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THE MODE OF ACTION OF PROGUANIL AND RELATED ANTIMALARIAL DRUGS

BY

J. F. RYLEY

From Imperial Chemical Industries Limited, Biological Laboratories, Morley, Wilmslow, Cheshire

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During recent years considerable interest has been aroused by the ability of a number of substances to antagonize pteroylglutamic acid in both microbial and mammalian systems. Discussing the antagonistic effects of substituted 2:4-diaminopyrimidines, Falco, Hitchings, Russell, and VanderWerff (1949) pointed out that these substances showed a formal resemblance to proguanil ("Paludrine"), and might therefore be expected to have antimalarial activity also. They suggested, too, that by virtue of this similarity proguanil should display pterolyglutamic acid antagonism in their bacterial cultures. Development of the idea that 2:4-diaminopyrimidines might possess antimalarial activity led to the synthesis of pyrimethamine (2:4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine; "Daraprim"), one of the most active compounds of the series, as an antimalarial drug.

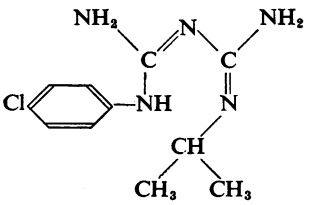
Soon after the introduction of proguanil as an antimalarial, Hawking (1947) and Hawking and Perry (1948) produced evidence to suggest that the drug itself did not possess antimalarial activity, but that it was metabolized in the body to an active compound. One such active metabolite was isolated from the urine of proguanil-treated rabbits by Carrington, Crowther, Davey, Levi, and Rose (1951), and shown to be 2:4-diamino-1-*p*-chlorophenyl-1:6-dihydro-6:6-dimethyl-1 : 3 : 5-triazine, which bears a marked structural resemblance to pyrimethamine (see formulae).

The present study was undertaken to obtain further information on the bearing of the metabolite on the antimalarial action of proguanil, and on the correlation, if any, between antimalarial activity and interference by substances active in the pteroylglutamic acid system. Other drugs, related to proguanil and its metabolite, have been used to obtain additional information. The structural formulae of the substances are given on the next page.

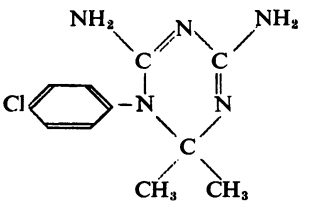
METHODS

L. casei was cultivated in a medium containing a charcoal-treated tryptic digest of casein, supplemented with inorganic salts, glucose, and growth factors; 1 l. of the final growth medium contained the equivalent of 25 g. casein, $MgSO_4.7H_2O$ 800 mg., $MnSO_4.4H_2O$ 160 mg., $FeSO_4.(NH_4)_2SO_4.6H_2O$ 56 mg., glucose 10 g., L-cysteine 100 mg., adenine 7 mg., guanine 2 mg., thiamin, nicotinic acid, Ca pantothenate and pyridoxin 1 mg. each, riboflavine 0.4 mg., and biotin 1 μg. The medium was prepared in a concentrated form, and distributed in 3.5-ml. amounts in 5 × $\frac{5}{8}$-in. tubes plugged with absorbent cotton-wool covered with surgical gauze. 0.5 ml. pteroylglutamic acid (PtG) was added to each tube to give $10^{\frac{1}{2}}$- (i.e., 3.16-) fold final dilutions over the range 10^{-5} to 10^{-11} M (i.e., 4.4 to 0.0000044 μg./ml.). The tubes were autoclaved for 10 min. at 10 lb./sq. in., cooled, and 0.5 ml. drug solution, sterilized by Seitz filtration, was added. The tubes were inoculated with 0.5 ml. of a dilute suspension of bacteria and incubated in an atmosphere of 5% CO_2–95% N_2 (v/v) for 40 hours at 37° C. Growth was measured using the Hilger Spekker absorptiometer and a 0.5 cm. cell. Curves were plotted correlating the growth obtained with $\log_{10}$ (PtG concentration) in the absence and in the presence of variable quantities of drug. Two tubes were used for each concentration of PtG, so that each growth curve was based on 28 tubes. Curves were plotted representing growth in the presence of different fixed concentrations of drug; the drugs were used in $10^{\frac{1}{2}}$-fold dilutions, five different concentrations being examined with one control in any particular experiment.

