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EFFECTS OF SOME ANTIMALARIALS AND RELATED SUBSTANCES ON INTRALYSOSOMAL PROTEOLYSIS

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Abstract—The antimalarials primaquine, chloroquine, quinine, and quinacrine and the related substances 6-chloro-9-(3′-dimethylamino-2′-hydroxypropylamino)-2-methoxyacridine and 9-amino acridine inhibited intralysosomal proteolysis at pH 8 in mouse kidney subcellular suspensions containing [^{125}I]-labeled albumin-filled phagolysosomes at concentrations from 5×10^{-5} (quinacrine) to about 2.5×10^{-4} M (quinine). All these substances inhibited rat liver cathepsin B_1 but had no effects on cathepsins A, C or D. These inhibitions were greater on the alkaline side of the pH optimum of cathepsin B_1. The drugs also partially inhibited the stimulatory effect of ATP on intralysosomal proteolysis at pH 8 which we have interpreted previously to reflect an energy-dependent proton pump for maintenance of intralysosomal acidity. Preincubation of [^{125}I]-labeled albumin-filled phagolysosomes for 10 min at pH 8 in media containing 0.1 mM quinacrine or chloroquine or 0.5 mM quinine sulfate, followed by washing to remove these drugs, partially inhibited the stimulatory effect of ATP on further incubation in antimalarial-free media but had no effects on intralysosmal proteolysis. Preincubation in 1 mM chloroquine completely abolished the stimulatory effect of ATP but also partially inhibited proteolysis. Antimalarials also inhibited rat liver lysosomal (tritosomal) membrane ATPase suggesting that this activity may be related to the stimulatory effects of ATP on intralysosomal proteolysis. Quinine was the most effective of the inhibitors tested. These studies suggest that [^{125}I]-labeled albumin-filled mouse kidney phagolysosomes may provide a convenient model system for rapid screening of drugs potentially useful for the treatment of diseases caused by organisms possessing a lysosome-like digestive system.

Chloroquine and other antimalarial drugs have been shown to accumulate in lysosomes of higher animals *in vivo* [1–3] and *in vitro* [4]. Wibo and Poole [1] have also shown that chloroquine is an inhibitor of cathepsin B_1, a major protease in lysosomes. Chloroquine is a weak base with a pK of 8.1 [5]. In the uncharged form, the drug may diffuse across cell membranes into an acid compartment where it becomes protonated and thus would not be able to diffuse back across the membrane [6]. de Duve *et al.* [7] point out that since chloroquine has a second basic group of pK 10.1, it is improbable that at physiological pH the resulting monoprotonated drug could diffuse across lysosomal membranes at the observed rates without the participation of a permease. However, the drug does inhibit intralysosomal proteolysis of exogenous proteins in tissue slices [8] and macrophages [7] as well as endogenous proteins in rat fibroblasts [1].

Homewood *et al.* [6] have proposed a theory on the cytotoxicity of antimalarial drugs based on an accumulation of chloroquine in the digestive system of the plasmodium similar to that which occurs in lysosomes. According to this theory, accumulation would cause an inhibition of hemoglobin digestion due to the resulting pH rise. Accumulation of chloroquine in parasitized erythrocytes to many times plasma levels and significantly less accumulation in erythrocytes infected by resistant strains [9, 10] provides some support for the hypothesis. However, evidence against such a mechanism has been obtained by Williams and Fanimo [11].

To our knowledge antimalarials other than chloroquine have not been tested as inhibitors of lysosomal enzymes, nor have these drugs been tested directly on their capacities to inhibit intralysosomal proteolysis in cell-free systems. In the present study, we have examined the inhibitory effects of a number of antimalarials and related substances on purified rat liver cathepsin preparations, on intralysosomal proteolysis in mouse kidney phagolysosomes and on the stimulation of this proteolysis by ATP.

