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Quaternary Ammonium Compounds Efficiently Inhibit *Plasmodium falciparum* Growth In Vitro by Impairment of Choline Transport

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Hemicholinium 3, decamethonium, and decyltrimethylammonium previously have been demonstrated to be efficient inhibitors, with 50% inhibitory concentrations of 4×10^{-6}, 10^{-6}, and 7×10^{-7} M, respectively. We show that lengthening of the alkyl chain of decyltrimethylammonium by successive additions of two carbon atoms up to hexadecyltrimethylammonium results in a very low 50% inhibitory concentration of 5×10^{-7} M for dodecyltrimethylammonium. Furthermore, hemicholinium 3 and decamethonium exerted their antiplasmodial activity, regardless of the developmental stage of the parasite, whereas decyltrimethylammonium was particularly lethal for the mature forms. After infected erythrocytes with radioactive choline were supplied, the determination of the water-soluble choline-containing compounds, as well as the assay of choline kinase activity, showed that the specific inhibition of phosphatidylcholine biosynthesis is related to the impairment of choline entry into erythrocytes. Thus, the impairment of the transport of choline, a natural polar head group of phospholipids, appears to be lethal for *Plasmodium falciparum* in vitro and could be a reasonable approach for a new malaria chemotherapy.

The urgent need for new therapeutic approaches to *Plasmodium falciparum* malaria often has been pointed out (18) because of the resistance of both mosquitoes and parasites to various pesticides and conventional drugs (27). We have shown previously that phospholipid (PL) metabolism could constitute an ideal target for a new chemotherapy (25) because of its magnitude (9, 21, 24, 26) and specificity, because little or no PL biosynthesis occurs in mature mammalian erythrocytes (23). In our previous studies we showed that the lethal effect of quaternary ammonium compounds on *P. falciparum* in vitro involved the inhibition of phosphatidylcholine (PC) biosynthesis from choline, the natural polar head group of PC (2). Furthermore, we have shown that no alteration in the biosynthesis of the other PL, or of proteins or nucleic acids, occurred. In this study we establish that this inhibition of PC biosynthesis is due to an impairment of choline transport into infected erythrocytes. In addition, we show that the bis-onium compounds act regardless of the developmental stage of the parasite, whereas the monoammonium compound that was tested, decyltrimethylammonium, appears to be most lethal for the maturing parasites (i.e., the schizont form).

MATERIALS AND METHODS

Chemicals. Bromide salts of decyltrimethylammonium (DTMA), dodecyltrimethylammonium, tetradecyltrimethylammonium, hexadecyltrimethylammonium, decamethonium (DM), and hemicholinium 3 (HC3) were obtained from Sigma Chemical Co., St. Louis, Mo.; [*methyl*-^{14}C]choline chloride was purchased from Amersham Corp. (United Kingdom); RPMI 1640 medium was obtained from GIBCO Laboratories (France); modified RPMI 1640 without choline, inositol, and serine was provided by Eurobio (France). AB^+ human blood or AB^+ human serum came from the Blood Bank of Montpellier. All reagents were of analytical grade.

Obtaining *P. falciparum*-infected erythrocytes. The Nigerian strain of *P. falciparum* (W. H. Richard, Wellcome Research Laboratories, Beckenham, England [Div. Burroughs Wellcome Co.] [17]) was maintained by serial passage in AB^+ human erythrocytes suspended in complete medium [medium A: RPMI 1640 supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer and 10% AB^+ serum] at 37°C by the petri dish candle jar method (13). In some experiments, parasites that were synchronized twice by 5% sorbitol treatments (15) were used to infect freshly washed, uninfected erythrocytes.

Obtaining *Plasmodium knowlesi*-infected erythrocytes. Splenectomized *Macaca fascicularis* monkeys (weight, 3 to 6 kg; Sanofi, Montpellier, France), were infected with cryopreserved (20) *P. knowlesi* Washington, variant 1 (from G. Mitchell, Guy's Hospital, London). On days 6 to 9, highly infected blood cells were collected and then washed with basic medium (medium B: RPMI 1640 supplemented with 25 mM HEPES [pH 7.4] containing 40 μM choline and inositol and 140 μM serine). Leukocytes were eliminated by passage through a cellulose powder column (CF 11; Whatman, Inc., Clifton, N.J.) (10). After two washes with medium B, the infected erythrocytes were ready for biochemical experiments.