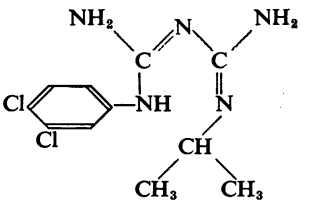
A normal strain of *Plasmodium gallinaceum* was used, and also a strain made resistant to 2 mg. proguanil (all doses are expressed as mg. drug given orally b.i.d. × $3\frac{1}{2}$ to a 50-g. chick). This resistant strain had been passaged in the presence of proguanil over a period of $10\frac{1}{2}$ months. The sensitivity of these two strains to the various drugs under test was determined by the standard therapeutic test of Davey (1946); the minimum effective dose (MED) was taken to be the lowest dose which would reduce the parasite count below 10 per 500 erythrocytes (count in untreated controls 300–400 parasites per 500 erythrocytes).



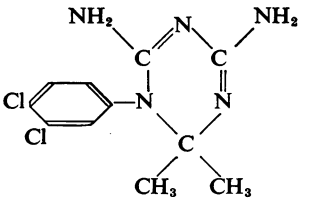
Proguanil



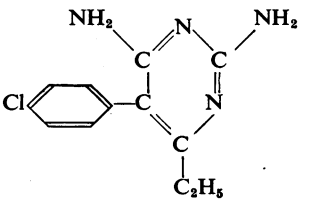
10,580 (Carrington *et al.*, 1951)



5943 (Curd, Davey, Hendry, and Rose, 1950)



10,732 (Carrington *et al.*, 1951)



Pyrimethamine (Falco, Goodwin, Hitchings, Rollo, and Russell, 1951)

Previous studies of the effects of antimalarial drugs on the erythrocytic stages of malaria parasites *in vitro* have been done using the rocker-dilution technique of Geiman, Anfinsen, McKee, Ormsbee, and Ball (1946) or a modified Bass and Johns method (e.g., Hawking, 1947). In the present work it was found convenient to use a method developed by my colleague, Dr. D. G. Davey. The method has not been described before and it is therefore given here in detail. The culture tubes, modified from a design due to Lindbergh (Parker, 1938), are illustrated diagrammatically in Fig. 1. The tubes were filled with

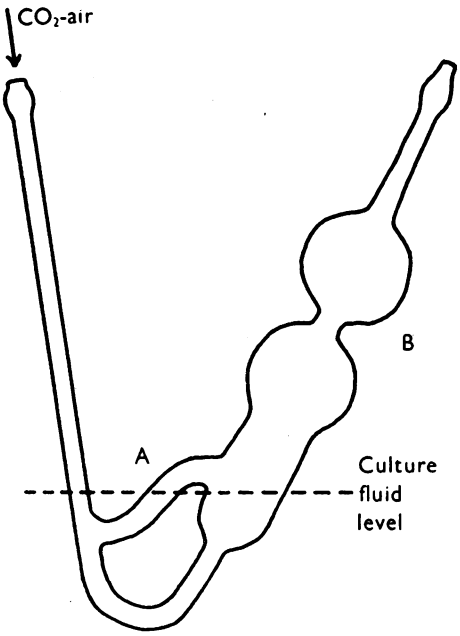


FIG. 1.—Culture tube for malaria parasites. ×½.

erythrocytic culture to the level indicated in the figure (with the particular tubes in use, this required 4 ml. of culture). By blowing a stream of gas mixture down the inlet tube, small slugs of culture were carried up the gas lift A. In this way the culture was kept continually mixed, and in equilibrium with the gas phase. During aeration the serum in the culture medium caused a certain amount of frothing; this froth was broken down in the bulbs B. For culture experiments, 12 of the tubes were connected in series with rubber tubing. The whole assembly was sterilized by autoclaving, and culture components were introduced by means of sterile syringes through the rubber connecting tubes (sterilized locally by means of 70% ethanol). The gas mixture of 5% CO_2–95% air (v/v) was sterilized by passage through a long column of cotton-wool, and was saturated with water vapour by bubbling through two wash-bottles of water. Cultures were incubated at 38° C. for 24 or 40 hours.

