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Possible chloroquine-induced modification of N-acetylation of isoniazid and sulphadimidine in the rat

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Chloroquine, a 4-aminoquinoline derivative widely used in the treatment of malaria [1] affects a wide range of biochemical processes in the living cell [2–4]. For instance, it is known to alter the permeability of the lysosomes resulting in increased levels of hydrolytic enzymes [5]. It also interferes with protein synthesis [6]. We have previously reported the chloroquine-induced alterations in rat hepatic microsomal components resulting in decreased activity of some of the drug oxidizing enzymes which we suggested could arise from changes in the endoplasmic reticulum membrane fluidity [7].

Following an interest in the consequences of the combined administration of isoniazid (INH) or sulphadimidine (SDM) with chloroquine to tuberculosis patients during

periods of malarial attacks common in our community, and our continued interest in the interference of chloroquine with the drug metabolizing enzyme system, we have studied the influence of therapeutic dose chloroquine administration on the N-acetylation, the major route of metabolism for INH and SDM.

Materials and Methods

Chemicals. Isoniazid(4-pyridine carboxylic acid hydrazide) and sulphadimidine[4-amino-*N*-(4,6-dimethyl-2-pyrimydinyl)-benzensulphonamide] were bought from the drug stores. They were extracted and recrystallized with ethanol. Purity was checked by determining the melting point of the crystals and ultraviolet absorption.

Acetyl derivatives of the drugs were synthesized by suspending 1 g of the drug in 10 mL of warm glacial acetic acid to which 2 mL of acetic anhydride was added with stirring. The crystals that formed on cooling were recrystallized from ethyl acetate as colourless needles and were characterized by their melting points and ultraviolet absorptions.

Chloroquine was purchased as injectable chloroquine phosphate (40 mg base/mL) a product of IPORG (F.R.G.).

Animals and pretreatment. For each drug, three groups, each with six mixed male and female Wistar albino rats, weighing between 125–200 g were kept in plastic cages. They were fed with commercial rat chow (Ladokun Feeds Nigeria Limited) and liberally supplied with water.

Two groups were treated with chloroquine 10 mg/kg body weight administered intraperitoneally for either 4 consecutive days for the multiple dose chloroquine treatment group or for the single dose chloroquine treatment group the same dose was given only once. The third group was kept as control and received physiological saline.

Rats were transferred to metabolic cages on the last day of chloroquine treatment and 24 hr after the last treatment, urine was collected from all rats and used as reference, then test animals were given (i.p.) either 10 mg/kg body weight INH or 40 mg/kg body weight SDM. Urine was collected after 24 hr in each case.

Analysis of urine samples. INH in urine was analysed by the method of Kelly and Poet [8]. Acetylisoniazid (AcINH) in the urine was determined by the colorimetric method of Ventakaraman *et al.* [9], using chloramine T and measuring absorbances at 550 nm.

Free and total SDM were estimated by the method of Varley [10] and basically involved a micro-modification of the Bratton and Marshall reaction [11]. Absorbances were read at 540 nm. The amount of SDM excreted in the acetylated form was obtained by determining the total SDM before and after hydrolysis with 1 mL 2 M HCl as above, from which the acetyl derivative was represented by the difference.

In vitro *assay of* N-*acetyltransferase activity.* Liver supernatants (15,000 *g*) were prepared from both chloroquine pretreated and control rats by centrifugation in 0.1 M phosphate buffer. The procedure of Johnson and Corte [12] was used to estimate the *N*-acetyltransferase activity using INH as substrate.

Results and Discussion

The results obtained after administration of the drugs alone and on prior treatment with chloroquine demonstrated that chloroquine increased the urinary excretion of free drug from 26.8 to 32.4% for INH (Table 1) and from 34.8 to 40.9% (Table 2) for SDM under multiple-dose chloroquine pretreatment conditions.

