Brit. J. Pharmacol. (1953), **8**, 93.

PROGUANIL—THE ISOLATION OF A METABOLITE WITH HIGH ANTIMALARIAL ACTIVITY

BY

A. F. CROWTHER AND A. A. LEVI

From Imperial Chemical Industries Ltd., Research Laboratories, Hexagon House, Blackley, Manchester, 9

(RECEIVED SEPTEMBER 26, 1952)

The existence of a metabolite, wholly or partly responsible for the antimalarial activity of "Paludrine" [proguanil, N^1-*p*-chlorophenyl-N^5-*iso*propyldiguanide (I ; R = H)], has been suspected for some years. Hawking and Perry (1948) showed that whilst the serum from animals dosed with proguanil was active *in vitro* against *Plasmodium gallinaceum*, the drug itself had no demonstrable effect on the exo-erythrocytic forms of the parasite grown in tissue cultures or on the blood forms of *P. cynomolgi* (see also Hawking, 1947, and Tonkin, 1946). Incubation of proguanil with minced liver was said to produce *in vitro* activity. Hawking and Perry therefore suggested that proguanil was modified in the body to form a product that was directly responsible for antimalarial activity. Madinaveitia and Raventos (1949) demonstrated that antimalarial drugs in general were adenosine antagonists and that their antimalarial activity was paralleled by the degree of this antagonism. The inhibition of the auriculo-ventricular block produced in guinea-pigs by the injection of adenosine was used to measure this effect, which was also manifest in heart-lung preparations and could therefore be examined under *in vitro* conditions. For instance, mepacrine was shown to antagonize adenosine both when administered to intact guinea-pigs and when added to normal guinea-pig blood in a heart-lung preparation. Proguanil, however, showed the effect in the intact guinea-pig only after some delay following its administration, and had no action on the heart-block in the isolated preparation. Therefore Madinaveitia and Raventos also postulated the existence in the body of an active metabolite of proguanil. This metabolic change might also be responsible for the greater antimalarial activity of proguanil when given orally than when given intravenously (Davey, 1946).

After a prolonged search for this hypothetical metabolite a highly active compound has eventually been obtained from the urine of rabbits dosed with proguanil. This communication describes in greater detail the isolation and characterization of this metabolite as recorded in a preliminary note (Carrington, Crowther, Davey, Levi, and Rose, 1951).

Isolation of an Active Proguanil Metabolite

Most of the experiments have been carried out with rabbits. One of the first necessities was a method of estimating the drug in its original and metabolized forms. Spinks and Tottey (1946) described a method for the estimation of proguanil in tissues and body fluids which depended on the acid hydrolysis of the drug. In this method the samples were made strongly alkaline and the bases liberated were extracted by benzene and hydrolysed by an acid treatment. The *p*-chloroaniline so formed was diazotized and coupled with β-sulphato-ethyl-*m*-toluidine and the resultant azo compound estimated colorimetrically. The results obtained were in substantial agreement with the amount of proguanil that could be isolated from the benzene extract. In order to measure the total amount of material derived from *p*-chloroaniline, including unchanged proguanil, this method was modified by omitting the extraction with benzene and hydrolysing the whole urine, or other extract. For this purpose it was found more reliable to use alkaline hydrolysis under pressure.

Partly in order to confirm the validity of this analytical method, experiments were performed in which the bromo analogue of proguanil, N^1-*p*-bromophenyl-N^5-*iso*propyldiguanide, labelled with radio-active bromine, was administered to rabbits. The information obtained from counts on various urine fractions was of assistance in the detection and isolation of metabolites. Details of these experiments will be reported in a later communication.

Some of the samples taken at various stages in the extractions were too toxic for biological assay but whenever possible antimalarial activity against

P. gallinaceum in chicks was determined and a rough measure of the relative concentration of active material was deduced by comparison with the *p*-chloroaniline content of the samples.

