activity depends on substituents in the 6 and 7 positions. Greatest activity is shown in the dialkyl compounds with straight or

branched chains containing three to six carbon atoms. Equivalent activity is exhibited by the dibenzyl compound and by one N-methylindolo compound.

2. All the compounds studied showed higher activity against strains requiring preformed pteroylglutamic acid than against strains able to synthesize this nutrient.

3. Sulphathiazole potentiates the inhibitory effect of 2 : 4-diamino-6 : 7-dibenzylpteridine upon strains of *Str. faecalis* not requiring pteroylglutamic acid.

4. Five per cent (v/v) human urine does not appear to antagonize the inhibitory effect of dibenzylpteridine on *Str. faecalis* ; 5 per cent oxalated horse blood may exert slight antagonism.

5. The activities *in vitro* of eighteen 2 : 4-diaminopteridines against a strain of *Staph. aureus* are reported. Activity is greatest in the dibenzyl and di*cyclo*hexylmethyl compounds and in the 6 : 7-dialkyl compounds when the side-chains are unbranched. In the di-*n*-alkyl series peak activity occurs in the di-*n*-butyl and di-*n*-amyl compounds.

6. Dibenzylpteridine salts, administered intraperitoneally to mice, exhibit toxicity similar to that of sulphathiazole. All mice tolerated 2.0 g. of either drug per kg.

7. Single doses of dibenzylpteridine phosphate, given by mouth or intraperitoneally after infective inoculation, prolonged the lives of mice infected with *Staph. aureus.*

8. Dibenzylpteridine phosphate acts synergistically with sulphathiazole both *in vitro* and in protecting mice against infections of *Staph. aureus.*

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