Studies on intralysosomal proteolysis were performed with mouse kidney phagolysosomes filled with [^{125}I]-labeled bovine serum albumin. When subcellular particulate suspensions containing these phagolysosomes are incubated in 0.25 M sucrose containing a sulfhydryl reducing agent, the labeled albumin is degraded to a trichloroacetic acid-soluble form to the extent of 25–40 per cent of the total radioactivity during 40 min of incubation. Rates of proteolysis in samples from the same animal do not vary more than 2–4 per cent, and breakage of the particles by detergents or hypotonicity inhibits the proteolysis to less than 5 per cent of the total acid-insoluble protein in the suspensions [12]. Alkaline buffers (pH 8) inhibit intralysosomal proteolysis in the suspensions about 50 per cent [13, 14], and additions of ATP restore the rates to those observed at pH 5 [8, 13]. We have interpreted the stimulatory effect of ATP as an energization of a proton pump or of some system which functions to maintain acidic conditions in the phagolysosome [8, 13, 15], but the exact mechanism of the stimulation is not yet known.

MATERIALS AND METHODS

Animals. Adult male or female albino rats and mice were propagated in the animal facilities of this institution by random breeding.

Enzyme assays. Cathepsin B_1 was assayed according to Barrett [16] using α-*N*-benzoyl-DL-arginine-β-naphthylamide HCl at pH 6. The enzyme catalyzes hydrolysis of this substrate and the 2-naphthylamine released is determined colorimetrically, after coupling with fast garnet GBC, by absorption at 520 nm. One nmole substrate hydrolyzed/min at 37° was defined as one unit of activity. Cathepsin D was assayed as described by Barrett [17] using hemoglobin at pH 3. Absorptions of trichloroacetic acid-soluble filtrates, taken at intervals during incubation at 37°, were measured at 280 nm. One unit of activity was an increase in absorption of 1.0 optical density unit/hr. Cathepsin C was assayed by a procedure similar to that used for cathepsin B_1 using glycyl-phenylalanyl-β-naphthylamide as the substrate [18]. Cathepsin A was assayed using *N*-carbobenzoxy glutamyl phenylalanine [19, 20]. The reaction product, phenylalanine, was determined colorimetrically with ninhydrin reagent.

Lysosomal (tritosomal) membrane ATPase activity was assayed by measuring phosphate liberated from ATP according to Fiske and Subbarow [21]. Reaction mixtures containing 2.5 μmoles ATP (disodium salt neutralized to pH 8 with NaOH), 5 μmoles $CaCl_2$, 50 μmoles Tris–acetate buffer, pH 8, and sufficient once-washed (0.2 M KCl) rat liver tritosome membrane preparation to allow a measurable linear rate were incubated at 37° in volumes of 1 ml with and without inhibitors. Samples of 0.2 ml were removed at zero time and at intervals for 40 min and added to 4.8 ml of 10% trichloroacetic acid. These were centrifuged to sediment any precipitated protein and the clear supernatant solutions were decanted and assayed for phosphate. Blanks containing all reagents but no membranes were incubated and assayed similarly. These controls produced negligible or no color in the Fiske and Subbarow phosphate assays.

Preparation of lysosomal proteases. Cathepsins B_1, D and C were purified from rat liver tritosomes. Tritosomes (Triton WR-1339-filled phagolysosomes) were prepared essentially by the procedure of Trouet [22] and Leighton *et al.* [23]. This method was modified by using 34.5% sucrose rather than 45% resulting in significantly reduced succinic dehydrogenase activities in the purified tritosomes, suggesting less mitochondrial contamination.* The purified tritosomes were broken by repeated freezing and thawing in a dry ice–acetone bath. These broken tritosomes were then centrifuged at 114,000 *g* for 60 min and the clear brown supernatant fluid was used as the starting material for enzyme purification. This supernatant was filtered through a 2.5 × 100 cm column of Sephadex G-75. Purification of cathepsin B_1 was approximately 800-fold and that of cathepsin D was about 400-fold. Cathepsin C was purified from tritosome extracts according to the method of McDonald *et al.* [24].