Concomitant with this increased free drug excretion was a decrease in the amount of acetylated metabolites excreted in both cases with values decreasing from 9.3% of dose in normals to 2.9% in chloroquine pretreated animals for INH, while for SDM it decreased from 35.8 to 23.9% (Tables 1 and 2). These results were found to be statistically significant ($P < 0.001$) with the Student's *t*-test.

If chloroquine was given as a single dose before administration of the drugs, a similar pattern was observed and the excretion of free drugs equally rose from 29.3 to 32.3% (normal to chloroquine-pretreated) for INH (Table 1), and 35.4 to 40.5% for SDM (Table 2). The depression in acetylated metabolite was also obtained with values dropping from 9.1 to 4.9% for INH (Table 1) and 40.2 to 30.5% for SDM (Table 2).

However, these effects were less pronounced than with the multiple chloroquine pretreatment condition. These results indicate that chloroquine has an inhibitory effect on the acetylation of the antitubercular drug, INH and the antibacterial SDM, *in vivo*.

The similarity in the pattern of chloroquine inhibition of these drugs as reported in this work is significant and corroborates earlier findings by investigators who have demonstrated that both INH and SDM are metabolized by the same hepatic enzyme, *N*-acetyltransferase [13–15]. For both drugs therefore, the established pattern of metabolism involves primarily an *N*-acetylation to form the acetylated derivative, followed by excretion of the unchanged drug. For INH, there is the additional pathway of hydrolysis for both INH and the acetylated metabolite to isonicotinic acid (INA) [16] (Fig. 1).

Further work to investigate the mechanism by which chloroquine increases the excretion of free drug while simultaneously decreasing that of the acetylated metabolite was carried out in stages as follows: from Fig. 1, it becomes apparent that chloroquine could be exerting its influence essentially at either or both steps in the metabolic route of INH as indicated:

—in one, it could be acting at step 1 by directly inhibiting

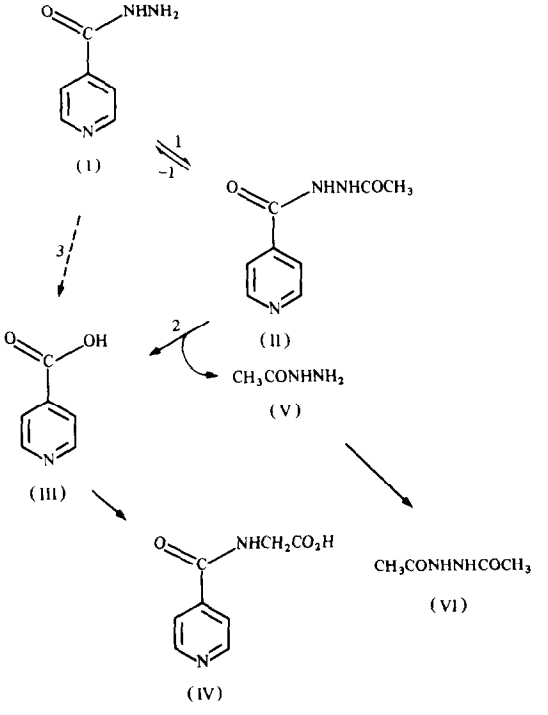

Fig. 1. Metabolism of isoniazid to the acetyl derivative and other metabolites. (I) Isoniazid (INH); (II) acetylisoniazid (AcINH); (III) isonicotinic acid (INA); (IV) isonicotinylglycine (INAG); (V) acetylhydrazine; (VI) diacetylhydrazine. Step 1, *N*-Acetyltransferase reaction; Step −1, deacetylation reaction; Step 2, hydrolysis of AcINH to INA; Step 3, hydrolysis of INH to INA.

Table 1. Effect of chloroquine pretreatment on 24-hr urinary excretion of isoniazid (10 mg/kg, i.p.)

