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THE MODE OF ACTION OF SULPHONAMIDES, PROGUANIL AND PYRIMETHAMINE ON *PLASMODIUM GALLINACEUM*

BY

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Despite the mass of information on the antimalarial action of sulphonamides, proguanil, and pyrimethamine (see Goodwin and Rollo, 1955), there is as yet no complete picture of the relationship between them, although they all probably act upon the same metabolic pathway in the synthesis of nucleoprotein. Hawking (1953a) in his review of protozoal chemotherapy pointed out: "Many different lines of work appear to be converging here towards a general explanation, but it will be necessary to achieve further elucidation of the metabolism of *p*-aminobenzoic acid (PAB) and of folic acid by the malarial parasite before all the different facts in the jigsaw puzzle can be fitted into place." In this paper an attempt is made to fill some of the gaps in our knowledge of cross-resistance, potentiation, and antagonism between antimalarial drugs, by reviewing and analysing the known facts, in the light of new data.

METHODS

The parent strain of *Plasmodium gallinaceum* was that maintained in these laboratories for many years by blood and occasional mosquito passage in young chicks. This and the other drug-treated strains have, during the course of the experiments, been passaged solely by blood inoculation. Five- or twelve-day-old chicks (Rhode Island Red–Light Sussex cross) were inoculated intravenously with approximately 50 million parasitized red blood cells. The antimalarial drugs were given orally either in solution or, if insoluble in water, in gum tragacanth suspension. Starting a few hours after inoculation, a total of seven doses was given over 3½ days. Infection was assessed from stained blood films on the fourth day after inoculation, when in untreated controls about 70–90% of the red blood cells were infected. The infected red cells in the test animals were counted and the results were expressed as percentages of the controls. That dose which reduces parasitaemia to 50% of the mean parasitaemia of untreated controls (ED50) was obtained from a 3- or 4-dose assay (Rollo, 1952). A group of five chicks was normally used at each dose level.

Cross-resistance.—A strain of *P. gallinaceum* (P.36), which was highly resistant to proguanil and to pyrimethamine, was obtained from Dr. D. G. Davey, of Imperial Chemical Industries. The sensitivity of this strain to sulphadiazine was tested in order to complete the picture of cross-resistance relationships reviewed by Thurston (1953). Two further strains were prepared by treating successive passages with subcurative doses of proguanil and pyrimethamine respectively. During each passage the chicks received a total of seven doses of the drug as described above. Both strains were passaged and treated in parallel and were tested periodically for cross-resistance during the early stages of the development of drug resistance.

Potentiation.—The potentiating effect of pyrimethamine upon the activity of proguanil was investigated by giving the drugs both singly, and together in various proportions, to groups of infected chicks. ED50's were determined from the dose-response curves and were plotted on a graph to demonstrate the effect of one drug upon the action of the other. Similar experiments were done using pyrimethamine with sulphadiazine, sulphaguanidine, succinylsulphathiazole, penicillin, or streptomycin. In some experiments the blood levels of the sulphonamides produced by single oral doses were determined by the method of Bratton and Marshall (1939).

Inhibition.—The effects of PAB and folic acid on the activity of sulphadiazine and pyrimethamine were determined. The PAB or folic acid was given intraperitoneally in aqueous solution or suspension 30 min. before each oral dose of the antimalarial drug except in one experiment with pyrimethamine in which PAB was given orally five times a day at three-hourly intervals; in addition, the chicks were fed on a diet containing 0.1% PAB. To ensure that the diet would be consumed at night when the chicks were not being dosed, the cages were darkened throughout the day and brightly lit during the night.

The inhibitory action of amino-an-fol (2, 4-diaminopteroylaspartic acid) was investigated in one experiment.

RESULTS

Cross-resistance.—The effect of sulphadiazine upon strain P.63 is shown in Table I. There was no evidence of resistance to sulphadiazine although this strain was highly resistant both to proguanil

and to pyrimethamine. Indeed, there is some evidence of hypersensitivity to sulphadiazine.