Most effort was directed towards the examination of the urine from the treated animals. After the addition of lead acetate to the urine, in order to precipitate much unwanted material, the greater part of the therapeutically active fraction remained in solution and was then almost completely extractable into butanol. After distilling off the butanol, dissolving the residue in water and making strongly alkaline, part of the material yielding *p*-chloroaniline on hydrolysis could be extracted by ether, but part remained in the aqueous phase. The activity of the ether-soluble bases was shown to be due solely to unchanged proguanil. Estimations of the antimalarial activity remaining in the aqueous phase were at first erratic until it was realized that the active substance was unstable under alkaline conditions. Neutralization was effected, thereafter, as early as possible, the active substance extracted with butanol, and the solvent again replaced by water. Addition of picric acid was found to precipitate all the active principle as its picrate. The yield of picrate calculated to an assumed molecular weight of 500 was 1–3% based on the proguanil administered to the rabbits.

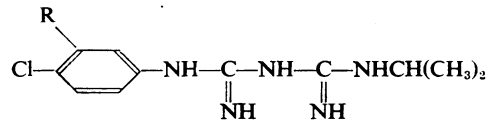
The faeces of the rabbits were also examined. After an ether treatment which did not remove any derivatives of *p*-chloroaniline, the faeces were extracted with methanol. The methanol solution was evaporated and *p*-chloroaniline derivatives were dissolved in dilute acid. This solution could then be treated as for the urine. In this way, a further 1% of picrate identical with that from urine was obtained.

The yield from the urine of human volunteers dosed with proguanil was about 5%.

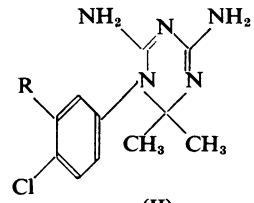
The picrate was readily converted into the free base, which was shown by our colleague Dr. D. G. Davey to be about ten times as active as proguanil against a blood-induced infection of *P. gallinaceum* in chicks. Like proguanil it was also effective against exo-erythrocytic forms of the parasite. Further, its activity was much reduced against proguanil-resistant strains of the parasite.

The metabolite has been shown to be 4:6-diamino-1-*p*-chlorophenyl-1 : 2-dihydro-2 : 2-dimethyl-1 : 3 : 5-triazine* (II ; R = H), and it has been found possible to synthesize this compound by a method which has proved general for dihydrotriazines of this type. Details of the synthesis together with an account of the work which established the structure of the metabolite will be published elsewhere.

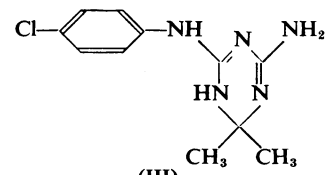
On treatment with alkali the metabolite was isomerized to give 4-amino-6-*p*-chloroanilino-1 : 2-dihydro-2 : 2-dimethyl-1 : 3 : 5-triazine (III), which was first prepared by Birtwell, Curd, Hendry, and Rose (1948), and which was inactive against *P. gallinaceum*. In acid solution the metabolite underwent facile hydrolysis to *p*-chlorophenyldiguanide (IV). The isolation of (III) and (IV) from the urine of monkeys dosed with proguanil has been reported by Crounse (1951). Under carefully controlled conditions we have only been able to isolate small amounts of (III) from rabbit urine, and it appears likely that these two "metabolites" arose as secondary products during Crounse's experiments.



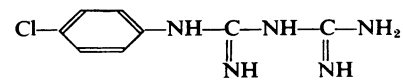
(I)



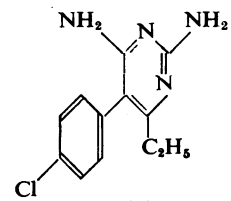
(II)



(III)



(IV)



(V)

* Patent protection pending.