The cultures contained approximately 1.2×10^8 erythrocytes per ml., suspended in a liquid phase consisting of equal parts of chicken serum and a solution containing 0.111 M NaCl, 0.005 M KCl, 0.002 M $MgCl_2$, 0.020 M $NaHCO_3$, 0.002 M phosphate buffer (*p*H 7.4), and 0.020 M glucose, which, when used in conjunction with a gas phase containing 5% CO_2, had a *p*H of 7.4. Blood was removed under aseptic

conditions from infected chickens, and was diluted with clean blood to give a parasitaemia of approximately 30% ; heparin was used as anticoagulant. This infected blood (1 vol.) was added to a mixture of sterile chicken serum and Ringer-glucose-bicarbonate solution (6 vol.), and 3.0 ml. of the resulting mixture was introduced into each culture tube. 1 ml. of Ringer-glucose-bicarbonate solution containing any drugs or growth factors to be studied was then added to each culture. All drug solutions and sera were sterilized by Seitz filtration, and the sterility of the cultures was checked by inoculation of a blood plate. It was found unnecessary to add antibiotics to the cultures to ensure sterility, as has been done by some previous workers.

0.2 ml. of culture was inoculated into 2 or 3 clean chickens (50 g.) before and after incubation, and blood smears taken from these chickens at 24-hr. intervals were examined for the presence of parasites. The infection was regarded as patent when at least three parasites were found in 100 oil-immersion microscope fields examined, and the course of infection was then followed for 3 or 4 days. By comparing this course of infection with that in the controls—provided by material from the cultures before *in vitro* incubation, or from cultures incubated in the absence of drug—it was possible to make an estimate of the prepatent-period delay, and hence of the survival of the parasites following incubation *in vitro* and after incubation in the presence of drug. It was found that an increase in prepatent period of 1.2 days (range 1.1 to 1.6 days in 7 experiments) corresponded to a mortality (or reduction in inoculum) of 90%, and an increase in prepatent period of 2.4 days (range 2.1 to 3.0 days in 7 experiments) to a 99% mortality. Taylor, Greenberg, Josephson, and Ray (1951) found a prepatent-period increase of 1.3 days corresponded to a mortality of 90%. The cultures at the end of the experiments were gently centrifuged, and smears made from the deposit were stained with Giemsa and examined.

Results

The Effect of Proguanil and Related Substances on the Growth-Promoting Properties of Pteroylglutamic Acid for Lactobacillus casei

L. casei grown in the standard PtG assay medium gave half-maximum growth with 2.25×10^{-10} M PtG, while maximum growth was obtained with approximately ten times this concentration of PtG. In the presence of drug concentrations of the order of 10^{-4} M or greater, no bacterial growth took place, no matter how much PtG was added ; in the presence of low drug concentrations (about 10^{-6} M), no inhibition of bacterial growth was observed. With the compounds 10,580, 10,732, and pyrimethamine it was found that intermediate concentrations of drug produced an inhibition of bacterial growth which could be completely reversed by the addition of suitable amounts of PtG. Curves representing bacterial growth in the presence of a fixed concentration of drug were similar in shape and parallel to the control curve, being displaced towards higher PtG concentrations. This competitive inhibition was usually observed over approximately a hundredfold range of drug concentration. Drug activity was expressed in terms of the inhibition index, which represents the ratio of concentrations of drug and growth factor at which their effects are just counterbalanced (based on a 50% reduction of growth). In contrast to the results obtained with pyrimethamine and the two triazines it was found that proguanil and 5943 exerted an " all or none " effect on bacterial growth ; in no experiment was it observed that either of these compounds produced a toxicity which was in any way reversible by added PtG. The results of these experiments are summarized in Table I.