Assays of drug effects on *P. falciparum* growth in vitro. Freshly washed erythrocytes were infected with a stock solution of *P. falciparum*-infected cells (4 to 10% parasitemia, i.e., percent infected erythrocytes), producing a 0.2 to 0.3% parasitemia. Each well of a 24-well plate (Becton Dickinson Labware, Oxnard, Calif. [Div. Becton Dickinson and Co.]) contained 0.3 ml of this parasitized erythrocyte suspension and 0.3 ml of medium A without (control) or with the drug at twice the desired concentration, bringing the hematocrit to 7%. One day later, media were carefully removed and replaced with 0.6 ml of medium A containing the drug at the appropriate concentration. After an additional period of 24 h, media were replaced by fresh medium A. On day 4, parasitemia of control and treated samples was monitored on blood smears with a 10% Giemsa Azure type B stain in phosphate buffer (pH 7.2). Growth inhibition in the presence of the various compounds was expressed as a

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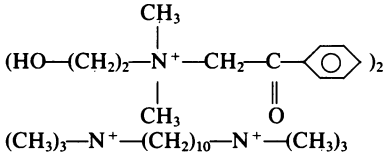
TABLE 1. General structures of quaternary ammonium compounds and IC_{50}s against *P. falciparum* in vitro

Compound	Chemical structure	IC_{50} (M)[a]
HC3		4×10^{-6}
DM	$(CH_3)_3—N^+—(CH_2)_{10}—N^+—(CH_3)_3$	10^{-6}
DTMA	$CH_3—(CH_2)_9—N^+—(CH_3)_3$	7×10^{-7}
Dodecyltrimethylammonium	$CH_3—(CH_2)_{11}—N^+—(CH_3)_3$	5×10^{-7}
Tetradecyltrimethylammonium	$CH_3—(CH_2)_{13}—N^+—(CH_3)_3$	9×10^{-7}
Hexadecyltrimethylammonium	$CH_3—(CH_2)_{15}—N^+—(CH_3)_3$	8×10^{-7}

[a] IC_{50}s (after 48 h of drug application) were derived from plots of percent inhibition versus the log of the drug concentration. Each value is the mean of at least two independent experiments carried out in triplicate.

percentage of control parasitemia. Each experiment was performed independently two times with various concentrations of drugs in triplicate.

Biochemical studies. Metabolism of radioactive precursors was monitored in *P. knowlesi*-infected erythrocytes preincubated for various times in the presence of different concentrations of each of the test compounds. Preincubation was performed in medium B, at a hematocrit of 26 to 35%, in a shaking water bath at 37°C. These suspensions were then incubated with radioactive choline in enriched medium containing 25 mM HEPES buffer (pH 7.4), 1 mM ATP, 30 μM coenzyme A, and 0.5 mM of the essential fatty acids in plasma, i.e., palmitic, stearic, oleic, and linoleic acids in a molar ratio of 1.7:0.7:1.3:1.3, respectively, bound to fat-free bovine serum albumin (17 to 20 mg/ml) (medium C), resulting in a final hematocrit of 13 to 27%. After a 30-min incubation at 37°C, reactions were stopped at 4°C. The cells were then pelleted at 10,000 × *g* min and washed at 4°C, once with 4 ml of medium B and twice with 4 ml of 0.9% NaCl containing 40 μM cold choline.

Cellular lipids were extracted by the method of Folch et al. (7), as modified previously (19), and then fractionated by thin-layer chromatography as described previously (25). The aqueous extracts of the procedure described by Folch et al. (7) were harvested and evaporated at 37°C under N_2. Residues were suspended in ethanol-water (1:1); choline and its metabolites were then separated by silica gel thin-layer chromatography developed in CH_3OH–0.5% NaCl–25% NH_4OH (100:100:10 [vol/vol/vol]) (22). After visualization of appropriate standards with iodine vapor, radioactive spots were counted with a Packard 460 CD liquid scintillation spectrometer. In the case of aqueous soluble metabolites containing choline, 1.6 ml of 15% CH_3COOH was added to the scraped silica gel before the scintillation cocktail (Packard no. 299) was added. No measurable radioactivity was found, apart from the spots corresponding to the metabolites mentioned in the figures. The amount of labeled precursor incorporated into cellular lipids, or into choline metabolites, was computed on the basis of the amount of radioactivity incorporated into the various metabolites and the specific radioactivity of the precursor in the incubation medium. Results are expressed as nanomoles per 10^7 cells per time.