Preparation of tritosome membranes. Membranes remaining after tritosome disruption by freezing and thawing were sedimented by centrifugation for 1 hr at 114,000 *g*. The pellets were resuspended in cold 0.2 M KCl to detach any residual peripheral proteins, centrifuged again, and the supernatant fluids were decanted and discarded. The sedimented membranes were kept frozen until needed. These membranes contained no detectable succinic dehydrogenase activity when assayed according to Shamberger [25] and no Na^+, K^+-dependent ATPase activities.

Intralysosomal proteolysis in cell-free preparations. Adult albino mice were injected intravenously with 1–2 mg of [^{125}I]-labeled, formaldehyde-treated bovine serum albumin. Phagolysosomes containing the injected protein were centrifuged from kidney homogenates, and intralysosomal proteolysis was assayed in these organelles as described previously [13, 14, 26]. Reaction mixtures contained 0.25 M sucrose, 5 mM $MgCl_2$, 25 mM pH 8 borate buffer, 50 mM β-mercaptoethanol, 2.5 mM ATP previously neutralized to pH 8 with NaOH, and the drug to be tested. Controls contained the same ingredients with ATP and no drug or neither ATP nor drug. Intralysosomal proteolysis was measured by the conversion of trichloroacetic acid-insoluble to acid-soluble radioactivity. Results were expressed as per cent of the total radioactivity converted to the acid-soluble form during 40 min of incubation at 35°.

Drugs, chemicals and other materials. All chemicals used were reagent grade. Powdered hemoglobin, crystalline bovine serum albumin, substrates for cathepsin assay, chloroquine [7-chloro-4-(4-diethylamino-1-methylbutylamino)-quinoline], quinacrine [6-chloro-9-(4-diethylamino-1-methylbutylamino)-2-methoxyacridine], 9-amino acridine and Sephadex G-75 were obtained from Sigma Chemical Co., St. Louis, MO. Quinine sulfate [6-methoxy-(5-vinyl-2-quinuclidinyl)-4-quinolinemethanol sulfate], primaquine [8-(4-amino-1-methylbutylamino)-6-methoxyquinoline] and 6-chloro-9-(3′-diethylamino-2-hydroxypropylamino)-2-methoxyacridine (CDMA) were purchased from Aldrich Chemical Co., Milwaukee, WI. Triton WR-1339 was obtained from Ruger Chemical Co., Irvington, NJ, and [^{125}I]iodide was purchased from New England Nuclear Corp., Boston, MA.

RESULTS

Figures 1 and 2 show the effects of quinacrine and quinine sulfate concentrations on the intralysosomal proteolysis of [^{125}I]-labeled albumin at pH 8 in the presence and absence of ATP. The results show that these substances inhibited intralysosomal proteolysis and appeared to abolish or reduce the stimulatory effects of ATP at concentrations of about 5×10^{-5} to 10^{-4} M. Similar studies were performed with chloroquine, CDMA, primaquine and 9-amino acridine, and approximately the same results as those shown in Figs. 1 and 2 were obtained. In some cases ATP appeared to stimulate proteolysis to the same extent at all concentrations of drug, i.e. chloroquine and CDMA, but quinine, primaquine and quinacrine all abolished the stimulatory effects of ATP at higher concentrations.

The effects of the antimalarials shown in Figs. 1 and 2 suggest that the drugs inhibited the stimulatory effects of ATP and perhaps accumulated in the phagolysosomes to concentrations high enough to inhibit cathepsin B_1. A second possibility was that the intralysosomal pH was raised by the basic properties of the drugs.

* R. L. Elliott and J. L. Mego, unpublished observations.