TABLE I

PtG AND THYMINE ANTAGONISM WITH *LACTOBACILLUS CASEI*

Drug	Drug Concentrations (M) Showing			Inhibition Index*	
	Full Growth	Competitive Inhibition	Complete Inhibition	PtG	Thymine
Proguanil	$3{\cdot}16 \times 10^{-4}$	—	10^{-3}	—	—
10,580 ..	$3{\cdot}16 \times 10^{-7}$	10^{-6} to 10^{-4}	10^{-3}	6,500	2·0
5943 ..	$3{\cdot}16 \times 10^{-5}$	—	10^{-4}	—	—
10,732 ..	$3{\cdot}16 \times 10^{-7}$	10^{-6} to $3{\cdot}16 \times 10^{-5}$	$3{\cdot}16 \times 10^{-4}$	6,500	0·9
Pyrimethamine	10^{-7}	$3{\cdot}16 \times 10^{-7}$ to $3{\cdot}16 \times 10^{-5}$	10^{-4}	1,800	0·1

* $\frac{\text{Concentration of drug}}{\text{Concentration of growth factor}}$

Thymine Antagonism with Lactobacillus casei

L. casei is able to grow to a limited extent in the presence of thymine and adenine, but in the absence of PtG. Growth curves were obtained for bacteria cultivated in the presence of 10^{-5} M guanine, 5×10^{-5} M adenine, and thymine in $10^{\frac{1}{2}}$-fold dilutions over the range 3.16×10^{-3} to 3.16×10^{-5} M. It was found that half-maximum growth was obtained with about 10^{-4} M thymine, and maximum growth with about ten times this concentration ; in the presence of thymine, maximum growth was about half that obtained in the presence of optimal amounts of PtG. In the thymine systems, competitive inhibition or irreversible toxicity was observed at the same drug concentrations found effective in the PtG systems. Inhibition indices were calculated as before ; the approximate values are given in Table I. As in the

PtG systems, proguanil and 5943 were found to be either non-toxic or to produce a toxicity not reversed by the addition of more growth factor; there was again no evidence of competitive inhibition with these two drugs.

The Effect of Proguanil and Related Compounds on Plasmodium gallinaceum

Drug Sensitivity in vivo.—When assayed against the normal strain of *P. gallinaceum in vivo* the metabolite 10,580 was found to have ten times the activity, while 10,732 showed a hundred times the activity of proguanil; 5943 showed five times the activity, and pyrimethamine fifty times the activity of proguanil. With the resistant strain, however, fully effective doses were not tolerated by chicks. Thus, 10,732 gave an MED of 3 mg., while 5943 and 10,580 showed slight activity over the ranges 2–4 mg. and 5–7 mg. respectively. With the maximum tolerated doses (over the 5-day period of the test), proguanil at 3 mg. and pyrimethamine at 2.5 mg. were completely inactive. These results are summarized in Table II.

TABLE II

IN VIVO DRUG SENSITIVITY OF *P. GALLINACEUM*

(Figures represent the MED for proguanil, 5943, 10,580, and 10,732 as the hydrochlorides, or pyrimethamine as the free base)

	Normal Strain	Resistant Strain
Proguanil	0·25	>3
10,580	0·025	>7
5943	0·05	>4
10,732	0·0025	3
Pyrimethamine	0·005	>2·5

Morphological Effects Produced by Proguanil in vivo.—A number of 6-day-old chicks were inoculated with 50,000,000 parasites, and blood smears taken twice a day. When the parasitaemia reached a level of about 6% twice-daily dosing with 1 mg. proguanil was started. Initially, parasites were observed in all stages of development; the chromatin stained bright pink, and the pigment granules were clustered together. Within 6½ hours of the first dose of drug it was observed that, although young parasites were still normal in appearance, the chromatin of the larger forms was no longer in discrete particles and no mature parasites were seen. Twenty-two and a half hours after drug treatment had been started, all the parasites were of a large form, with indistinct, pale, and undivided chromatin; pigment granules were scattered throughout the parasites. At 30 hours the appearance of the parasites was much the same, and vacuolation of the cytoplasm was becoming apparent. At 46½ hours the parasites were found to be much vacuolated, with an irregular outline, and were degenerating. In the presence of drug, the degree of parasitaemia declined but little during the period of observation, while in the untreated birds the parasites multiplied normally and continued to show all stages of development with brightly staining chromatin.