Choline kinase assay. Choline kinase activity was determined in a final volume of 200 μl containing 125 mM Tris hydrochloride (pH 7.9), 10 mM $MgCl_2$, 10 mM ATP, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, and 0.5 mM [^{14}C]choline (0.9 Ci/mol). The reaction was started by the addition of 20 μl of cellular extract corresponding to 0.1×10^7 to 1.7×10^7 infected cells. After 45 min at 37°C, the reaction was stopped at 4°C by adding 100 μl ethanol. Each reaction mixture was applied to a column (0.55 by 2.5 cm) of Dowex AG 1X8 (OH^- form), 100-200 mesh, ion-exchange resin (Bio-Rad Laboratories, Richmond, Calif.). The column was washed with 10 ml of water, and the phosphorylcholine was eluted with 0.5 ml of 1 N NaOH followed by 1.5 ml of 0.1 N NaOH.

RESULTS

Effects of quaternary ammonium compounds on *P. falciparum* growth in vitro. We have previously (2) tested the in vitro effects on *P. falciparum* of typical quaternary ammonium compounds, i.e., HC3, a well-known inhibitor of choline transport in erythrocytes (16), and DM, both of which contain two charged groups, as well as DTMA, which contains one charged group. These three compounds exhibited good antiplasmodial activity, with 50% inhibitory concentrations (IC_{50}; corresponding to the drug concentration which reduces control parasitemia by 50%) of 4×10^{-6}, 10^{-6}, and 7×10^{-7} M, respectively. Because the lowest IC_{50} was obtained with DTMA, we tested three other compounds that also have a single quaternary ammonium but that have alkyl chains that are longer. Optimal antiplasmodial activity was obtained with an alkyl chain containing 12 carbon atoms, i.e., dodecyltrimethylammonium, the IC_{50} of which was very low (5×10^{-7} M); an increase in the alkyl chain length by 2 or 4 carbon atoms led to a slightly higher IC_{50}. In Table 1 is shown the structures of the different analogs and their IC_{50}s.

Inhibition as a function of the developmental stage of *P. falciparum*. The effects of DTMA and DM contact with the synchronized parasites for various periods and with the different developmental stages of *P. falciparum* are shown in Fig. 1 and 2, respectively.

The developmental stage of the parasites (Fig. 1A and 2A), as well as the corresponding parasitemias of untreated infected control cells (Fig. 1B and 2B), are illustrated for each drug. In these experiments, DTMA and DM were present in the incubation media at 3×10^{-6} and 2×10^{-5} M, respectively, for 48, 24, or 12 h at different periods of parasite maturation, as indicated by the horizontal thick bars in Fig. 1C and 2C.

Exposure to DTMA for 48 h killed practically all of the parasites (Fig. 1C, experiment 2). When applied for 24 h, the drug was similarly lethal to parasites, provided that contact occurred during the schizont stage and the reinvasion step (experiment 3). Inhibition was significantly decreased when DTMA was added for 24 h to the parasites in the ring form or