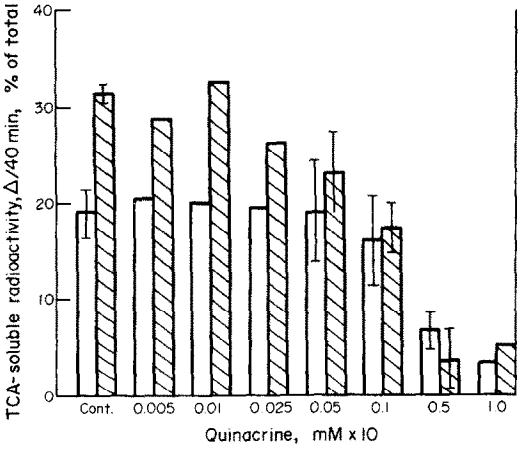

Fig. 1. Effects of quinacrine concentration on hydrolysis of formaldehyde-treated [^{125}I]-labeled albumin at pH 8 with (crosshatched bars) and without ATP in phagolysosomes from mouse kidneys. Labeled protein-filled phagolysosomes centrifuged (500–30,000 *g*) from mouse kidney homogenates 30 min after intravenous injections of radioactive protein were incubated at 35° in media containing 0.25 M sucrose, 5 mM $MgCl_2$, 50 mM β-mercaptoethanol, 25 mM borate buffer, pH 8 (control), 2.5 mM ATP previously neutralized to pH 8 (control plus ATP) and an antimalarial, at the concentrations shown. Trichloroacetic acid-soluble (TCA-soluble) radioactivity was measured at zero time and after 40 min of incubation. This radioactivity was calculated as per cent of the total in the samples and the resulting zero time value was subtracted from the 40-min per cent figure. Each set of bars (± ATP) represents the means of two to six experiments. Standard deviations from the means are shown only for those results which are the means of at least four experiments. Some absolute values for the percentage data are as follows (acid-soluble/total counts per min at zero time and after 40 min in the samples removed from the reaction mixtures): 5×10^{-7} M quinacrine, 345/3840–1051/3576; with ATP, 365/3579–1399/3443; 10^{-4} M quinacrine, 413/2675–479/2562; with ATP, 420/2482–550/2477.

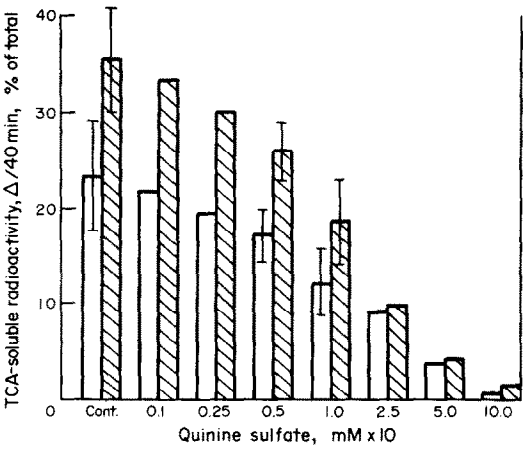

Fig. 2. Effects of quinine sulfate concentrations on hydrolysis of formaldehyde-treated [^{125}I]-labeled albumin at pH 8 with (crosshatched bars) and without ATP in phagolysosomes from mouse kidneys. Details of the experiment are described in the legend to Fig. 1 and in Materials and Methods. Some absolute values for the percentage data are as follows (acid = soluble/total counts per min at zero time and after 40 min in the samples removed from the reaction mixtures): 10^{-5} M quinine sulfate, 436/2490–822/2085; with ATP, 408/2047–1086/2027; 10^{-3} M quinine sulfate, 261/2352–282/2314; with ATP, 244/2515–278/2423.