Since as already indicated the antimalarial activity of the dihydrotriazine (II ; R = H) appeared to be of a very similar type to that of proguanil itself, it was now possible to conclude that proguanil owed its potency to the formation of the metabolite ; it was therefore to be expected that other active diguanides would undergo a similar metabolic change. To confirm this view the most potent compound of the diguanide series, N^1-3 : 4-dichlorophenyl-N^5-*iso*propyldiguanide (I ; R = Cl) (Curd, Davey, Hendry, and Rose, 1950 ; Crowther, Curd, Davey, Hendry, Hepworth, and Rose, 1951), was selected for study and administered to rabbits. The urine was extracted in the same way as before. The picrate finally isolated was identical with the picrate of a synthetic specimen of 4 : 6-diamino-1-(3 : 4-dichlorophenyl)-1 : 2-dihydro-2 : 2-dimethyl-1 : 3 : 5-triazine* (II ; R = Cl). To illustrate the relationship in activity between the dihydrotriazines and the corresponding diguanides it might be mentioned here that this member of the series (II ; R = Cl) was approximately 10 times as active against *P. gallinaceum* as the proguanil metabolite (II ; R = H) and therefore about 100 times as active as proguanil itself.

Experimental

Rabbits were dosed orally with a 1% solution of proguanil hydrochloride at the rate of 50 mg./kg. once daily. No toxic symptoms were manifest at this dose, but at 70 mg./kg. delayed deaths occurred. No significant rise in glucuronide or ethereal sulphate in the urine was detected. In fresh urine the amount of diazotizable amine was negligible, but measurable amounts appeared on prolonged storage. This was true of many of the extracts. Proguanil itself was stable under these conditions.

Determination of p-*Chloroaniline Found on Hydrolysis of Urine Fractions.*—The samples were diluted with 0.5N-sodium hydroxide to contain, after hydrolysis, 20–200 μg./ml. of *p*-chloroaniline. Extracts in water-immiscible solvents were first evaporated to dryness. The diluted solution (3 ml.) was heated in a sealed ampoule in a pressure vessel at 120–125° C. for 16–20 hours. (Longer heating of urine samples under these conditions gave no increase in the estimated amount of *p*-chloroaniline.) After cooling, two 1 ml. samples were withdrawn and each diluted with 2 ml. of 0.5N-hydrochloric acid. Both were diazotized and one was coupled with β-sulphato-ethyl-*m*-toluidine (Spinks and Tottey, 1946). The other served as a blank control. The amount of *p*-chloroaniline was estimated colorimetrically, comparison being made with the curve constructed from results obtained with standard solutions of *p*-chloroaniline. The accuracy of the method under routine conditions was not high, but it proved adequate for following fractionation procedures and it was possible to obtain a reasonable material balance after a series of urine treatments.

Acid hydrolysis to *p*-chloroaniline was substantially complete in 4 to 6 hours, but erratic results were obtained in extracts containing high concentrations of salts and especially in the presence of lead.

*Isolation of 4 : 6-Diamino-1-*p*-chlorophenyl-1 : 2-dihydro-2 : 2-dimethyl-1 : 3 : 5-triazine (II ; R = H) from the Urine of Rabbits Dosed with Proguanil.*—Powdered lead acetate was added to the urine (e.g., 10 l.) from several rabbits until further addition caused no more precipitation. (During collection urine was stored at 0–5° C. over chloroform.) The suspension was filtered through Kieselguhr. The filtrate was extracted with butanol (3 × 3 l.). The combined extracts were evaporated *in vacuo* to a volume of 0.5–1 l. Water (about 2 l.) was added and the solution again evaporated *in vacuo*, to remove the butanol, to a volume of about 1 l. The solution was made faintly acid with hydrochloric acid and filtered from the precipitated lead chloride. Hydrogen sulphide was passed into the filtrate and the lead sulphide was removed. (When the acid treatment was omitted and all the lead was precipitated as the sulphide much loss of material occurred through irreversible adsorption.) The solution was freed from excess hydrogen sulphide by a current of air, and basified with concentrated sodium hydroxide solution, the temperature being kept at 0–5° C. by the addition of ice. The solution was extracted with ice-cold ether (300 ml.). The combined ether solutions (A) were reserved for later examination. The aqueous part was cooled by the addition of ice, neutralized with hydrochloric acid, and shaken with butanol (3 × 400 ml.). The butanol extracts were combined and washed with a minimum of dilute sodium hydroxide solution to remove any phenols present. Dilute hydrochloric acid was added gradually to the butanol part with shaking until both phases were neutral. The butanol layer was separated, evaporated *in vacuo*, water was added, and the solution again evaporated. The aqueous solution (100–200 ml.) thus obtained, which had high antimalarial activity, was treated with saturated alcoholic picric acid (10–20 ml.) and stirred. After standing overnight the picrate which had separated was collected and crystallized from ethanol to give canary-yellow needles of 4 : 6-diamino-1-*p*-chlorophenyl-1 : 2-dihydro-2 : 2-dimethyl-1 : 3 : 5-triazine picrate, m.p. 206° C. (Found: C, 42.3 ; H, 3.65 ; N, 22.8, 22.75 ; Cl, 7.3. $C_{11}H_{14}N_5Cl$, $C_6H_3O_7N_3$ requires C, 42.5 ; H, 3.5 ; N, 23.3 ; Cl, 7.4%).