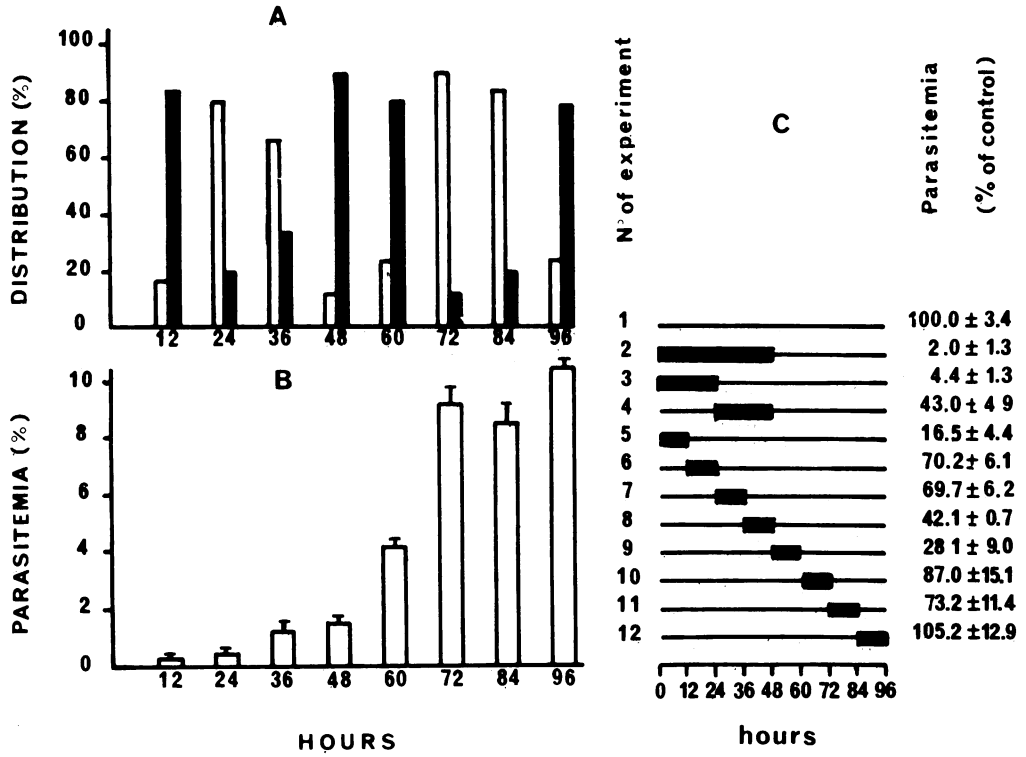


FIG. 1. Inhibition of *P. falciparum* growth by DTMA as a function of parasite development. Washed erythrocytes were infected at time zero with a parasitized cell suspension that was synchronized twice. The medium of each petri dish was changed every 24 h, unless the drug was added or removed. Data are the results of triplicate cultures in each set of experiments (except for the control, $n = 12$). (A) Relative percent distribution of *P. falciparum* stages in control cultures. Symbols: □, early parasite stage (ring forms); ■, mature forms (trophozoites and schizonts). (B) Percent parasitemia in control cultures ± standard error. (C) Scheme of experiments on inhibition by 3×10^{-6} M DTMA. Horizontal thick bars indicate the periods of incubation in the presence of drug. Parasitemia in each experiment, expressed as percentage of the control measured after 108 h of cultivation, is given on the right. Experiment 1 corresponds to control cells (final parasitemia = 9.8 ± 0.6%).

when they were getting mature (experiment 4). Application of DTMA to cultured parasites for only 12 h at different intervals led to more precision concerning the stage that was most sensitive to the drug (experiments 5 through 12). Exposure of early developmental stages (ring forms) only very slightly reduced parasitemia (experiments 6 and 7, and 10 and 11). The absence of strong inhibition in experiments 6 and 10 also suggests that the drug does not interfere with schizont rupture and the invasion process. On the other hand, a strong decrease in parasitemia (42% of control) occurred during the maturation of the parasite (experiment 8), reaching a maximum when the drug was in contact with the mature forms (schizont form) (experiments 5 and 9). The greater inhibition seen in experiments 5 and 9 indicates that DTMA acts on parasite maturation and its schizont form and remains ineffective on early stages. The experiments involving 12 h of contact between DTMA and cells demonstrated that the suppressive action of this drug depends on the specific period of drug application.

Similar experiments show that DM exhibits its antiplasmodial effect regardless of the developmental stage of the parasite (Fig. 2). When DM was present during the first 24 h, all of the parasites were killed (Fig. 2C, experiments 3, 5, and 6). After the first schizogony, the same residual parasitemia, i.e., 10 to 15% of control, was observed, irrespective of the 12-h contact period between parasites and drug (experiments 7 to 10). Nevertheless, microscopic examination revealed damaged or even dead parasites, suggesting that they had been affected by the drug. In the last two experiments (11 and 12), only a moderate effect of DM on parasite growth occurred. Thus, in addition to the absence of DM selectivity for a particular parasite developmental stage, our results indicate that a mere 12-h period of DM treatment can drastically affect parasite viability.

HC3 applied at 5×10^{-5} M to *P. falciparum*-infected erythrocytes was also lethal to parasites, whatever their developmental stage, provided that, as noted in the case of DM, the parasites remained in culture for at least one cycle after the drug had been removed (data not shown).