In a similar experiment in which the chickens were treated with 4 mg. mepacrine it was found that the parasites reacted to the drug somewhat more quickly, and little evidence of parasitaemia was found 30 hours after the first dose of drug. In contrast to the effects produced by proguanil, mepacrine caused a shrinkage and vacuolation of the cytoplasm, followed by a contraction and fragmentation of the parasite to give a number of dark-staining masses of irregular shape.

Antimalarial Activity in vitro.—When maintained *in vitro* under the condition described above some parasites were able to survive for at least 40 hours. Observations on the increase in prepatent period showed that during 24 hours' incubation there was a loss in culture viability of about 60%, while for a 40-hour period of incubation the loss in viability was about 90%. Culture survival was, however, variable from experiment to experiment; with 24-hour cultures mortality varied from 0 to 90%, and with 40-hour cultures from 50 to 99.9%. Although the cultures showed a decrease in the number of viable parasites as incubation proceeded, it was nevertheless observed, from examination of stained films, that most of the parasites continued to develop *in vitro*. It appeared that parasites were able to grow and develop, in many cases to maturity, but on liberation from the mature schizonts the merozoites were usually unable to invade fresh red cells. Thus from this cause alone there would be a steady loss in viability of the "cultures."

Although the parasites in culture were clearly not under optimal conditions, the antimalarial effects of a number of substances could nevertheless be observed *in vitro*. In the presence of an active compound the mortality might be considerably increased and, moreover, the treated parasites often showed morphological differences from the control. Thus 0.5 mg./l. of mepacrine or chloroquine, or 20 mg./l. of pamaquin, were able to increase the prepatent period, compared with the control, by 1.2 days or more, showing that there had been at least a 90% mortality among the parasites which would have survived in the absence of drug. Furthermore, examination of stained preparations showed that the drugs had produced changes in the parasites similar to those

observed *in vivo*; the parasites were shrunken, dark-staining, and degenerate in appearance.

Proguanil and related compounds also showed antimalarial activity *in vitro*; morphological changes in the parasites were similar to those produced *in vivo*, and viability was usually reduced—though at higher drug concentrations than those necessary to produce morphological changes. These effects were noticeable in 24-hour cultures, and usually became more pronounced with further incubation. Proguanil, at concentrations of 5 mg./l. or more, reduced parasite viability by 90% compared with the control, and at concentrations down to 1 mg./l. usually caused the production of significant numbers of "inhibited" parasites, having pale, diffuse, and undivided chromatin. Differential parasite counts showed that the number of parasites with divided chromatin was very much smaller than that in the controls. The metabolite of proguanil, 10,580, was able to produce significant numbers of "inhibited" parasites in concentrations as low as 0.01 mg./l.; the lethal effects of this compound were rather variable.

5943 was found to be a little more active than proguanil, producing morphological changes in concentrations down to about 0.5 mg./l., and lethal effects down to about 5 mg./l. The metabolite 10,732 was the most active compound examined; lethal effects were apparent at a concentration of 0.01 mg./l., and inhibited parasites were produced by concentrations of 0.001 mg./l., and on occasions by even less than this. Pyrimethamine suppressed chromatin division, and led to the production of medium-sized forms with pale and diffuse chromatin in concentrations as low as 0.005 mg./l.; the lethal effect of this compound was rather variable, but concentrations of 0.5 mg./l. usually were sufficient to give a 90% increase in mortality.

With these low drug concentrations there was a suppression of chromatin division, although the parasites continue to increase in size, and a variable but significant number of large parasites with undivided, pale, and diffuse chromatin were produced. With high drug concentrations, of the order of 100 mg./l., the effect was of a different type, the parasites becoming shrunken, dark-staining, and degenerate, in a similar manner to those treated with mepacrine.