The mother-liquor from the picrate still contained about 10% of the material hydrolysable to *p*-chloroaniline present before the separation of the picrate. It appeared to have little or no antimalarial activity and was not further investigated.

The crude picrate (367 mg.) suspended in 2N-hydrochloric acid (12 ml.) was exhaustively extracted with ether. The aqueous part was filtered through decolorizing carbon and cooled in ice. 10N-Sodium hydroxide was added drop by drop until no more solid

* Patent protection pending.

separated. The crystals were collected and washed with a very little ice-cold water, and dried *in vacuo* over phosphorus pentoxide; 114 mg., m.p. 144° C. The base was dissolved in chloroform (10 ml.), previously saturated with water, and filtered. Ether (5 ml.) and light petroleum (b.p. 40–60° C.) (5.5 ml.) were added to precipitate colourless prisms of the base (II; R=H); 56.4 mg., m.p. 143° C. (A repeat experiment gave m.p. 146° C.). (Found: C, 52.1; H, 5.55; N, 26.8, 27.0. $C_{11}H_{14}N_5Cl$ requires C, 52.5; H, 5.6; N, 27.8%). Ultraviolet absorption in 0.01N-hydrochloric acid: λ max., 240 mμ; log. ε, 4.09.

The base was soluble in cold water, being reprecipitated on the addition of sodium hydroxide, soluble in ethanol, and butanol. It was insoluble in ether, benzene, and dry chloroform and gave no complex on treatment with ammoniacal copper sulphate solution.

It was fully active against a blood-induced infection by *P. gallinaceum* in chicks when given orally at 0.01–0.025 mg./50 g., b.i.d. × 3½.

The ether extract (A) from several batches of the urine concentrate was evaporated to a small bulk and extracted with dilute hydrochloric acid. The aqueous part was neutralized by aqueous ammonia. The solid precipitated was collected, washed with water, and dried. It consisted of N^1-*p*-chlorophenyl-N^5-*iso*propyldiguanide hydrochloride (proguanil hydrochloride) (I; R=H) (cf. Spinks and Tottey, 1946). The filtrate was made strongly alkaline by the further addition of ammonia. An aqueous solution of copper sulphate was added to precipitate the rest of the proguanil as its copper complex. The mixture was filtered. There were no other detectable bases in the precipitate when freed from copper. The total recovery of proguanil represented about 10% of the administered drug. Hydrogen sulphide was passed through the filtrate, the precipitated copper sulphide removed, and the solution, which was devoid of antimalarial activity, was treated with picric acid. The precipitated picrate was collected and crystallized from ethanol to give orange-yellow needles in small amount of 4-amino-6-*p*-chloroanilino-1:2-dihydro-2:2-dimethyl-1:3:5-triazine picrate, m.p. 235° C. alone and mixed with an authentic specimen. Crounse (1951) gave m.p. 238° C.