Mechanism of action of quaternary ammonium compounds. In a previous study (2) we demonstrated that, irrespective of preincubation time, in the presence of various drug concentrations DM and HC3 selectively inhibit PC biosynthesis without any alteration of DNA or protein biosynthesis. With respect to DTMA, results obtained within short preincubation times indicate that the earliest effect is also an alteration of PC biosynthesis.

To determine the way in which these compounds alter the Kennedy pathway, we studied the incorporation of [^{14}C]choline into the various metabolites that leads to the presence of PC inside infected erythrocytes (i.e., intracellular choline, phosphorylcholine, CDP-choline, PC). In preliminary experiments, no radioactive CDP-choline was detected in infected erythrocyte suspensions, suggesting that this metabolite was immediately transformed into PC. Furthermore, we found two rate-limiting steps in the de novo

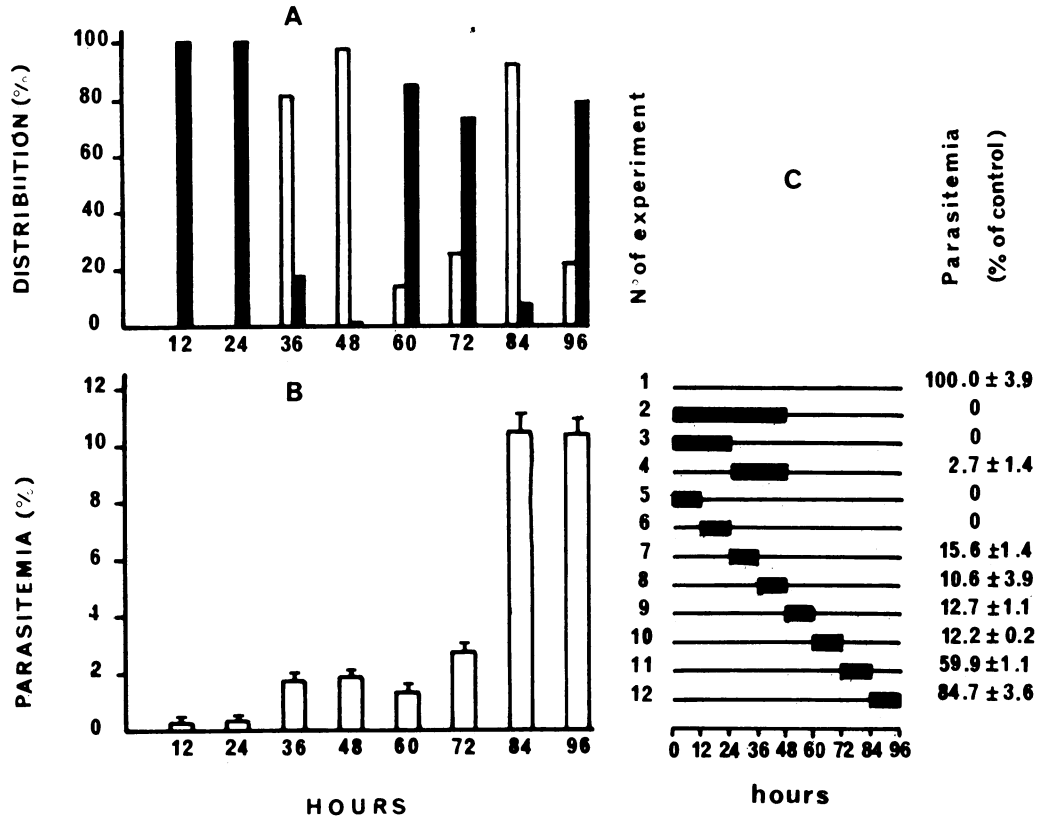


FIG. 2. Inhibition of *P. falciparum* growth by DM as a function of parasite development. Experiments were performed as described in the legend to Fig. 1, except that the DM concentration was 2×10^{-5} M and the final control parasitemia was 12.4 ± 1.7%. Symbols and panels are as described in the legend to Fig. 1.

biosynthesis of PC, in the choline transport into infected erythrocytes, and in the transformation of phosphorylcholine into CDP-choline catalyzed by cytidylyltransferase (EC 2.7.7.15), (M. L. Ancelin, H. J. Vial, and J. R. Philippot, manuscript in preparation).