Treatment administered	Mean rat weight (g)	Dose (mg/INH)	Amount excreted in urine in 24 hr as (mg): Free INH	AcINH	INH Acetylated*	Total	% of dose excreted in 24 hr: Free INH	INH Acetylated	Total INH
M.D.									
Control	153.00 ± 25.00	1.53 ± 0.25	0.41 ± 0.07	0.19 ± 0.05	0.14 ± 0.03	0.55 ± 0.09	26.88 ± 3.59	9.26 ± 1.23	36.00 ± 2.84
Test	154.00 ± 18.00	1.54 ± 0.18	0.50 ± 0.06	0.06 ± 0.03	0.04 ± 0.02	0.54 ± 0.07	32.43 ± 2.68	2.90 ± 1.26†	35.14 ± 3.32
S.D.									
Control	151.00 ± 10.00	1.50 ± 0.10	0.44 ± 0.05	0.18 ± 0.04	0.14 ± 0.03	0.58 ± 0.06	29.31 ± 2.82	9.13 ± 1.30	38.51 ± 2.70
Test	148.00 ± 12.00	1.48 ± 0.12	0.48 ± 0.04	0.10 ± 0.04	0.07 ± 0.02	0.55 ± 0.05	32.25 ± 1.93	4.96 ± 0.81†	37.13 ± 1.32

Values are given as the mean ± SD for at least six experiments (involving six rats each).
M.D. and S.D. represent multiple- and single-dose chloroquine pretreatments, respectively.
* Actual amount of INH acetylated was calculated from recovered AcINH in the urine.
† Values statistically significant at $P < 0.001$, using the Student's *t*-test.

Table 2. Effect of chloroquine pretreatment on 24-hr urinary excretion of sulphadimidine (40 mg/kg, i.p.)

Treatment administered	Mean rat weight (g)	Dose (mg/SDM)	Amount excreted in urine in 24 hr as (mg): Free sulphadimidine	N^4-AcSDM	Total SDM	% of dose excreted in 24 hr: Free SDM	N^4-AcSDM	Total SDM
M.D.								
Control	203.00 ± 42.00	8.07 ± 1.71	2.85 ± 0.69	2.90 ± 0.67	5.71 ± 1.36	34.80 ± 1.83	35.81 ± 1.42	70.48 ± 2.89
Test	184.00 ± 28.00	7.35 ± 1.13	3.01 ± 0.48	1.76 ± 0.35*	4.77 ± 0.79	40.94 ± 2.93	23.98 ± 3.08*	64.92 ± 4.84
S.D.								
Control	194.00 ± 33.00	7.75 ± 1.30	2.75 ± 0.60	3.13 ± 0.70	5.87 ± 1.35	35.40 ± 5.00	40.17 ± 5.70	75.60 ± 10.50
Test	190.00 ± 24.00	7.90 ± 1.00	3.20 ± 0.48	2.43 ± 0.50	5.63 ± 0.90	40.54 ± 2.80	30.50 ± 4.50	71.10 ± 5.50

Values are given as the mean ± SD for at least six experiments (involving six rats each).
M.D. and S.D. represent multipe- and single-dose chloroquine pretreatments, respectively.
* Values are significant at $P < 0.05$, using the Student's *t*-test.

Table 3. Effect of chloroquine pretreatment on 24-hr urinary excretion of *N*-acetylisoniazid (10 mg/kg, i.p.)

Treatment administered	Mean rat weight (g)	Dose (mg/*N*-AcINH)	Amount excreted in urine in 24 hr as (mg): Free INH	*N*-AcINH	Total *N*-AcINH	% of dose excreted in 24 hr: Free INH	*N*-AcINH	Total *N*-AcINH
M.D.								
Control	181.00 ± 19.00	1.80 ± 0.20	0.34 ± 0.04	0.59 ± 0.01	0.92 ± 0.02	18.68 ± 2.55	35.28 ± 5.64	50.96 ± 7.70
Test	172.00 ± 23.00	1.72 ± 0.23	0.18 ± 0.06	0.71 ± 0.20	0.89 ± 0.22	10.49 ± 3.48*	41.13 ± 8.48	51.62 ± 10.20
S.D.								
Control	142.00 ± 9.00	1.42 ± 0.09	0.21 ± 0.05	0.49 ± 0.07	0.70 ± 0.05	14.90 ± 3.57	34.42 ± 6.40	49.30 ± 6.50
Test	151.00 ± 13.00	1.51 ± 0.13	0.20 ± 0.02	0.56 ± 0.09	0.76 ± 0.08	13.00 ± 1.80	37.15 ± 4.50	50.15 ± 4.25