Isolation of (II; R=H) from the Faeces of Rabbits Dosed with Proguanil.—The faeces were collected, air-dried, ground to a powder with anhydrous sodium sulphate, and exhaustively extracted with ether. Negligible amounts of *p*-chloroaniline derivatives were removed. The solid was treated with methanol by percolation and the extract was evaporated *in vacuo* to leave an aqueous solution and some tarry material. The residue was triturated repeatedly with dilute hydrochloric acid and the extracts were treated as in the isolation of the metabolite from urine from the stage at which the lead salts had been removed. The crude picrate was crystallized from ethanol to give canary-yellow needles, m.p. 206° C. undepressed on admixture with the picrate of (II; R=H). Ultraviolet absorption after conversion to a solution of the hydrochloride, in 0.01N-HCl: λ max., 240 mμ; log. ε, 4.07. The antimalarial activity was comparable with that of the metabolite obtained from the urine. The yield of the picrate was about 1% based on the administered proguanil.

Isolation of (II; R=H) from the Urine of Human Volunteers Dosed with Proguanil.—Two volunteers took a total of 6 g. of proguanil hydrochloride at the rate of 1 g. per day each for 3 days. The pooled urine was treated exactly as described for rabbit urine above. The yield of crude picrate of (II; R=H) was 608 mg. representing 5% based on the administered dose. The picrate, crystallized from *iso*propanol, had m.p. 209–212° C. and did not depress the m.p. of the picrate of (II; R=H). It was converted into the base which was crystallized from moist chloroform, ether and light petroleum (b.p. 40–60° C.) to give pure (II; R=H), m.p. 145° C., undepressed in admixture with authentic material. The base showed the expected antimalarial activity.

*Isolation of 4:6-Diamino-1-(3:4-dichlorophenyl-1:2-dihydro-2:2-dimethyl-1:3:5-triazine (II; R=Cl) from the Urine of Rabbits Dosed with N^1-3:4-Dichlorophenyl-N^5-*iso*propyldiguanide Hydrochloride.*—The drug was given at a dose of 30 mg./kg. once daily. Higher doses caused a high proportion of deaths amongst the rabbits. The urine was extracted as in the proguanil experiments. The yield of crude picrate corresponded to 0.75% based on the administered diguanide. Crystallization from aqueous 2-ethoxyethanol gave canary-yellow needles of the picrate of (II; R=Cl), m.p. 197–198° C. alone and mixed with an authentic synthetic sample. Ultra-violet absorption after conversion to a solution of the hydrochloride, in 0.01N-HCl: λ max., 240 mμ; log. ε, 4.09, in 0.1N-NaOH (after heating at 100° C. for 20 minutes in 0.1N-NaOH): λ max., 260 mμ; log. ε, 4.25. [The corresponding figures for an authentic synthetic sample were, in 0.01N-HCl: λ max., 240 mμ; log. ε, 4.10, in 0.1N-NaOH (after heating at 100° C. for 20 minutes in 0.1N-NaOH): λ max., 262 mμ; log. ε, 4.23.]

Discussion

Several workers have attempted to elucidate the mode of action of proguanil by examining the effect of *p*-aminobenzoic acid and of pteroylglutamic acid on the properties of the drug (for references see Greenberg and Richeson, 1951). With the isolation of a highly active metabolite, however, coupled with the earlier reports that proguanil had no antimalarial activity *in vitro*, it now seems clear that the inhibition of the bactericidal action of proguanil on *Lactobacillus casei in vitro* by pteroylglutamic acid (Falco, Hitchings, Russell, and VanderWerff, 1949) has no bearing on the antimalarial effect of the drug, nor indeed have the results of any work *in vitro*. On the other hand, the inhibition of the action of proguanil *in vivo* against *P. gallinaceum* by pteroylglutamic acid (Greenberg, 1949) and against *P. berghei* by

p-aminobenzoic acid (Thurston, 1950) must have some significance concerning the mode of action of the metabolite. It is unlikely that either pteroylglutamic acid or *p*-aminobenzoic acid interferes with the formation of the metabolite since the former had no effect on the action of proguanil against *P. berghei* (Thurston, 1950) and the latter had no effect on the action against *P. gallinaceum* (Bishop and McConnachie, 1948).