In Fig. 3 it is shown that, starting at 6×10^{-6} M, DM decreased intracellular radioactive choline even after a short preincubation period of 30 min. Radioactive phosphorylcholine, as well as PC, was concomitantly decreased. At 5×10^{-5} and 4×10^{-4} M, the synthesis of these two products inside the erythrocytes was much more severely affected (about 70% decrease at 4×10^{-4} M) and significantly more reduced than that of intracellular choline. At 2×10^{-6} M, HC3 and DTMA did not significantly decrease the level of water-soluble choline metabolites and biosynthesized PC, but at 2×10^{-5} and 2×10^{-4} M, they exhibited the same profile of inhibition as DM. This indicates that the quaternary ammonium compounds impair the entry of choline into erythrocytes, regardless of the time of preincubation with the drugs.

As in untreated, infected erythrocyte suspensions, no radioactive CDP-choline was detected when the drugs were added. Furthermore, no modification of the ratio of [^{14}C]PC/[^{14}C]phosphorylcholine was noted. Thus, neither the transformation of CDP-choline into PC nor, probably, the formation of PC from phosphorylcholine are affected by any of the drugs. Therefore, the only steps that could be inhibited are anterior to the transformation of phosphorylcholine into CDP-choline.

To determine whether these compounds affect choline phosphorylation by choline kinase, this enzymatic activity was characterized in *P. falciparum* and *P. knowlesi*. No physicochemical difference was found between *P. falciparum* and *P. knowlesi* choline kinase (1). In Fig. 4 it is shown that DM, HC3, and DTMA are competitive inhibitors of choline kinase activity; 4×10^{-4} M DM, 10^{-3} M HC3, and 2×10^{-3} M DTMA inhibited choline kinase activity by 22, 25, and 40%, respectively. The consequent K_i values were 0.49, 1.3, and 1.8 mM, respectively, one order of magnitude higher than the K_m of the enzyme for choline (79 ± 20 μM).

DISCUSSION

A large increase in erythrocytic PL is observed after infection by *Plasmodium* spp., the malarial parasite, regardless of the species (9, 21, 24, 26). Because of its magnitude and its absence from the host erythrocyte, we assume that PL metabolism is essential for parasite life and therefore a good target for inhibiting parasite growth. Because PC is one of the most abundant PLs (40 to 50% of total PLs), we looked for drugs that could interfere with PC biosynthesis. Certain quaternary ammonium compounds have been shown to be effective in stopping parasite growth in vitro by inhibiting PC biosynthesis at very low IC_{50}s of 7×10^{-7}, 10^{-6}, and 4×10^{-6} M, respectively (2). In this study we show that a variation of the number of carbon atoms in the alkyltrimethylammonium alkyl chain results in an optimal antiplasmodial activity in dodecyltrimethylammonium, with an IC_{50} of 5×10^{-7} M. The addition of lethal doses of DTMA, DM, or HC3 for pulse-inhibitory periods (12, 24, or 48 h) to a synchronous culture showed that DTMA only

