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INHIBITION OF LIVER ALCOHOL DEHYDROGENASE BY PRIMAQUINE AND 8-AMINO-6-METHOXYQUINOLINE COMPOUNDS

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Abstract—The effect of primaquine, chloroquine and other aminoquinoline derivatives on the enzymatic activity of crystalline horse liver alcohol dehydrogenase and partially purified human liver alcohol dehydrogenase has been studied. While chloroquine inhibits neither enzyme, primaquine inhibits both the horse and the human enzyme reversibly and noncompetitively with respect to NAD. The inhibition constant is 2 μM for the horse enzyme and 30 μM for the human enzyme. Spectrophotometric studies of the interaction of primaquine with the horse liver enzyme indicate that primaquine binds to a specific hydrophobic site on the protein. The stoichiometry of binding measured by spectrophotometric titrations is 2 moles primaquine/mole of enzyme and the dissociation constant of the complex is 3 μM. The effects of other 8-amino-6-methoxyquinoline compounds upon horse and human liver alcohol dehydrogenases suggest that the aliphatic side chain, as well as the terminal primary amino group of the side chain, contributes importantly to the tight binding of primaquine to these enzymes.

INVESTIGATIONS of the mechanism of action and the metabolic effects of antimalarial drugs have revealed that these compounds can interfere with many and diverse biochemical processes. Chloroquine, primaquine and other quinoline derivatives, quinine and quinacrine, all or individually have been shown to inhibit nucleic acid and protein synthesis,[1–6] the uptake of amino acids by cells,[7] glycolysis,[8] lipolysis,[9] as well as the activity of a number of specific enzyme systems.[10–15] The recent report that chloroquine inhibits yeast alcohol dehydrogenase[12] led us to investigate the effects of the quinoline family of antimalarial compounds upon mammalian liver alcohol dehydrogenase,† particularly since this enzyme is well known to serve a key role in the metabolism of alcohol in man.

The present report shows that, while chloroquine does not inhibit horse and human LADH, primaquine and other 8-amino-6-methoxyquinolines are potent inhibitors. Spectrophotometric titrations demonstrate that primaquine binds firmly to horse LADH in stoichiometric amounts, apparently at a specific hydrophobic site on each of the two identical polypeptide chains of the molecule.

MATERIALS AND METHODS

Crystalline horse LADH was purchased from Calbiochem (A grade) as a suspension of crystals in 10% ethanol and 0·02 M sodium phosphate, pH 7. The suspension was

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† Abbreviations used: LADH, liver alcohol dehydrogenase; NAD, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide.

dialysed four times against a 100 vol. excess of 0·1 M sodium phosphate, pH 7·4, at 4°, and the solution was clarified by centrifugation. The specific activity of the enzyme was 15·5 ΔA_{340}/min/mg of protein. Human LADH was isolated and purified from a single liver, obtained at autopsy, as described previously.[16] The liver was normal both by gross and by microscopic examination. The human enzyme, purified approximately 150-fold, had a specific activity of 1·7 ΔA_{340}/min/mg protein. β-NAD (Sigma Chemical Company, grade III) was 99 per cent pure. Dehydrated ethanol was analytical reagent grade. Chloroquine, primaquine, pamaquine, pentaquine, isopentaquine, 8-amino-6-methoxyquinoline, 8-acetamido-6-methoxyquinoline, 8-(4′-aminobutylamino)-6-methoxyquinoline, 8-(3′-amino-1′-methoxypropylamino)-6-methoxyquinoline and 4-(4′-amino-1′-methylbutylamino)-7-chloroquinoline were obtained from the Division of Medicinal Chemistry, Walter Reed Army Institute of Research, as the phosphate salts and were dissolved in water. All other chemicals were reagent grade. Deionized, distilled water was used throughout.

The specific activities of the enzymes were measured spectrophotometrically at 23° by observing the increase in absorbance at 340 mμ when NAD is reduced by ethanol. The assay conditions were those described by Dalziel.[17] Protein concentrations of the enzymes were estimated spectrophotometrically using $A_{280} = 0{\cdot}46\ cm^{-1}$ for a 1 mg/ml solution of horse LADH[18] and $A_{280} = 0{\cdot}61\ cm^{-1}$ for a 1 mg/ml solution of human LADH.[16] The molar concentration of horse LADH was calculated based on a molecular weight of 80,000.[19] The concentration of NAD was determined spectrophotometrically using $A_{260} = 18{\cdot}0\ mM^{-1}\ cm^{-1}$.