Attempts were made to prevent the *in vitro* antimalarial effects of proguanil and 10,732 by the addition to the cultures of pteroylglutamic acid and a number of other compounds. Proguanil was used at a concentration of 5 mg./l., and 10,732 at 0.013 mg./l. (4×10^{-8} M), concentrations which, on most occasions, produced typical effects on both morphology and viability. Pteroylglutamic acid (10^{-8}, 10^{-6} or 10^{-5} M), *Leuconostoc citrovorum* factor (10^{-8} or 10^{-6} M), adenosine (10 or 50 mg./l.), or a mixture of adenine (15 mg./l.), guanine (15 mg./l.), and thymine (10 mg./l.), were added to cultures in the presence of drug, but on no occasion was there any antagonism of antimalarial action due to the presence of these substances.

To give support to the contention that the drug effects observed *in vitro* were not artefacts, similar experiments were carried out using the resistant strain of the parasite. Proguanil and 5943 caused a shrinkage and degeneration of the parasite rather than the production of inhibited forms, while 10,580, 10,732, and pyrimethamine had little effect on morphology, except perhaps to cause a slight scattering of the pigment granules. Concentrations of drug up to 100 mg./l. were tested, and it was found that lethal effects were only produced at concentrations higher than those necessary with the normal strain. Thus proguanil was active at 10 mg./l., or more, and 5943 at 25 mg./l. 10,580 showed no lethal effect at 100 mg./l. in 24 hours, but was effective at 10 mg./l. after 40 hours' incubation. 10,732 was active at 10 mg./l. after 24 hours, or 1 mg./l. after 40 hours, while pyrimethamine showed corresponding activities at 30 mg. and 2.5 mg./l.

Discussion

It is now generally believed that pteroylglutamic acid is used by many living cells in the elaboration of a substance catalytically active in the synthesis of a number of purines, pyrimidines, and amino-acids. Using *Lactobacillus casei*, Hitchings, Falco, VanderWerff, Russell, and Elion (1952) studied the ability of a series of 2:4-diamino-pyrimidines to antagonize PtG in the growth of this organism. They noted that one of the most active compounds of the series was pyrimethamine, which had an inhibition index of 2,300. The present work confirms the antagonistic action of this compound (inhibition index found 1,800), and shows that the triazines 10,580 and 10,732, which are similar in structure to pyrimethamine, are also active antagonists. When Falco *et al.* (1949) originally suggested a connection between antimalarial activity and the ability to antagonize PtG, they suggested that proguanil ought to show this antagonistic activity. Indeed, they demonstrated antagonism by PtG and also by purines. Although their curves show some reversal of drug toxicity against *L. casei* at certain proguanil concentrations, they do not demonstrate competitive

inhibition of the kind associated with pyrimethamine and the triazines. We have been unable to confirm the antagonistic action of proguanil, or of the dichloro analogue 5943; these compounds either produced a toxic action on the bacteria which could not be reversed by PtG, or had no toxic effect at all.

With the 2:4-diaminopyrimidines or diaminotriazines, one molecule of PtG is able to reverse the toxic action of several thousand molecules of drug. The present study shows that there is a more nearly equimolecular relationship between these compounds and thymine, and it seems likely that the drugs produce their antimalarial effect, not by antagonizing PtG but rather by interfering with reactions involving thymine or its derivatives. This being so, PtG would exert an antagonistic action, not by competing directly with the drug, but by acting catalytically in the synthesis of increased amounts of thymine.

Proguanil, the triazines, and diaminopyrimidines are all rather slow in producing a therapeutic response in cases of malaria, and can readily give rise to resistant strains of parasites. This seems to suggest that they exert their effects on the anabolic rather than on the catabolic systems of the parasites, and that growth of the parasite in the presence of drug is necessary for activity to be revealed. The activity of this group of drugs seems to involve an inhibition of nuclear growth and division. Black (1946) found that *P. falciparum,* maintained *in vitro* in the presence of serum from patients treated with proguanil, continued to develop to the early schizont stage, but that development then ceased, the chromatin failed to divide, and the parasites slowly degenerated. Thurston (1951) observed that the development of *P. berghei, in vivo,* in the presence of proguanil, was unaffected up to the schizont stage, but that normal nuclear division did not take place. Similarly McFadzean (1951) observed that schizogony of *P. cynomolgi* was inhibited in the presence of proguanil, abnormal forms containing elongate chromatin fragments being produced. The present study suggests that, with *P. gallinaceum,* proguanil, or its active metabolite, is able to inhibit the synthesis of nuclear material; both the parasites and their nuclei seem to increase in size in the presence of drug, but, as chromatin synthesis is inhibited, the nucleus fails to divide, and its staining power is decreased.