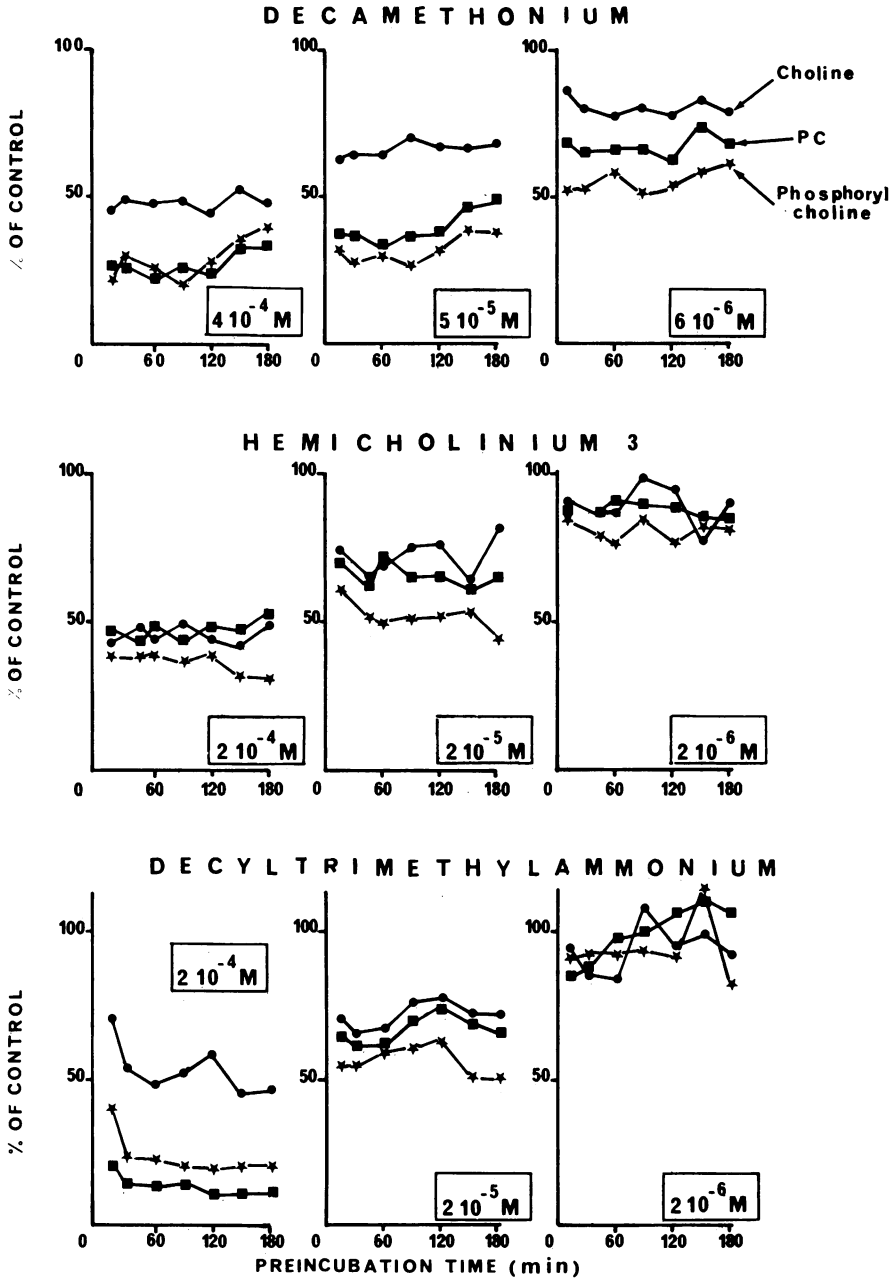


FIG. 3. Effects on labeled metabolites in the choline Kennedy pathway. Preincubations were carried out at 13 to 17% hematocrit in medium C containing the indicated concentration of drug at 37°C. After the addition of 0.7 Ci of [^{14}C]choline per ml, cells were further incubated for 30 min. The controls correspond to cells preincubated without drug. Each point represents the mean of triplicate samples. DM was in contact with 2.5 × 10^8 infected cells at 24.5% parasitemia, HC3 was in contact with 1.4 × 10^8 infected cells at 19.7% parasitemia, and DTMA was in contact with 5.9 × 10^8 infected cells at 69% parasitemia.

appears to be specific for the developmental stage of the parasite. Treatment with this drug was lethal to maturing parasites, i.e., the late trophozoite or schizont stages, but almost inoperative on young ring forms. In contrast, DM and HC3, which contain two quaternary ammoniums, act irrespective of the developmental stage of the parasite, without any detectable specificity.

Quaternary ammonium compounds are reported to penetrate the erythrocyte membrane with difficulty, first getting into the outer leaflet of the lipid bilayer and then gradually translocating into the inner leaflet (11, 12). Nevertheless, the antiplasmodial effect of these compounds is not linked to their detergent and hemolytic properties or to a mere alteration of host cell membranes, as reported previously (2).

DM and HC3, as well as DTMA, selectively inhibit the Kennedy pathway, which is responsible for the biosynthesis of PC from choline. We have shown (unpublished data) that in untreated *Plasmodium*-infected erythrocytes, de novo PC biosynthesis is directly related to the intracellular phosphorylcholine pool. No radioactive CDP-choline is detected because it is immediately transformed into PC. Because no radioactive CDP-choline accumulates when DM,