Values are given as the mean ± SD for at least four experiments (involving six rats each).
M.D. and S.D. represent multiple- and single-dose chloroquine pretreatments, respectively.
* Values not statistically significant at $P > 0.05$, using the student's *t*-test.

Table 4. Effect of chloroquine pretreatment on 24-hr urinary excretion of N^4-acetylsulphadimide (40 mg/kg, i.p.)

Treatment administered	Mean rat weight (g)	Dose (mg/N^4-AcSDM)	Amount excreted in urine in 24 hr as (mg): Free SDM	N^4-AcSDM	Total SDM	% of dose excreted in 24 hr: Free SDM	N^4-AcSDM	Total SDM
M.D.								
Control	159.00 ± 4.00	6.36 ± 0.10	0.91 ± 0.22	3.61 ± 0.40	4.52 ± 0.20	14.30 ± 3.70	56.70 ± 5.18	70.99 ± 2.30
Test	170.00 ± 10.00	6.78 ± 0.40	0.36 ± 0.13	5.02 ± 0.20	5.37 ± 0.18	5.26 ± 1.80	74.00 ± 3.90	79.20 ± 3.00
S.D.								
Control	144.00 ± 4.00	5.75 ± 0.20	0.80 ± 0.11	3.17 ± 0.10	3.96 ± 0.21	13.82 ± 1.62	55.07 ± 0.20	68.88 ± 1.70
Test	155.00 ± 4.00	6.20 ± 0.14	0.79 ± 0.12	3.80 ± 0.35	4.59 ± 0.34	12.69 ± 2.02	61.31 ± 5.50	74.00 ± 5.50

Values are given as the mean ± SD for at least four experiments (involving six rats each).
M.D. and S.D. represent multiple- and single-dose chloroquine pretreatments, respectively.

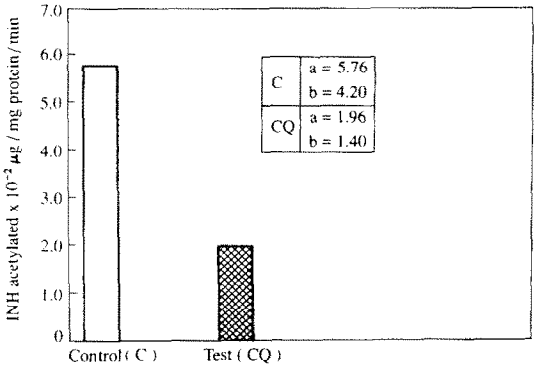

Fig. 2. A bar chart representation of the inhibitory effect of chloroquine on the activity of rat hepatic *N*-acetyltransferase (EC 2.3.1.5). Enzyme activity values are expressed in: (a) $\times 10^{-2}$ μg INH acetylated/mg protein/min. (b) $\times 10^{-4}$ μmole INH acetylated/mg protein/min. CQ, chloroquine treated; C, control experiment.

the *N*-acetyltransferase enzyme activity. This would lead to decreased acetylation and a corresponding accumulation of unmetabolized drug to account for the observed pattern of excretion;

—in the other possibility, chloroquine could be acting at step −1 by enhancing the rate of hydrolysis (deacetylation) of the acetylated metabolite back to the free drug. This would imply that reaction step 1 was inherently reversible and that chloroquine promotes the reverse reaction.