Since cathepsins are acid hydrolases with pH optima around 5, a rise in pH would inhibit proteolytic activity. Wibo and Poole [1] have shown that chloroquine inhibits cathepsin B_1, but their results indicate that relatively high concentrations (about 5 mM) are required for significant inhibition at pH 6. We first investigated the inhibitory effects of chloroquine and other antimalarials using a crude enzyme prepared by osmotic shock of rat liver lysosomes [14]. The acridines, quinacrine and CDMA, were even more effective inhibitors than chloroquine when [^{125}I]-labeled albumin was the substrate rather than α-*N*-benzoyl-DL-arginine-β-naphthylamide (BANA) in this system. This suggested that other proteases or peptidases involved in albumin degradation might have been sensitive to the inhibitory effects of antimalarials. For this reason, we also investigated the effects of the drugs on cathepsins A and C. Although the hydrolysis of glycyl-phenylalanyl-β-naphthylamide, the substrate for cathepsin C, was inhibited to a small extent by CDMA, quinacrine and quinine in the crude enzyme system, these drugs had no effect on partially purified rat liver cathepsin C using the same substrate. We subsequently discovered that purified cathepsin B_1 also hydrolyzed the substrate for cathepsin C at a low rate which would account for these results. Cathepsin A was not affected by any of the drugs tested in this study.

The pH optimum of cathepsin B_1 is about 6 with BANA as substrate but appreciable activity is present at pH 5 or 7. A test of inhibitory effects of antimalarials on cathepsin B_1 at pH 5–7 revealed that the inhibition was pH dependent. All the drugs tested were more effective inhibitors on the alkaline side of the optimum

Table 1. Effects of pH on the inhibitions of cathepsin B_1 activity by antimalarials and related substances in a purified rat liver enzyme preparation*

Inhibitor (1 mM)	Experiment	% Inhibition pH 5	pH 6	pH 7
Chloroquine	1	3	16	49
	2	6	18	46
CDMA	1	19	19	39
	2	24	26	46
Quinacrine	1	21	20	41
	2	19	29	43
Quinine	1	14	11	19
	2	4	8	16
Aminoacridine	1	17	18	37
	2	0	15	29

*Reaction mixtures contained enzyme, 2.3 mM BANA, 1 mM EDTA, and 75 mM phosphate buffer in 2 ml with and without 1 mM inhibitor. Experiment 1 (2.5 μg enzyme) was incubated for 40 min at 37° and Experiment 2 (4.2 μg enzyme) was incubated for 10 min. Quinine sulfate and aminoacridine were dissolved in ethanol and separate controls containing the same concentrations of ethanol were run for these assays. The following nmoles of BANA were hydrolyzed in the controls during the incubation periods: Exp. 1, pH 5: 125, pH 6: 143, and pH 7: 83; plus ethanol, pH 5: 103, pH 6: 127, and pH 7: 41. Exp. 2, pH 5: 48, pH 6: 83, and pH 7: 52; plus ethanol, pH 5: 33, pH 6: 63, and pH 7: 29.

Table 2. Inhibition of rat liver tritosome membrane ATPase activity by some antimalarials and related substances*

Inhibitor (1 mM)	% Inhibition	
	Expt. 1	Expt. 2
Chloroquine	35	39
Quinacrine	76	79
Primaquine	46	27
Quinine	73	100
CDMA	76	59
Aminoacridine	38	33

* Reaction mixtures contained enzyme, 2.5 mM ATP (neutralized to pH 8), 50 mM Tris–acetate buffer (pH 8) and 5 mM $CaCl_2$ with and without (controls) inhibitor in a volume of 1 ml. Experiment 1 (90 μg protein) was incubated for 1 hr at 37° and Experiment 2 (42 μg protein) was incubated for 30 min. Quinine sulfate and aminoacridine were dissolved in ethanol and separate controls containing ethanol were run for these assays. At zero time and at the end of the incubation period, 0.5 ml of each reaction mixture was added to 4.5 ml of cold 10% trichloroacetic acid. These were centrifuged, decanted, and assayed for inorganic phosphate. The following μmoles phosphate were hydrolyzed from ATP in the controls during the incubation periods: Expt. 1: 3.14; plus ethanol: 2.12. Expt. 2: 0.76; plus ethanol: 0.64.