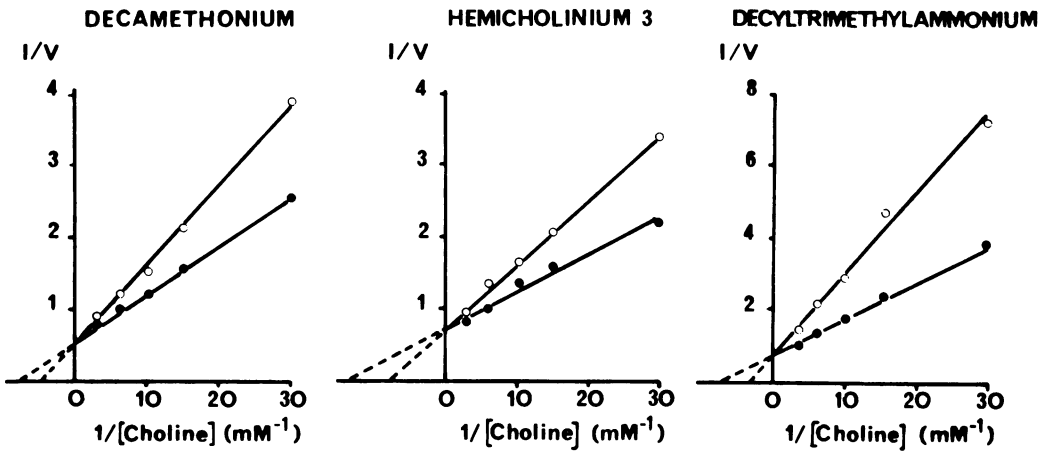


FIG. 4. Effects of quaternary ammonium compounds on choline kinase activity. Incubation was maintained for 30 min at 37°C with (○) or without (●) drug. The DM concentration was 4×10^{-4} M, and the corresponding homogenate fraction contained 4.8×10^6 infected cells. The HC3 concentration was 10^{-3} M with 7.2×10^6 infected cells. The DTMA concentration was 2×10^{-3} M with 6.7×10^6 infected cells.

HC3, or DTMA is added, the decreased PC biosynthesis cannot be ascribed to an inhibition of the enzyme that catalyzes the last step in the Kennedy pathway, i.e., choline phosphotransferase (EC 2.7.8.2). Furthermore, no modification of the PC/phosphorylcholine ratio was noted when these analogs were in contact with infected erythrocytes; thus CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15), which transforms phosphorylcholine into CDP-choline, is not involved in the effect of DM, HC3, or DTMA. Hence, only the action of the drugs on choline transport into erythrocytes, phosphorylation of choline by choline kinase, or both can be involved in the decreased PC biosynthesis that was observed.

In the case of HC3, both of these effects could be involved, because it has already been reported to be an inhibitor of choline penetration in other cellular systems, as well as an inhibitor of choline phosphorylation (3, 8, 16). Our results show that DM, HC3, and DTMA are competitive inhibitors of choline kinase in *Plasmodium*-infected cells. Nevertheless, their affinity for the enzyme is very low because their K_i values are one order of magnitude higher than the K_m value of choline kinase for choline. Furthermore, because choline kinase is a parasite cytosol enzyme (1), quaternary ammonium-containing compounds would have to enter the erythrocytes to decrease phosphorylcholine formation by the inhibition of the kinase. This hypothesis is unlikely in the case of normal uninfected erythrocytes (16) because these amphiphile cationic compounds are reported to have difficulty in crossing the membrane.

On the other hand, the choline level is decreased by all of these compounds (Fig. 3). Evidence for a choline carrier model of transport has been reported in normal erythrocytes (4, 5, 14) and it is well known that these quaternary ammonium compounds are inhibitors of this choline transport system (4, 5) without crossing the erythrocytic membrane (16).

It should be noted that the concentrations needed to effectively decrease choline entry into infected cells were higher than the observed IC_{50}s for antiplasmodial activity. This rather poor correlation is due to the fact that the metabolic studies were carried out over short periods (≤3 h 30 min), whereas antiplasmodial activity was measured after one parasite cycle, i.e., 48 h of contact between drugs and infected cells.

Thus, these choline analogs containing quaternary ammonium compounds specifically interfere with PC metabolism by blocking choline transport into infected erythrocytes. Because no concomitant interference with DNA, protein, or PL other than PC biosynthesis has been registered, at least in the case of DM and HC3, it can be assumed that transport blockage is responsible for their good antiplasmodial action and that, in view of their low IC_{50}s, choline availability is crucial for *Plasmodium* growth.

Because of the well-known cholinergic properties of these compounds (6), they cannot be assayed in vivo, but their in vitro activity is a clear illustration of the principle of chemotherapy that we propose and could lead to the development of a new chemotherapeutic treatment for malaria.

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