A Cary 14 recording spectrophotometer equipped with expanded (0–0·1) slide wire was employed for the inhibition and the kinetic studies, the measurement of absorption spectra and difference absorption spectra, and the spectral titrations. All measurements were made at 23° and pH 8·5, using tris-HCl as the buffer. The concentration of tris-HCl employed for the inhibition and the kinetic studies was 0·06 M and for the spectral studies, 0·1 M.

RESULTS

Horse liver alcohol dehydrogenase. The enzymatic activity of horse LADH is unaffected by chloroquine [4-(4′-diethylamino-1′-methylbutylamino)-7-chloroquinoline] even when its concentration in the reaction mixture is 2mM. Pre-incubation of the enzyme with 2 mM chloroquine for 60 min also does not alter activity. Neither does 4-(4′-amino-1′-methylbutylamino)-7-chloroquinoline inhibit horse LADH.

In contrast, the 8-amino-6-methoxyquinoline compounds do inhibit LADH activity, some of them strongly. Thus primaquine [8-(4′-amino-1′-methylbutylamino)-6-methoxyquinoline] inhibits the enzyme instantaneously and the degree of inhibition increases as a function of the concentration of the compound added to the reaction mixture (Fig. 1). The inhibition is reversible by dilution and the amount of activity thus regained is exactly that which would be expected on the basis of the activity–inhibitor concentration profile in Fig. 1. Fifty per cent inhibition is attained with 2 μM primaquine, suggesting an enzyme–inhibitor dissociation constant of 2 μM under the conditions of the enzyme assay. The inhibition is not time dependent: enzyme activity immediately after exposure to primaquine is identical with that observed after 90 min of incubation. Furthermore, inhibition by primaquine is un-

altered either by the addition of 5 mM Zn^{2+} to the reaction mixture or by the presence of 0·14 mM chloroquine.

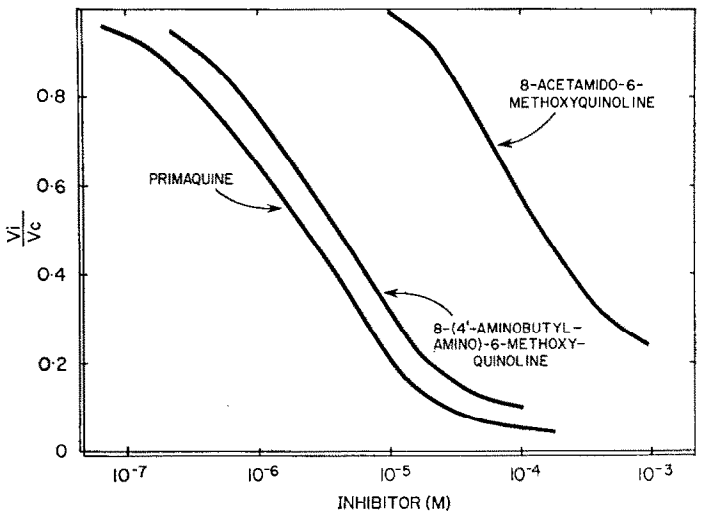

FIG. 1. Inhibition of horse LADH by 8-amino-6-methoxyquinoline compounds: activity–inhibitor concentration profiles of primaquine, 8-(4′-aminobutylamino)-6-methoxyquinoline and 8-acetamido-6-methoxyquinoline. V_i/V_c is the ratio of the initial velocities in the presence (V_i) and in absence (V_c) of the inhibitor. Conditions: NAD, 0·45 mM; ethanol, 8·6 mM; enzyme, 1·1 μg/ml; 0·06 M tris-HCl, pH 8·5, 23°.