The pattern of development of resistance in the two other strains which had been treated in parallel with proguanil or pyrimethamine is shown in Table II.

TABLE I

THE ACTION OF SULPHADIAZINE ON NORMAL AND PROGUANIL-PYRIMETHAMINE RESISTANT STRAINS OF *P. GALLINACEUM*

Strain	Drug	Dose (mg./kg.)	Parasitaemia* (%)
Normal ..	Proguanil	2·0	12
	Pyrimethamine	0·06	<1
		0·03	78
		0·015	98
	Sulphadiazine	60	4·5
		30	42
		15	72
Proguanil-pyrimethamine resistant (P.63)	Proguanil	50	92
	Pyrimethamine	6·0	103
	Sulphadiazine	30	<1

* Parasitized cells in the treated birds shown as a percentage of the parasitized cells in the untreated controls. Hence the lower the percentage the more effective is the particular dose.

TABLE II

CROSS-RESISTANCE TESTS BETWEEN PROGUANIL- AND PYRIMETHAMINE-TREATED STRAINS OF *P. GALLINACEUM*

Strain	Drug	Dose (mg./kg.)	% Parasitaemia* Passage No. 1	3	6	7	9
Proguanil-treated	Proguanil	50					120
		25					86
		10			39	93	
		4		25			
		2	7·2	39			
		1	39				
	Pyrimethamine	0·12					2·8
		0·06		19	34	57	71
		0·03		84			107
Pyrimethamine-treated	Proguanil	2		<2	25	34	6·5
		1		28			26
	Pyrimethamine	0·06	11	17	35	32	<3
		0·03	61	140			26
		0·015	88				

* As in Table I.

The proguanil-treated strain soon acquired high resistance, and at the same time slight but definite cross-resistance, to pyrimethamine. At this time, the strain treated under the same conditions with pyrimethamine had not become distinguishable from the parent strain in sensitivity to either drug.

Potentiation.—Table III shows the effect of giving both proguanil and pyrimethamine in different proportions to groups of infected chicks.

The ED50's are plotted in Fig. 1. Points lying below the straight line joining the ED50's of the

TABLE III

THE COMBINED ACTION OF PROGUANIL AND PYRIMETHAMINE ON *P. GALLINACEUM*

Proguanil (mg./kg.)	% Parasitaemia, Pyrimethamine (μg./kg.) —	7·5	15	30	60	ED50 Pyrimethamine with Appropriate Dose Proguanil† (μg./kg.)
—	100*		65	24	<1·2	22
0·25			72	11	<1·2	20
0·5	81	68	56	7·1		16
1·0	58	62	20	<1·2		†
2·0	11					

* Untreated controls; percentage parasitaemia as in Table I.
† ED50 proguanil alone 1 mg./kg.

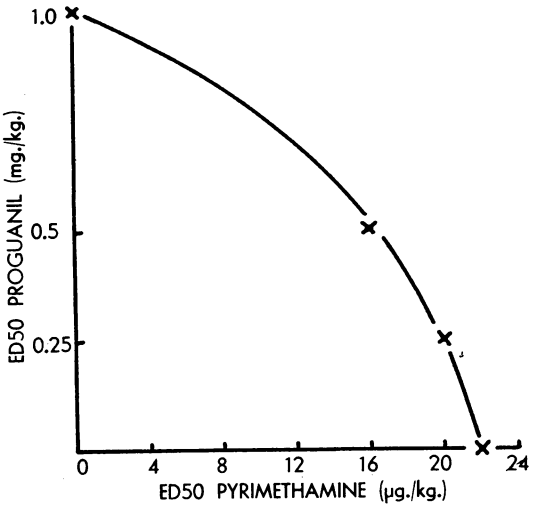


FIG. 1.—ED50's (doses reducing parasitaemia to 50% of the mean parasitaemia of untreated controls) of proguanil and pyrimethamine administered both singly and together in various proportions, in chicks infected with *P. gallinaceum*. ED50's estimated graphically from dose-response curves. For interpretation see text. Note lack of potentiation.