of this enzyme. Table 1 shows the effects of pH on the inhibition of purified rat liver cathepsin B_1 by antimalarials. The same results were obtained using a crude rat liver enzyme with [^{125}I]-labeled albumin or BANA as substrates. According to Homewood *et al.* [6], antimalarials might accumulate in lysosomes because they become protonated and thus are unable to diffuse back through the membrane. The drugs, therefore, would be expected to raise intralysosomal pH since they are weak bases. The combined effects of decreased acidity in lysosomes and more effective inhibition of enzyme activity by antimalarials on the alkaline side of the pH optimum should exert a potent inhibitory effect of cathepsin B_1 at relatively low concentrations. An accumulation of antimalarials in phagolysosomes resulting in a rise in pH and increased inhibitory effects on cathepsin B_1 thus appears to be a satisfactory explanation for the inhibitory effects of antimalarials on intralysosomal proteolysis (Figs. 1 and 2). However, this does not explain why these drugs appear to abolish the stimulatory effects of ATP on intralysosomal proteo-

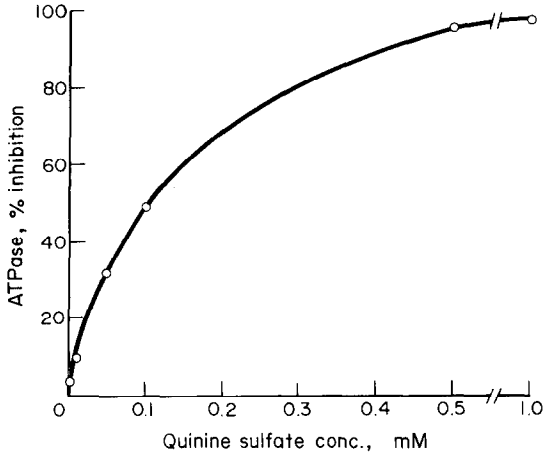

Fig. 3. Inhibition of rat liver lysosome membrane ATPase activity by quinine sulfate. About 50 μg of twice-washed (0.2 M KCl) tritosomal membrane protein was incubated in media containing 2.5 mM neutralized (pH 8) ATP, 50 mM Tris–acetate buffer, pH 8, 5 mM $CaCl_2$ and quinine sulfate dissolved in ethanol. The control contained the same volume of ethanol but no quinine. A sample containing an equivalent quantity of sodium sulfate was also run in the above experiment but this control showed no inhibition of activity. Samples (0.5 ml) were removed at zero time and after 30 min at 37°. These were added to 4.5 ml of 10% trichloroacetic acid, centrifuged and the supernatant decanted and assayed for phosphate according to Fiske and Subbarow [21]. The control sample hydrolyzed 0.26 μmole phosphate during 30 min.

Table 3. Effect of preincubation of [^{125}I]-labeled albumin-filled mouse kidney phagolysosomes in the presence of some antimalarials on intralysosomal proteolysis and of the stimulatory effects of ATP*

Expt.	Antimalarial	Concn (mM)	Acid-soluble radioactivity (% of the total) produced in 40 min at 35°: No additions	pH 8	pH 8 with ATP
1	Chloroquine	None	23.8	11.4	31.8
		1	13.2	5.2	5.7
2	Chloroquine	None	30.6	15.4	37.7
		0.1	32.6	14.2	23.5
3	Quinacrine	None	24.0	12.2	26.9
		0.05	27.2	15.9	18.4
		0.1	24.0	12.5	15.9
4	Quinine	None	18.8	11.6	26.8
		0.1	17.9	11.1	25.1
		0.5	20.4	8.5	19.5