TABLE 1. EFFECT OF 8-AMINO-6-METHOXYQUINOLINE COMPOUNDS ON LIVER ALCOHOL DEHYDROGENASE ACTIVITY*

Substituent on the 8-amino group	Activity (% control)	
	Horse LADH	Human LADH
—H	92	37
—C(=O)—CH_3	73	56
—$(CH_2)_4$—NH_2	15	53
—CH(CH_3)—$(CH_2)_2$—NH_2	24	60
—CH(CH_3)—$(CH_2)_3$—NH_2 (Primaquine)	6	33
—CH(CH_3)—$(CH_2)_3$—N(CH_2CH_3)—CH_2CH_3 (Pamaquine)	88	80
—$(CH_2)_5$—NH—$CH_2CH_2CH_3$ (Pentaquine)	64	66
—CH(CH_3)—$(CH_2)_3$—NH—$CH_2CH_2CH_3$ (Isopentaquine)	80	93

*Conditions: NAD, 0·45 mM; ethanol, 8·6 mM; horse LADH, 1·1 μg/ml, or human LADH, 18 μg/ml; inhibitors, 70 μM; 0·06 M tris-HCl, pH 8·5, 23°.

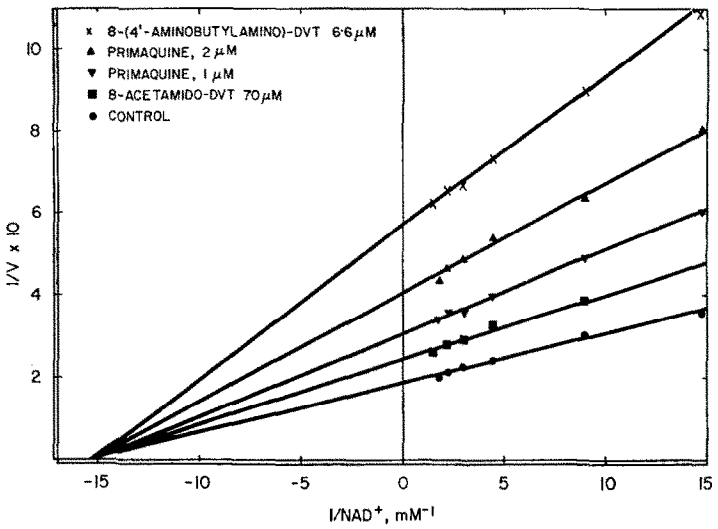

FIG. 2. Double reciprocal plot of the kinetics of inhibition of horse LADH by 8-amino-6-methoxyquinoline derivatives. The steady state rates were measured at varied NAD concentrations in the absence of inhibitors (●) and in the presence of 6·6 μM 8-(4'-aminobutylamino)-6-methoxyquinoline (×), 2·0 μM primaquine (▲), 1·0 μM primaquine (▼), or 70 μM 8-acetamido-6-methoxyquinoline (■). Conditions: enzyme, 1·1 μg/ml; ethanol, 8·6 mM; 0·06 M tris-HCl, pH 8·5, 23°.

8-(4'-Aminobutylamino)-6-methoxyquinoline and 8-(4'-amino-1'-methylpropylamino)-6-methoxyquinoline are almost as potent inhibitors of horse LADH as is primaquine; other 6-methoxyquinoline derivatives examined are less effective (Table 1). 8-Amino-6-methoxyquinoline itself is the least potent of the inhibitors examined. The shapes of the activity–inhibitor concentration profiles for 8-(4'-aminobutylamino)-6-methoxyquinoline and the 8-acetamido derivative are similar to that for primaquine (Fig. 1), suggesting that the mode of inhibition by the 8-amino-6-methoxyquinoline derivatives is similar to that for primaquine.

This is confirmed by kinetic studies performed with primaquine, 8-(4'-aminobutylamino)-6-methoxyquinoline and 8-acetamido-6-methoxyquinoline. All three compounds are fully noncompetitive with the coenzyme, NAD (Fig. 2), but at the concentrations employed, they appear almost noncompetitive with the substrate, ethanol (Fig. 3). The inhibition constants, K_I, calculated from these data are 2 μM, 4 μM and 0·2 mM for primaquine, 8-(4'-aminobutylamino)-6-methoxyquinoline and 8-acetamido-6-methoxyquinoline respectively. The inhibition kinetics by primaquine are unaltered by the addition of 70 μM chloroquine to the reaction mixtures.