TABLE IV

THE COMBINED ACTION OF SULPHADIAZINE AND PYRIMETHAMINE ON *P. GALLINACEUM*

Sulphadiazine (mg./kg.)	% Parasitaemia, Pyrimethamine (μg./kg.) —	1·9	3·8	7·5	15	30	60	ED50 Pyrimethamine with Appropriate Dose Sulphadiazine (μg./kg.)†
—	100*				84	62	4	34
0·19				116	78	52	<1·5	26
0·38				82	46	18	<1·5	14
0·75				79	22	<1·5		11
3			57	13	1·5			4·1
6	97							
12	91	40	3	<1·5				1·7
24	57							

* Untreated controls; percentage parasitaemia as in Table I.
† ED50 sulphadiazine alone 26 mg./kg.

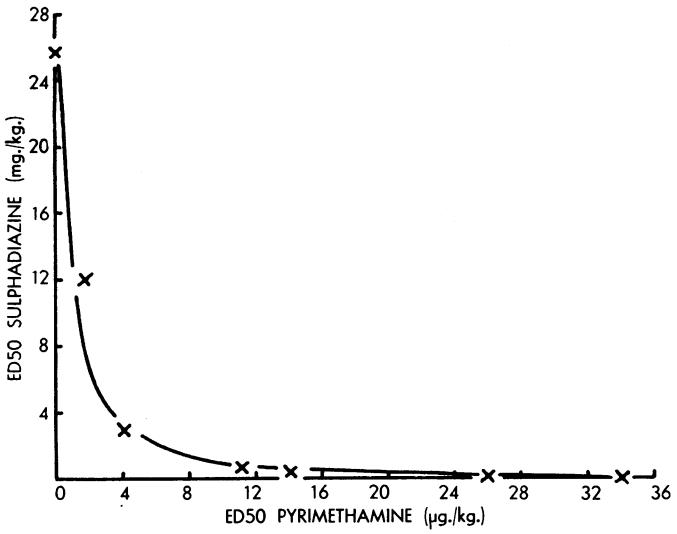


FIG. 2.—ED50's of pyrimethamine and sulphadiazine, administered both singly and together in various proportions, in chicks infected with *P. gallinaceum*. Conventions as in Fig. 1. Note marked potentiation.

two drugs given alone indicate potentiation. The action of pyrimethamine was not potentiated by proguanil (Fig. 1), but was greatly potentiated by sulphadiazine (Table IV, Fig. 2). The point on the curve nearest the origin indicates the optimum combination of doses which will produce the measured effect. Here this proportion is 1/7th of the ED50 of sulphadiazine with 1/8th of the ED50 of pyrimethamine.

The potentiating action of sulphadiazine might possibly be due to an action upon intestinal organisms which normally synthesize metabolites essential to the malarial parasite. The effect of giving poorly absorbed sulphonamides and antibiotics which act upon the intestinal flora is shown in Table V. These results show clearly that the potentiating effect was due, not to the effect in the gut, but to the sulphonamide in the blood. Succinylsulphathiazole was found in the blood only at a very low level and did not potentiate, whereas sulphaguanidine reached a much higher level and did potentiate. The antibiotics had no potentiating effect.

TABLE V

THE EFFECT OF POORLY ABSORBED SULPHONAMIDES AND ANTIBIOTICS ON THE ANTIMALARIAL ACTION OF PYRIMETHAMINE ON *P. GALLINACEUM*

Pyrimethamine (mg./kg.)	Controls	Sulphaguanidine (10 mg./kg.)		Succinylsulphathiazole (10 mg./kg.)		Penicillin 50 ku./kg.	Streptomycin 50 ku./kg.
		Antimalarial Effect	Peak Blood Level (Single Dose)	Antimalarial Effect	Peak Blood Level (Single Dose)	Antimalarial Effect	Antimalarial Effect
None	100*	102	0·28 mg.% at 2 hours†	100	Trace†	77	106
0·03	19	< 1·3		23		36	36

* Untreated controls; percentage parasitaemia as in Table I.
† A single oral dose of sulphadiazine at 10 mg./kg. gave a peak blood level of 0.84 mg.% at 1 hr.