* Mice were injected intravenously with [^{125}I]-labeled albumin (2 mg, 5.4 × 10^6 counts/min) and killed 30 min later. Kidney subcellular particles (500–30,000 *g*) containing [^{125}I]-labeled protein-filled phagolysosomes were preincubated at 35° in 0.25 M sucrose, pH 8 (25 mM borate buffer), with and without antimalarial. The particles were then washed twice in 20 ml of cold 0.25 M sucrose and incubated in 0.25 M sucrose–5 mM $MgCl_2$–0.05 M β-mercaptoethanol (no additions); sucrose, Mg, mercaptoethanol and pH 8 borate buffer (pH 8); sucrose, Mg, buffer, mercaptoethanol and 2.5 mM ATP (pH 8, ATP) in 6-ml volumes. Samples were taken at zero time and after 40 min, and analyzed for trichloroacetic acid-soluble radioactivity. The acid-soluble radioactivity was calculated as per cent of the total counts/min in the sample and the percentage of zero time was subtracted from that at 40 min in each experiment. Absolute values for some of the percentage data are as follows, with acid-soluble/total counts/min at zero time followed by acid-soluble/total counts/min at 40 min, per 2-ml sample removed from the reaction mixtures: control for expt. 1: no additions, 707/6631–2106/6109; pH 8, 592/6738–1374/6790; pH 8 with ATP, 646/6862–2706/6572. Chloroquine (1.0 mM): no additions, 763/8837–1850/8488; pH 8, 787/10886–1290/10443; pH 8 with ATP, 482/5866–777/5589. Chloroquine: (0.1 mM): no additions, 719/5504–2388/5227; pH 8, 725/5611–1540/5689; pH 8 with ATP, 713/5467–1948/5335. Quinacrine (0.05 mM): no additions, 762/7443–2803/7495; pH 8, 685/7104–1771/6933; pH 8 with ATP, 780/7111–2153/7335. Quinacrine (0.1 mM): no additions, 624/7142–2258/6898; pH 8, 599/6250–1385/6246; pH 8 with ATP, 624/6623–1770/6986.

lysis at pH 8. Although rat liver lysosmal membranes contain ATPase activity [27], there is no evidence that this ATPase is related to the stimulatory effects of ATP on intralysosomal proteolysis. Table 2 shows that antimalarials and related compounds were effective inhibitors of the rat liver tritosomal membrane ATPase. Quinine sulfate was the most effective inhibitor but substantial effects were also obtained by CDMA and quinacrine at 1 mM concentrations. Chloroquine and primaquine were least effective. Figure 3 shows the effects of quinine concentration on ATPase activity.

The inhibition of lysosomal membrane ATPase by antimalarials suggested that the drugs might have interacted directly with the energy-dependent system responsible for stimulation of intralysosomal proteolysis at pH 8. To test this possibility, [^{125}I]-labeled albumin-filled mouse kidney phagolysosomes were preincubated in various concentrations of chloroquine, quinacrine or quinine sulfate in 0.25 M sucrose at 35° for 10 min. The suspensions were then diluted with equal volumes of ice-cold 0.25 M sucrose and centrifuged. Pellets were resuspended with 20 ml of cold sucrose, recentrifuged and decanted to remove all drug from the media. Intralysosomal proteolysis and the effects of ATP at pH 8 were then assayed in the particles. Results of these experiments are shown in Table 3. Intralysosomal proteolysis was unaffected at 0.1 mM concentrations of chloroquine or quinacrine (Table 3, no additions) but the stimulatory effects of ATP were reduced significantly. At a 1 mM concentration of chloroquine, intralysosomal proteolysis was partially inhibited and the stimulatory effect of ATP was completely abolished. Although quinine was the most effective inhibitor of rat liver lysosomal (tritosome) membrane ATPase, this drug proved to be the least effective inhibitor of the ATP effect in the preincubation experiments. Little or no effect was evident even at concentrations of 0.5 mM quinine. The most likely explanation for this was that quinine was removed easily from the phagolysosomes by washing.