To discern further the nature of the interaction of primaquine with horse LADH, the absorption spectral properties of the primaquine in the presence and absence of the enzyme have been studied. At pH 8·5, primaquine exhibits a strong absorption band in the near ultraviolet wavelength region centered at about 259 mμ. Upon addition of the enzyme, the absorption spectrum undergoes a red-shift and an increase in absorptivity (Fig. 4A). The difference spectrum of primaquine in the presence and absence of the enzyme demonstrates that primaquine exhibits an absorption band centered at 273 mμ when it is bound to horse LADH (Fig. 4B). The absorption spectrum of primaquine undergoes a similar change when it is exposed to organic solvents: a

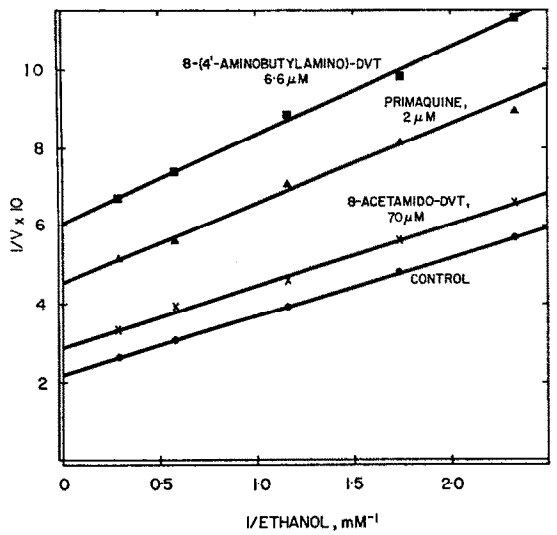

FIG. 3. Double reciprocal plot of the kinetics of inhibition of horse LADH by 8-amino-6-methoxyquinoline derivatives. The steady state rates were measured at varied ethanol concentrations in the absence of inhibitor (●) and in the presence of 6·6 μM 8-(4'-aminobutylamino)-6-methoxyquinoline (■), 2·0 μM primaquine (▲), or 70 μM 8-acetamido-6-methoxyquinoline (×). Conditions: enzyme, 1·1 μg/ml; NAD, 0·45 mM; 0·06 M tris-HCl, pH 8·5, 23°.

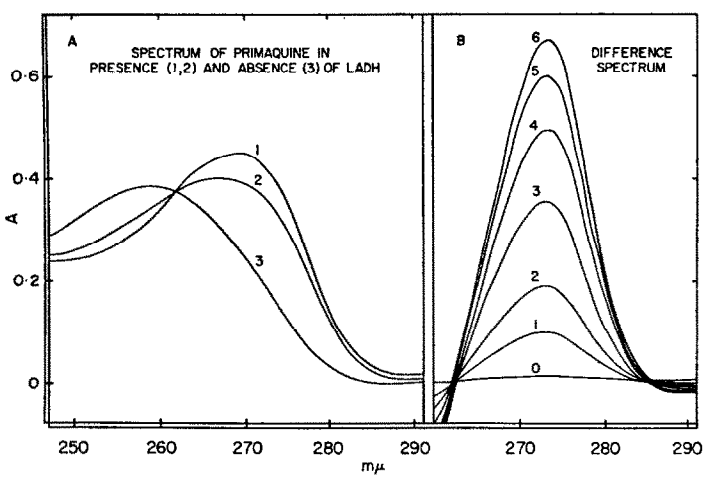

FIG. 4. (A) Absorption spectrum of 20 μM primaquine in the absence of horse LADH (curve 3) and in the presence of 6·7 μM (curve 2) and 26·7 μM LADH (curve 1). Conditions: 0·1 M tris-HCl, pH 8·5, 23°. The absorbance of the enzyme was subtracted optically in curves 1 and 2. (B) Four-cell difference spectrum of primaquine in the presence and absence of horse LADH. Two cells, one containing 27·5 μM enzyme in 0·1 M tris-HCl, pH 8·5, and the other containing only 0·1 M tris-HCl were placed in both the reference and the sample beams of the spectrophotometer. Curve (0) was recorded when primaquine was added to the cuvettes containing the buffer in the reference and the sample beams. Curves 1–6 were recorded when 8, 16, 32, 48, 64 and 80 μM primaquine, respectively, was added to the cuvette containing enzyme in the sample beam, but not to the cuvette containing buffer in the reference beam.

red-shift and hyperchromicity occur when dioxane is added to a solution of primaquine in 0·1 M tris-HCl, pH 8·5. In 100% dioxane, the absorption maximum is at 269 mμ and absorptivity increases 15 per cent. Consistent with the kinetic data, the spectral changes that occur upon mixing 8 μM enzyme with 30 μM primaquine are unaltered when 50 μM NAD is added to the solution.