Inhibition.—The inhibitory action of PAB and folic acid upon the activity of sulphadiazine and pyrimethamine is shown in Table VI. This inhibition was essentially competitive in nature. The effect of PAB upon pyrimethamine was barely significant ($P=0.1$) because of the large variance of the results. Repetition of the experiment gave similar figures. The degree of antagonism was small, perhaps because pyrimethamine persists in the blood and is therefore difficult to antagonize with a compound which is probably rapidly eliminated. Further work is necessary to investigate this aspect of the problem.

Results obtained using amino-an-fol with sulphadiazine are also included in Table VI; it acted in the same manner as folic acid. Although chromatographic examination of the sample did not disclose any PAB, there were impurities present and these may have affected the result.

TABLE VI

COMPETITIVE ANTAGONISM OF SULPHADIAZINE BY PAB AND OF SULPHADIAZINE AND PYRIMETHAMINE BY PGA IN *P. GALLINACEUM*

Antagonist		Antimalarial: Sulphadiazine (mg./kg.) None	15	30	60	120	Pyrimethamine (mg./kg.) None	0·03	0·06
PAB (mg./kg.)	None	100*	49	2·5			100*		6·3
	0·25			41					
	1·0				2·5				
	4·0			85					
	16·0	90			85				
	30·0						103		15·9
Folid acid (mg./kg.)	None	100*	76	< 1·2			100*	58	2·8
	10							71	15
	25			98	53				
	50				89	52			
	100	93				80	97	92	32
Amino-an-fol (mg./kg.)	None	100*	87	22	6·9				
	20	107		99	54				

* Untreated controls; percentage parasitaemia as in Table I.
(Each rectangle enclosed in bold rules contains the results of a separate experiment, i.e., this table is made up from 5 experiments.)

Discussion

Mode of Action of Sulphonamides.—It is commonly recognized from studies on bacteria that sulphonamides containing an $NH_2.C_6H_4.SO_2.NH$– group act by competing with PAB. The antimalarial action of sulphonamides may also depend on a similar mechanism because PAB reverses it (Maier and Riley, 1942) and because PAB is important for the metabolism of malarial parasites, particularly *P. berghei,* which is extremely sensitive to sulphadiazine (Hawking, 1953b).

Resistance and Cross-resistance.—There are at least four possible explanations of the mechanism of sulphonamide resistance. (1) The resistant organism may produce sufficient PAB to carry on the inhibited metabolic reaction. Thus Landy, Larkum, Oswald, and Streightoff (1943) found that resistant staphylococci produce up to 100 times more PAB than did normal strains. (2) The inhibited reaction may be by-passed by a related action not affected by sulphonamides ; or may be made unnecessary by the direct utilization of the end-product of the reaction (Work and Work, 1948). (3) The resistant strain may develop enzymes for the conversion of sulphanilamide into an essential metabolite, as suggested by the results of Emerson and Cushing (1946) with sulphonamide-resistant neurosporae. (4) The permeability of the cell membrane may be altered so that the sulphonamides are selectively excluded ; or spatial rearrangement of cell receptors may restrict the attachment of the sulphonamide molecule but not that of PAB.

Thurston (1953) put forward the tentative suggestion that sulphadiazine resistance in *P. berghei* may be due to the increased production of PAB. Nevertheless, from recent but still incomplete work, showing that malarial infections—particularly with *P. berghei*—(Hawking, 1953b), do not progress normally in the absence of dietary PAB, it seems unlikely that malarial parasites can synthesize PAB. The experimental conditions are critical and any departure from a strict PAB-deficient diet may give conflicting results (Maegraith, 1954) ; other substances besides PAB may be involved in the suppression of malaria by milk (Maegraith, 1953 ; Refaat and Bray, 1953).