DISCUSSION

The antimalarials bind strongly to tissue components although the degree of binding varies from drug to drug [28]. For example, quinacrine binds more strongly than chloroquine or quinine and chloroquine has a higher affinity for tissues than quinine. Chloroquine, quinacrine, quinine and primaquine all bind to nucleic acids and this is considered to be the site responsible for tissue binding and cytotoxicity [28, 29]. Evidence exists that these substances accumulate in lysosomes [1–3] which may account for some tissue binding. Results obtained in the present studies are in agreement with this evidence since concentrations of antimalarials inhibiting intralysosomal proteolysis were 10- to 100-fold less than those required to inhibit free cathepsin B_1, even at pH 7. Furthermore, chloroquine at concentrations as high as 10 mM has little effect on intralysosomal proteolysis in the absence of pH 8 buffer [8], suggesting that the drug must be in the monoprotonated form to diffuse into phagolysosomes. Preincubation studies (Table 3) also provides some support for this hypothesis. Quinine has the lowest tissue binding affinity *in vivo* and this drug appeared to be most readily removed from phagolysosomes by washing. Quinacrine and chloroquine, however, retained their capacities to inhibit the stimulatory effects of ATP, suggesting higher affinities for phagolysosomes.

The inhibitory effects of antimalarials on both the activity of rat liver membrane ATPase and the stimulatory effect of ATP on intralysosomal proteolysis at pH 8 suggest that the ATPase may be related to the ATP effect. However, quinine was the most effective inhibitor of the membrane ATPase and the quinacrines appeared to be more effective inhibitors of the stimulatory effects of ATP on intralysosomal proteolysis. The reason for this is not clear at the present time. It seems reasonable to assume that the stimulatory effects of ATP on intralysosomal proteolysis are a consequence of ATP hydrolysis but this has not been established. There is no doubt that the antimalarials inhibited the stimulatory effects of ATP on intralysosomal proteolysis at concentrations not affecting proteolytic activity. This was evident both in the preincubation experiments (Table 3) and in the experiments in which intralysosomal proteolysis was measured in the presence of the drug, particularly in the case of quinacrine (Fig. 1).

The accumulation of chloroquine in lysosomes is well known [1, 4, 7] and evidence exists that other antimalarials also become concentrated in these organelles [2, 3]. Wibo and Poole [1] have shown that chloroquine inhibits the activity of cathepsin B_1, a major protease in lysosomes. These findings explain the action of chloroquine as an inhibitor of intracellular proteolysis [1, 7, 8]. The present studies show that chloroquine and other antimalarials inhibit intralysosomal proteolysis in a cell-free system and that quinine, quinacrine, primaquine, CDMA and 9-amino acridine are also effective inhibitors of cathepsin B_1. Furthermore, all the antimalarials tested, as well as some related substances (9-amino acridine and CDMA), behaved similarly with respect to inhibition of intralysosomal proteolysis and lysosomal ATPase activity. These substances, therefore, may share some common mechanism which may be related to their cytotoxic effects on the malaria plasmodium. For example, chloroquine and the other antimalarials may inhibit hemoglobin degradation in lysosomes of the malaria parasite, as proposed by Homewood *et al.* [6]. Indeed, chloroquine induces a vacuolization in the malaria plasmodium [30, 31] similar to that produced in cells of higher animals [7].

The usual maximum concentration of chloroquine attained in the plasma during therapy is about 10^{-6} M [32] and levels reached in infected erythrocytes may be 100 times this [33] or about 10^{-4} M. The effects noted in the present studies were significant at concentrations of quinacrine of about 10^{-5} M, and 10^{-4} M concentrations almost completely inhibited intralysosomal proteolysis (Fig. 1). Similar effects were noted at these concentrations of chloroquine [8] but primaquine and quinine required significantly higher levels (about 1 mM) to completely inhibit proteolysis. However, quinine inhibited the stimulatory effect of ATP at a concentration of about 2.5×10^{-4} M (Fig. 2).

Although the work of Williams and Fanimo [11] tends to rule out the theory of Homewood *et al.*, the possibility remains that antimalarials may somehow interfere with hemoglobin degradation by malaria para-

sites or perhaps with some ATP-dependent activity related to proteolysis similar to the one sensitive to antimalarials reported in the present study. If this proves to be the case, the measurement of intralysosomal proteolysis described in the present study and elsewhere [8, 12–15, 26] may provide a rapid and convenient model system for the screening of potential drugs for the treatment of diseases caused by organisms having a lysosome-like digestive system.

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