These spectral changes permit the determination of the stoichiometry of interaction of primaquine with the enzyme. Spectral titration of horse LADH with primaquine yields a molar ratio of inhibitor to enzyme of 2·0 (Fig. 5A). A molar ratio of 2·2 is obtained, utilizing the difference spectral changes for the titration (Fig. 5B). Thus two molecules of primaquine interact with each molecule of enzyme. From the data in Fig. 5B, the dissociation constant, K_S, for the enzyme–primaquine complex has been calculated, assuming that the two primaquine binding sites are independent. A value of 1·6–5·0 μM is obtained, in good agreement with the K_I for primaquine obtained from the kinetic studies.

Human liver alcohol dehydrogenase. Primaquine and the other 8-amino-6-methoxyquinoline derivatives examined also inhibit the partially purified human LADH (Table 1). With the exception of 8-acetamido-6-methoxyquinoline and 8-amino-6-methoxyquinoline, the effects of these compounds upon human LADH activity are not as pronounced as that upon the horse enzyme. As with the horse enzyme, inhibition of human LADH by primaquine is noncompetitive with NAD, and the K_I is 30 μM. However, the kinetics of inhibition of human LADH by 8-acetamido-6-methoxyquinoline and 8-amino-6-methoxyquinoline are mixed competitive–noncompetitive. Chloroquine and 4-(4′-amino-1′-methylbutylamino)-7-chloroquinoline do not inhibit human LADH.

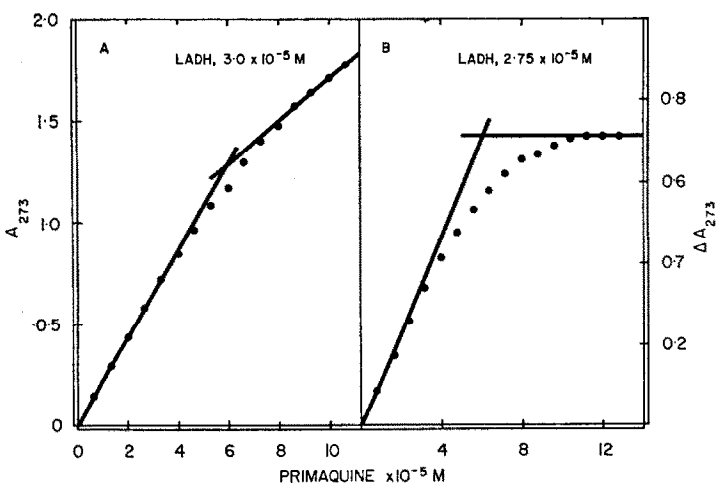

FIG. 5. (A) spectral titration of 30 μM LADH with primaquine. The absorbance at 273 mμ is plotted against the concentration of primaquine added to the enzyme solution. The absorbance of the enzyme was subtracted optically. Conditions: 0·1 M tris-HCl, pH 8·5, 23°. (B) Four-cell difference spectral titration of 27·5 μM LADH with primaquine. The change in absorbance at 273 mμ is plotted against the concentration of primaquine added to the enzyme. The conditions are as described under Fig. 4B.

DISCUSSION

The alcohol dehydrogenase from mammalian sources which has been studied the most extensively is the one from horse liver. Crystallized in 1948,[20] the enzyme has been shown to have two active enzymatic sites, each binding one molecule of the coenzyme, NAD(H).[21] It has broad substrate specificity, oxidizing not only ethanol and other aliphatic alcohols, but also certain steroid alcohols. The enzyme molecule contains four firmly bound zinc atoms, two of which are at the active sites and essential for catalytic activity.[19] Horse LADH exists in multiple molecular forms in the liver, but in commercial crystalline preparations of the enzyme such as the one employed in this study, one isoenzyme, the "EE" form,[22] predominates, apparently because of the selective purification by the isolation procedures employed.[23] When compared with the other isoenzymes, the EE isoenzyme exhibits higher activity towards ethanol than toward the steroid alcohol substrates. Consistent with the two independently functioning, active enzymatic sites of the enzyme, this isoenzyme has recently been shown to be composed of two identical polypeptide chains of molecular weight 40,000 each.[24]

The alcohol dehydrogenase from human liver has also been purified and studied in recent years.[25–27] It is similar to horse LADH in many of its enzymatic, physical and chemical properties and also exists in multiple molecular forms in the liver.[26] As with horse LADH, it has broad substrate specificity and contains firmly bound zinc atoms, essential for catalytic function.[25] The alcohol dehydrogenase from yeast, on the other hand, differs from the mammalian liver enzymes in many aspects: it has a molecular weight of 150,000 and four active enzymatic sites. It has a very limited substrate specificity, and activity toward ethanol is many times greater than that for the mammalian liver enzymes.[21] It is, therefore, not surprising that, while chloroquine is a potent inhibitor of yeast alcohol dehydrogenase,[12] it does not at all inhibit human and horse LADH.