It is strange that Taylor, Josephson, Greenberg and Coatney (1952) were unable to find activity with proguanil in concentrations below 50 mg./l., or pyrimethamine at 100 mg./l. However, the effective drug concentrations in their experiments probably differed from ours, since their cultures contained whole blood rather than serum-Ringer-glucose-bicarbonate. In the present work proguanil showed detectable activity in concentrations as low as 1 mg./l., and pyrimethamine had something like 200 times this activity. These *in vitro* drug effects were of the same type, although not necessarily as pronounced as those observed *in vivo*; the difference in degree is probably accounted for by the parasites in culture being under conditions far less favourable to growth than those found in the host. The cultures showed about the same degree of survival found by Taylor *et al.* (1951) using the rocker-dilution technique, but our cultures contained rather more trophozoites and fewer schizonts than theirs. *In vitro,* the five compounds showed relative activities in the same order as that observed *in vivo,* but the differences in activity were greater; thus 10,732 showed 100 times the activity of proguanil *in vivo,* and 1,000 times the activity *in vitro.* This is doubtless due to the fact that the drugs act as such *in vitro,* whereas *in vivo* they are subject to metabolic changes by the host. It is significant that proguanil itself *in vitro* has quite a marked antimalarial action; the concentrations necessary to produce this effect, however, are about ten times greater than concentrations likely to be encountered *in vivo.*

Although drug toxicity in bacterial systems can be overcome by PtG or thymine, no such reversal was observed in the cultures of malaria parasites. The parasites are possibly unable to use PtG as such, but require a "higher form" of folic acid, and, rather than utilize free purines or pyrimidines in the synthesis of nucleic acids, prefer to utilize the corresponding nucleosides or nucleotides. Preliminary estimations suggest that both normal and parasitized cells contain the same amount of deoxyribonucleic acid phosphorus; this would seem to indicate that the parasites synthesize their chromatin at the expense of that of the host cell. It is possible that the utilization of the chromatin of the host cell does not involve a complete breakdown to purines, pyrimidines, phosphate, and deoxyribose, in which case free nitrogenous bases would not be expected to antagonize drug activity. Madinaveitia and Raventós (1949) made the interesting but inexplicable observation that the block in the auriculo-ventricular contraction of the guinea-pig heart produced by adenosine was antagonized by most antimalarials tested with the exception of proguanil; adenosine, however, showed no antagonistic effect towards any of the drugs tested in our malaria parasite cultures.

Summary

1. The metabolite of proguanil, 2:4-diamino-1-*p*-chlorophenyl-1:6-dihydro-6 : 6-dimethyl-1 : 3 : 5-triazine (10,580), the corresponding dichloro analogue (10,732) and pyrimethamine, are antagonists of pteroylglutamic acid and thymine in the growth of *Lactobacillus casei*; no such antagonism is caused by proguanil itself or its dichloro analogue (5943).

2. Proguanil *in vivo* causes morphological changes in *Plasmodium gallinaceum* which suggest an inhibition of chromatin synthesis and nuclear division.

3. When proguanil (1 mg./l.), 5943 (0.5 mg./l.), 10,580 (0.01 mg./l.), 10,732 (0.001 mg./l.), or pyrimethamine (0.005 mg./l.) are incubated with erythrocytic cultures of *P. gallinaceum in vitro*, they cause morphological changes of the same type as those produced by these drugs *in vivo*. Drug concentrations about ten times as great are able *in vitro* to reduce culture viability by 90%.

4. A strain of *P. gallinaceum* made resistant to the maximum doses of proguanil tolerated by chickens showed resistance *in vitro* to proguanil, 5943, 10,580, 10,732, and pyrimethamine; drug concentrations effective against the resistant strain *in vitro* did not produce morphological effects that would suggest an interference with chromatin synthesis.

5. The antimalarial effects of these drugs *in vitro* are not antagonized by pteroylglutamic acid, thymine, or a number of other substances.

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