The similarity in structure between proguanil and the antimalarial 2:4-diamino-5-aryloxypyrimidines has been pointed out by Falco, Hitchings, Russell, and VanderWerff (1949). There is a much more remarkable resemblance between the structure of the proguanil metabolite (II; R=H) and the most active of the 2:4-diamino-5-aryl-pyrimidines (V) of Russell and Hitchings (1951) and Falco, Goodwin, Hitchings, Rollo, and Russell (1951).

Summary

1. A metabolite of "Paludrine" (proguanil) has been isolated from the urine and faeces of rabbits and from the urine of human volunteers dosed with the drug. The metabolite, 4:6-diamino-1-*p*-chlorophenyl-1 : 2-dihydro-2 : 2-dimethyl-1 : 3 : 5-triazine (II; R=H) had an activity about 10 times that of proguanil against blood-induced infections of *P. gallinaceum* in chicks and was also active against the exo-erythrocytic forms of the parasite.

2. Only insignificant amounts of its isomer, 4-amino-6-*p*-chloroanilino-1 : 2-dihydro-2 : 2-dimethyl-1:3:5-triazine (III), were present in the rabbit urine.

3. 4:6-Diamino-1-(3:4-dichlorophenyl)-1 : 2-dihydro-2:2-dimethyl-1:3:5-triazine (II; R=Cl), isolated from the urine of rabbits dosed with N^1-3:4-dichlorophenyl-N^5-*iso*propyldiguanide, was about 100 times as active as proguanil against the blood forms of *P. gallinaceum* in chicks.

References

Birtwell, S., Curd, F. H. S., Hendry, J. A., and Rose, F. L. (1948). *J. chem. Soc.*, 1645.

Bishop, A., and McConnachie, E. W. (1948). *Nature, Lond.*, **162**, 541.

Carrington, H. C., Crowther, A. F., Davey, D. G., Levi, A. A., and Rose, F. L. (1951). Ibid., **168**, 1080.

Crounse, N. N. (1951). *J. org. Chem.*, **16**, 492.

Crowther, A. F., Curd, F. H. S., Davey, D. G., Hendry, J. A., Hepworth, W., and Rose, F. L. (1951). *J. chem. Soc.*, 1774.

Curd, F. H. S., Davey, D. G., Hendry, J. A., and Rose, F. L. (1950). *Brit. J. Pharmacol.*, **5**, 438.

Davey, D. G. (1946). *Ann. trop. Med. Parasit.*, **40**, 52.

Falco, E. A., Goodwin, L. G., Hitchings, G. H., Rollo, I. M., and Russell, P. B. (1951). *Brit. J. Pharmacol.*, **6**, 185.

—— Hitchings, G. H., Russell, P. B., and VanderWerff, H. (1949). *Nature, Lond.*, **164**, 107.

Greenberg, J. (1949). *Proc. Soc. exp. Biol. Med.*, **71**, 306.

—— and Richeson, E. M. (1951). Ibid., **77**, 174.

Hawking, F. (1947). *Nature, Lond.*, **159**, 409.

—— and Perry, W. L. M. (1948). *Brit. J. Pharmacol.*, **3**, 320.

Madinaveitia, J., and Raventos, J. (1949). Ibid., **4**, 81.

Russell, P. B., and Hitchings, G. H. (1951). *J. Amer. chem. Soc.*, **73**, 3763.

Spinks, A., and Tottey, M. M. (1946). *Ann. trop. Med. Parasit.*, **40**, 101.

Thurston, J. P. (1950). *Lancet*, **259**, 438.

Tonkin, I. M. (1946). *Brit. J. Pharmacol.*, **1**, 163.