Previous work on cross-resistance between sulphonamides, proguanil and pyrimethamine upon several species of *Plasmodium* (Thurston, 1953), together with the results shown in Table I, lead to the conclusions that

(a) Strains resistant to sulphonamides are usually resistant to proguanil and to pyrimethamine.

(b) Strains resistant to proguanil are usually resistant to pyrimethamine and *vice versa.*

(c) Strains resistant to proguanil and to pyrimethamine are not usually resistant to sulphonamides.

Although some reported results conflict with these conclusions there is usually a reason for the discrepancy. Thus Thurston (1953) and Rollo (1951) reported strains of *P. cynomolgi* and *P. gallinaceum* respectively which, while resistant to proguanil, retained sensitivity to pyrimethamine. On the other hand, Robertson, Davey and Fairley (1952) and Singh, Ray, Basu and Nair (1952) reported strains of *P. gallinaceum* and *P. knowlesi* which became cross-resistant to pyrimethamine after treatment with proguanil. The strains used by Thurston and Rollo were " old " strains which retained a high degree of resistance to proguanil, but which had not been exposed to that drug for some time before testing with pyrimethamine. The strains used by Robertson *et al.* and Singh *et al.,* however, had been exposed to proguanil shortly before the cross-resistance tests were carried out. Proguanil may, therefore, under optimal conditions, give rise to resistance to pyrimethamine as well as to proguanil. Pyrimethamine resistance appears to develop less readily, and is more labile in character, than resistance to proguanil (Table II) ; if the strain is left without treatment, sensitivity to pyrimethamine may return.

Rollo (1951) showed that a strain of *P. berghei,* treated in 9 successive passages with sulphadiazine and rendered 4 times less sensitive to the drug than the parent strain, remained sensitive to pyrimethamine. A similar strain, prepared over a longer period by Thurston (1953), and showing a 100-fold increase in resistance to sulphadiazine, was cross-resistant to pyrimethamine. Rollo's strain, however, was only partially resistant, and it is possible that, with an increase in its resistance to sulphadiazine, cross-resistance to pyrimethamine would have appeared.

Bishop and McConnachie (1948) reported that a proguanil-fast strain of *P. gallinaceum* became resistant to sulphadiazine. All other workers have found that strains made resistant to proguanil have retained their sensitivity to sulphadiazine, and Bishop (1951) was unable to confirm the earlier result. This cross-resistance is not dependent upon the pyrimidine part of the molecule, for Bishop and McConnachie (1950a) showed that a strain of *P. gallinaceum* resistant to sulphanilamide was also highly resistant to proguanil.

Potentiation and Antagonism.—Greenberg (1949) showed that the action of proguanil is

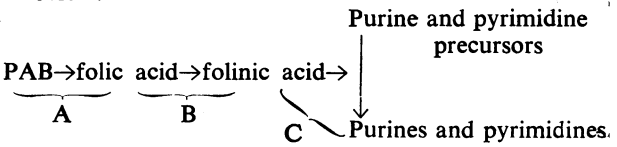
strongly potentiated by sulphadiazine and other PAB competitors which are themselves active against malaria. From the present work it is clear that a similar relationship exists between pyrimethamine and sulphadiazine, but that pyrimethamine and proguanil do not potentiate each other's action.

It is likely that potentiation occurs when two drugs act at different points on the same metabolic pathway, although it is possible that indirect effects—such as the reduction of available metabolite by the action of drugs on the intestinal flora—might produce the same result. It is also likely that an additive effect occurs when the drugs act at the same point or upon different pathways. Therefore, sulphadiazine probably acts at a different point from, but on the same pathway as, pyrimethamine or proguanil. Because of their relationship with sulphadiazine, and because their antimalarial action is antagonized by PAB, it is probable that proguanil and pyrimethamine act at the same point on the same pathway.