It was also probable that the two postulated possibilities co-operated to produce the observed result.

The possible existence of an enhanced deacetylation process by chloroquine (step −1) was investigated through administration of acetyl derivatives of the drugs. This effectively by-passed the *N*-acetyltransferase reaction (step 1).

Results obtained here demonstrated that a deacetylation pathway was active since both AcINH and AcSDM gave 18.7 and 14.3% of administered dose as deacetylated drugs, respectively, in the urine when administered alone (Tables 3 and 4). This result is in conformity with the report of Bridges and Williams [17] who had earlier implicated the deacetylation process as a significant factor in the acetylation metabolism in animals. On multiple dose chloroquine pretreatment, the percentage of free drugs excreted on administration of the acetylated derivatives was found to decrease significantly from 18.7 to 10.5% for AcINH and from 14.3 to 5.2% for AcSDM (Tables 3 and 4). Single dose chloroquine pretreatment only minimally inhibited the deacetylation reaction. Values of 14.9 and 13% were obtained for control and test, respectively, for AcINH, and 13.8 and 12.7% for AcSDM (Tables 3 and 4).

These results appear therefore to strongly suggest that the increased level of the free drugs coupled with a decreased acetylated metabolite observed after administration of either INH or SDM to chloroquine treated rats must be due to inhibition of the acetylation of the drugs rather than enhanced deacetylation of the acetylated metabolite. Furthermore, the higher values of AcINH over controls obtained when synthetic AcINH is administered shows that the decrease in the excretion of endogeneously derived AcINH was not due to a chloroquine enhancement of the hydrolysis stage (step 2) of INH metabolism.

Results from our *in vitro* studies tend also to confirm this view. Chloroquine inhibited the activity of the *N*-acetyltransferase enzyme in INH metabolism. The decrease in the acetylating ability of the enzyme due to chloroquine treatment was found to be as low as to 34% of control values (Fig. 2).

In summary, the effect of chloroquine, a 4-aminoquinoline antimalarial administered intraperitoneally as a multiple dose (10 mg/kg body weight daily for 4 consecutive days), or as a single dose (same dose, once) on the *N*-acetylation of the anti-tubercular drug, isoniazid and the antibacterial, sulphadimidine has been investigated *in vivo* and *in vitro* in the rat.

Chloroquine decreased the urinary excretion of the acetyl metabolites while increasing the excretion of the free drugs. These effects were chloroquine-dose related being more pronounced with the multiple dose treatment. Administration of the synthetic *N*-acetyl derivatives of both isoniazid and sulphadimidine indicated that chloroquine also decreased the urinary excretion of the acetylated forms but without increasing their hydrolysis to the free forms.

In vitro studies appear to confirm a direct inhibition of the hepatic *N*-acetyltransferase enzyme activity by chloroquine. The implication of these observations in combination therapy with these drugs should be noted.

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Mechanisms of interactions between organic anions and the organic cation transporter in renal brush border membrane vesicles

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Although data in the literature generally suggest that organic anions do not inhibit the renal transport of organic cations and vice versa [1], there are also studies suggesting that such interactions may occur [2–6]. In particular, a number of studies have demonstrated that probenecid, an organic anion, inhibits the renal transport of cimetidine, an organic cation, in isolated renal cortical preparations [2–5]. Recently, we carried out experiments to determine whether the observed interaction between cimetidine and probenecid was unique to these two compounds or whether organic anions may generally inhibit organic cation transport [5]. We also ascertained if the interactions between anions and cations occurred at the brush border membrane, the site of active organic cation transport in the proximal tubule. Our data suggested that both probenecid and furosemide inhibited the uptake of the model organic cation, N^1-methylnicotinamide (NMN*), in brush border membrane vesicles prepared from rabbit renal cortex. The interaction between probenecid and NMN was competitive. Since probenecid is structurally dissimilar from cimetidine, at least in terms of charge, we speculated that the interaction may involve hydrophobic displacement. The purpose of this study was to examine further the nature of the interactions between organic anions and organic cations.