Horse and human LADH have been shown to be inhibited by a variety of organic compounds. Chelating agents such as 1,10-phenanthroline, 8-hydroxyquinoline-5-sulfonic acid and bipyridyl inhibit by binding to zinc, and exhibit competition with NAD.[28] Pyrazole[16,29] and long-chain fatty acids[21] compete with ethanol for binding. Other inhibitors include thyroxine,[30] triiodothyronine,[31] folic acid, aminopterin and amethopterin.[32] The inhibition by primaquine apparently differs from that produced by the above mentioned inhibitors: while instantaneous and reversible by dilution, the inhibition is not reversed by the addition of Zn^{2+}, indicating that primaquine does not bind to the catalytically essential zinc atoms of the molecule. Moreover, the inhibition is competitive neither with NAD nor with ethanol (Figs. 2, 3).

The spectral studies demonstrate that the interaction of primaquine with LADH is specific and stoichiometric. The spectral changes that occur upon addition of the enzyme to primaquine (Fig. 4) parallel those which are observed when primaquine is placed in an organic solvent, indicating that primaquine binds to hydrophobic sites on the protein. The fully noncompetitive nature of the inhibition (Fig. 3) suggests that these sites may be at or near the active enzymatic centers and, consistent with the two active sites of the enzyme, only two moles primaquine bind per mole of enzyme (Fig. 5). Both the kinetic inhibition studies and the spectral titrations indicate a firm binding with a dissociation constant for the enzyme–primaquine complex of 2 μM. Comparison of the inhibitory effects of the various 8-amino-6-methoxyquinoline

derivatives examined indicates that the aliphatic side chain of primaquine and its terminal primary amino group contribute importantly to tight binding (Table 1). Since chloroquine and 4-(4'-amino-1'-methylbutylamino)-7-chloroquinoline do not inhibit at all, the position of the side chain relative to the nitrogen atom of the quinoline ring appears to be another critical determinant.

The inhibitory effect of primaquine and other 8-amino-6-methoxyquinoline derivatives on human LADH activity (Table 1) is consistent with the previous observations that human and horse LADH are similar in many of their properties. As with the horse enzyme, the inhibition of human LADH by primaquine is fully noncompetitive with respect to NAD. However, the K_I of primaquine for human LADH is only 30 μM as compared to a value of 2 μM for the horse enzyme. Whether this reflects intrinsic differences in the primaquine binding sites of the two enzymes, or is due to the presence of impurities in the human enzyme preparation, or both, has not been determined. The finding that the inhibition by 8-amino-6-methoxyquinoline and 8-acetamido-6-methoxyquinoline is mixed competitive–noncompetitive with respect to NAD suggests that there may indeed be differences in the interaction of some of the 8-amino-6-methoxyquinoline compounds with human LADH when compared with the horse enzyme.

Since the enzymatic data indicate that primaquine is not a strong inhibitor of the human enzyme, it would be expected not to interfere with alcohol metabolism *in vivo*. However, primaquine is being widely employed for malaria prophylaxis and it seemed important that this question be answered directly. Hence, the effect of 45 mg primaquine phosphate, the amount presently given for malaria prophylaxis was examined in six healthy human subjects.* Four hr after the administration of primaquine or a placebo tablet, the subjects ingested 90–120 ml of 50 per cent ethanol. The serum alcohol concentrations were measured serially by chromatographic analysis.[33] The rates of ethanol disappearance (0·13 $\pm$ 0·01 to 0·22 $\pm$ 0·01 mg/ml/hr for the six subjects) were unaltered by primaquine. One subject received 45 mg primaquine phosphate once weekly for 3 weeks and, when retested in the same manner each week, no change in the rate of alcohol disposal was observed. Thus, when given to healthy individuals in the dose and schedule employed for malaria prophylaxis, primaquine does not significantly alter alcohol metabolism in man.

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