The antagonism of sulphadiazine by both PAB and folic acid is competitive and can easily be demonstrated. This has been shown in mice infected with *P. berghei* by Thurston (1954) and in infections of *P. gallinaceum* (see above). Antagonism of proguanil or of pyrimethamine by PAB and folic acid is less effective and less easy to demonstrate. Greenberg (1953), using high doses of PAB and folic acid, has shown in *P. gallinaceum* that proguanil can be competitively antagonized. Thurston (1954), by using a continuous dosing technique, has shown that both proguanil and pyrimethamine can be antagonized by PAB and folic acid in mice infected with *P. berghei*. Thurston's data are too sparse to decide whether the antagonism is competitive or not, except with pyrimethamine and PAB, where competition is observed. Both PAB and folic acid antagonize—the latter competitively—the action of pyrimethamine on *P. gallinaceum* (see above).

Thus in every example in which adequate data have been collected, the compounds have acted as competitive antagonists to all three drugs. The fact that folic acid competitively antagonizes sulphadiazine suggests that folic acid is broken down either by the host or the malarial parasite itself to PAB or *p*-aminobenzoylglutamic acid. The folic acid antagonists amino-an-fol, aminopterin, and amethopterin, are without antimalarial activity (Coatney, Cooper, Eddy and Greenberg, 1953); the first of them has been shown above to antagonize the action of sulphadiazine. Recently, Greenberg (1954) has shown that folic acid, amino-an-fol, and several other folic acid antagonists, competitively antagonize the action of sulphadiazine on *P. gallinaceum*. Plasmodia are probably unable to utilize preformed folic or folinic acid and it is likely that they synthesize these from PAB (Goodwin and Rollo, 1955), which may be supplied by the breakdown of folic acid or of the folic acid antagonists. Greenberg (1954) considers that it is uncertain whether the antagonism of sulphadiazine by substances containing the PAB moiety occurs because the analogues act as sources of folic acid or because the analogues are broken down to PAB which inhibits the sulphonamide.

Possible Mode of Action of Sulphonamides, Proguanil and Pyrimethamine.—The biological systems upon which we must assume the action of these antimalarials to take place are shown below.



The facts to be discussed are: (1) strains resistant to proguanil and pyrimethamine retain full sensitivity to sulphonamides, (2) sulphonamide-resistant strains are cross-resistant to proguanil and pyrimethamine, and (3) sulphadiazine potentiates the action of proguanil and pyrimethamine. Although the first and third of these facts suggest that the loci of action of sulphonamides and proguanil-pyrimethamine are not the same, the second suggests a common locus.

The effect of sulphonamides on plasmodia is presumably due to interference with the utilization of PAB in reaction A. The other drugs may, however, act quite differently. The results of cross-resistance tests and the lack of complete antagonism by PAB and folic acid indicate that their action may be more than simple competition with a metabolite. Hitching's evidence (in Goodwin and Rollo, 1954) on the varying ability of folic and folinic acids to reverse the effects of pyrimethamine favours a blockage of reaction B; and if resistance to proguanil and pyrimethamine involved interference with their action at point B only, this would leave reactions A and C vulnerable to other drugs—for example, to the action of sulphonamides on reaction A. So far no drugs are known that interfere with reaction C in plasmodia. Resistance to sulphonamides could be attributed to a by-pass of the whole series of reactions from PAB to folinic acid, thereby render-

ing the parasite insensitive not only to drugs interfering with reaction A but also to drugs interfering at B. Such a by-pass is, however, unlikely because Bishop and McConnachie (1950b) showed that strains treated with sulphadiazine readily became resistant to high doses of proguanil before any sign of sulphonamide resistance became evident. Hitchings (personal communication) has suggested that, in this instance, treatment with sulphadiazine may have resulted in an increase of the efficiency of reaction B. Such an increase in efficiency has been observed in biological systems in response to folic acid antagonists (Broquist, Kohler, Hutchison and Burchenal, 1953). This would account for the early development of proguanil resistance if the action of this drug were upon reaction B. Resistance to sulphonamides would follow as progressively less PAB became necessary to supply reaction B. However, where an increase in the efficiency of reaction B has been observed with bacteria, it has been produced by the direct interference of folic acid antagonists. It seems less likely that such a change would be induced by interference at A.