Methods

Brush border membrane vesicles were prepared from rabbit renal cortex by a modification of the divalent cation precipitation procedure developed by Booth and Kenny [7]. The modified method has been described in detail previously [8]. The transport of [^{3}H]NMN (sp. act. 2.8 to 3.0 Ci/mmol, ICN, Irvine, CA) was studied in brush border membrane vesicles in which an outwardly directed proton gradient was imposed. Briefly, brush border membranes were resuspended in a buffer containing 10 mM HEPES and 150 mM KCl at pH 6.0 and adjusted to protein concentrations of 15–20 mg/mL. Vesicles (10 μl) were incubated with 20 or 40 μL (pH 7.4) buffer (10 mM HEPES, 150 mM KCl adjusted to pH 7.4 with KOH) containing [^{3}H]NMN (2.0 μM) alone or [^{3}H]NMN (2.0 μM) together with various unlabeled compounds. Incubations were carried out at 25° for 8 sec and 120 min. Michaelis–Menten studies examining the mechanism of interaction of furosemide with the organic cation transport system were carried out by determining the 8-sec uptake of NMN (0.25, 0.5, 1.0, 2.5, 5.0, 10 and 25 mM as 2.0 μM [^{3}H]NMN plus unlabeled NMN) in the presence and absence of 20 mM furosemide. Incubations were stopped by rapid filtering of the incubates over 0.3-μm cellulose nitrate filters (Millipore Corp., Bedford, MA) which had been placed on a vacuum filtration manifold (Hoefer Scientific Instruments, San Francisco, CA). The amount of radioactivity associated with the filters was determined by liquid scintillation counting. [^{3}H]NMN binds to vesicles only minimally [8], and no correction was made for this vesicle-associated radioactivity. In each experiment, three replicate determinations were made to generate a single point. Experiments in separate brush border membrane vesicle preparations were used to generate mean values. The data are reported as means ± SE. In the studies in which the effect of furosemide on the transport of NMN was evaluated, Lineweaver–Burk plots of the data obtained in each individual experiment were constructed to obtain the apparent K_m and V_{max}.

Results and Discussion

Figure 1 depicts the effects of probenecid and the three congeners, *p*-(monopropylsulfamyl)benzoic acid (probenecid monopropyl), *p*-sulfamyl benzoic acid (probenecid depropyl) and *p*-(dibutylsulfamyl)benzoic acid (probenecid dibutyl), on the uptake of NMN determined at 8 sec. Probenecid (both 3 and 10 mM), probenecid monopropyl (10 mM) and probenecid dibutyl (3 mM) significantly reduced the initial uptake of NMN (probenecid 10 mM, 0.19 ± 0.03 pmol/mg; probenecid 3 mM, 0.25 ± 0.08 pmol/mg; probenecid monopropyl 10 mM, 0.40 ± 0.04 pmol/mg; and probenecid dibutyl 3 mM, 0.41 ± 0.09 pmol/mg) versus control (0.87 ± 0.14 pmol/mg, $P < 0.05$). The 8-sec uptake of NMN in the presence of probenecid depropyl (10 mM) was not significantly different (0.69 ± 0.09 pmol/mg) from the control. None of the compounds inhibited the uptake of NMN at 2 hr suggesting that neither vesicle integrity nor volume was altered by the compounds.

The effect of furosemide (20 mM) on the initial uptake of NMN is depicted in a Lineweaver–Burk plot shown in Fig. 2. As shown, furosemide appeared to interact competitively with NMN. The apparent K_m of NMN was

* Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; NMN, N^1-methylnicotinamide; SITS, 4-acetamido-4′-isothiocyano-2,2′-disulfonic stilbene; and DIDS, 4,4′-diisothiocyano-2,2′-disulfonic stilbene.