The experimental findings could be explained by a two-point mode of action for proguanil and pyrimethamine. This would involve, firstly, an acceptor mechanism whereby the drugs are made available to interfere with the metabolic reaction and, secondly, a " lethal " point of action within the metabolic pathway. There may thus be cell receptors responsible for making PAB available to reaction A. Sulphonamides would also be accepted, thus making possible the blocking of reaction A.

Sulphonamide resistance may involve a decrease in cell permeability to the sulphonamide molecule, or a change in distribution or orientation of the receptors, so that, although PAB is still accepted, the sulphonamide molecule is rejected. If pyrimethamine and the active metabolite of proguanil are accepted or rejected by the same mechanism as sulphonamide, sulphonamide-resistance would be accompanied by resistance to these drugs, perhaps before the resistance to sulphonamide became evident.

Resistance to proguanil and pyrimethamine could involve a by-pass of their effect on reaction B, leaving unchanged the sensitivity of the strain to sulphonamide acting at A. On the other hand, a reorientation of PAB receptors, insufficient in extent to cause rejection of sulphonamide molecules, might yet be sufficient to cause rejection of the proguanil-metabolite and pyrimethamine. This would also explain the results in Table II if it were assumed that pyrimethamine has a firmer " foothold " upon the receptors than the proguanil metabolite has. Little change would be needed in the distribution or orientation of the receptors for the complete rejection of the proguanil metabolite, the smallness of the change being reflected in the ease with which resistance can be induced. Pyrimethamine-resistance, on the other hand, may involve a greater change. Further evidence of the effects of small changes of structure in molecules of the pyrimethamine type upon their action against resistant strains is given by Greenberg and Bond (1954) and by unpublished experiments by the author. Some of the phenoxy and benzyl analogues of the pyrimethamine series, when tested against strains of *P. gallinaceum* and *P. cynomolgi* highly resistant to the action of pyrimethamine, showed activity almost as high as against the pyrimethamine-sensitive parent strains of parasite. The analogues, resembling pyrimethamine closely in structure, would be expected to act at the same point in the metabolic reactions; and differences in activity upon resistant strains of parasite can best be accounted for by differences in degrees of acceptance resulting from differences in spatial structure.

From the evidence presented and discussed it is therefore postulated that both pyrimethamine and the metabolite of proguanil enter the metabolic pathway concerned with the uptake and utilization of PAB by the same acceptor mechanism as PAB; the drugs then exert their lethal action on the parasite by interfering with the conversion of folic acid to folinic acid.

Summary

1. A strain of *P. gallinaceum* resistant to both proguanil and pyrimethamine retained its sensitivity to sulphadiazine.

2. *P. gallinaceum* treated with proguanil quickly became resistant to that drug and slightly resistant to pyrimethamine. Identical treatment with pyrimethamine failed to induce resistance either to pyrimethamine or to proguanil.

3. Sulphadiazine strongly potentiated the action of pyrimethamine. There was no potentiation between proguanil and pyrimethamine.

4. The action of both sulphadiazine and pyrimethamine was competitively antagonized by folic acid.

5. A possible mechanism of action depicting the interrelationship of sulphadiazine, proguanil and pyrimethamine is put forward. Proguanil and pyrimethamine may have a twofold mode of

action involving, firstly, an acceptor mechanism whereby the drugs are made available to interfere with the metabolic reaction and, secondly, a " lethal " point of action within the metabolic pathway—probably by interfering with the conversion of folic to folinic acid.

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