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PHOSPHOLIPID METABOLISM AS A NEW TARGET FOR MALARIA CHEMOTHERAPY. MECHANISM OF ACTION OF D-2-AMINO-1-BUTANOL

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Abstract—A number of choline and ethanolamine analogs were evaluated as inhibitors of *P. falciparum* growth *in vitro*. 1-Aziridineethanol, DL-2-amino-1,3-propranediol and D- or L-2-amino-1-butanol were the most efficient inhibitors of parasite multiplication, with an IC_{50} of 50–80 μM, whereas numerous other analogs were less active. The effect of D-2-amino-1-butanol on various metabolisms of *P. knowlesi*-infected simian erythrocytes was studied by incubating these cells with different labeled precursors of phospholipids, nucleic acids, proteins, and with radioactive glucose. In the presence of radioactive glycerol, oleate or lysophosphatidylcholine, the appearance of radioactivity in an unnatural phospholipid indicated that 2-aminobutanol was incorporated into a new PL which accounted for up to 30–40% of the total biosynthesized lipids. This new phospholipid accumulated primarily at the expense of PE biosynthesis and decreased the decarboxylation of phosphatidylserine. These effects were not accompanied, over a large range of concentrations, by any parallel change in nucleic or protein synthesis, nor in glucose metabolism. These data demonstrate that the incorporation of analogs, instead of the natural polar head groups, into cellular phospholipids, and/or modification of phospholipid composition have a deleterious impact on the growth of *Plasmodium*. It follows that PL metabolism is a crucial process for *Plasmodium* growth and may constitute a potentially fruitful chemotherapeutic approach to malaria.

The worldwide spread of chloroquine-resistant *Plasmodium falciparum* malaria has been further complicated by the increasing prevalence of parasites resistant to combinations of sulfonamides and pyrimethamine, and it has become clear that new methods must be found to reduce the dramatic resurgence of malaria [1, 2]. One approach to designing chemotherapeutic agents against this disease is to identify a metabolic requirement specific for *Plasmodium* and attempt to stop parasite development by interfering with this metabolism.

The biosynthesis of phospholipids (PLs)§ is a crucial process, since PLs are essential components of all cellular membranes. The erythrocytic cycle of the malarial parasite is now well characterized by a large increase (as much as 500%) in host-cell phospholipid content, mainly phosphatidylcholine (PC) and phosphatidylethanolamine (PE), whereas the enzymatic machinery for PL generation and turnover are either lacking or incomplete in mature mammalian erythrocytes [3–5]. Our recent studies on *P. knowlesi*-infected monkey erythrocytes [6] and erythrocytic cultures of *P. falciparum* [7] have allowed us to identify and quantify the different metabolic pathways which provide PL to the parasite. These results show that PL biosynthesis only occurs in the constitutive metabolism of infected erythrocytes. Therefore, we decided to investigate this difference between host-cell and parasite metabolism in order to design new drugs capable of exterminating the parasites without injuring the host-cell.

Development of *Plasmodium* inside the erythrocyte is characterized by a persistent need for the polar head groups from plasma as a building block of PL molecules [6]. Thus, analogs of the polar head groups could either curtail the availability of these precursors or result in unusual PL which could exhibit antiplasmodial activity. For example, modifications of the polar head group in membrane PL by base analogs have been studied in intact animals [8–10] and isolated cells [11–13] with respect to the structure and function of biological membranes. Consequently, we performed experiments to test whether head group analogs of PLs can inhibit parasite development. Such studies could lead to a new class of therapeutic agents which inflict no damage on the cellular metabolism of the host cell, and could provide an alternative approach to controlling the proliferation of this disease. Preliminary to any search for elaborated analogs, we analyzed the antiplasmodial effect of fifteen commercially available choline and ethanolamine analogs known for their capacity to interfere with PL metabolism [8–13], or not yet tested. Our present results show that most of them prevent parasite maturation and that these agents, such as 2-amino-1-butanol, probably achieve their inhibitory effect against *P. falciparum in vitro* by direct interference with PL metabolism, which

§ Abbreviations: PL, phospholipid; PC, PE, PS, PI, phosphatidyl-choline, -ethanolamine, -serine, -inositol; TLC, thin layer chromatography; TCA, trichloroacetic acid.

is therefore a possible target for malaria chemotherapy.

MATERIALS AND METHODS

Chemicals. The various drugs came from Sigma (2-dimethylamino ethanol, DL-2-amino-1-propanol, DL-2-amino-1-3-propanediol and chlorocholine), from EGA-Aldrich, France (1-azidirineethanol, 2-diethylaminoethanol, D-2-amino-1-butanol, L-2-amino-1-butanol, DL-1-amino-2-propanol, 6-amino-1-hexanol and 2-chlorethylamine), from Ventron GMBH (3-amino-1-propanol), from Fluka (5-amino-1-pentanol), and from Hoechst-Behring (2-aminoethylphosphonic acid). L-[U-^{14}C]serine was purchased from CEA, France, [U-^{14}C] glucose, [1(3)-^{3}H] glycerol, [9,10(n)-^{3}H] oleic acid, [myo-2-^{3}H] inositol, [2-^{14}C]ethanolamine and [methyl-^{3}H] choline from Amersham, and [^{3}H (G)] hypoxanthine from NEN. RPMI 1640 was obtained from GIBCO (France), modified RPMI 1640 without choline, inositol and serine was provided by Eurobio (France). Outdated AB^{+} human blood or AB^{+} human serum came from the blood bank of Montpellier. All reagents were of analytical grade.

Determination of antiplasmodial activity. Drug effects on parasite growth were assayed on *P. falciparum* in culture. This parasite (Nigerian strain obtained from Dr W. H. Richard, Wellcome Res. Lab., Beckenham, U.K. [14]) was cultivated in AB^{+} human erythrocytes suspended in complete medium composed of RPMI 1640 supplemented with 25 mM Hepes buffer, pH 7.4, and 10% AB^{+} serum using the Petri-dish candle-jar method [15]. Stock cultures of infected cells were maintained in 1.5 ml of an 8% erythrocyte suspension in 3.5 cm plastic Petri dishes.

A single suspension of parasitized cells was prepared for each experiment by appropriately diluting the harvest from a stock solution (parasitemia of 4–10%) with freshly washed uninfected cells to give a 0.2–0.3% initial parasitemia. Cultures were then placed in a 24-well plate (Falcon) by depositing 0.3 ml of the parasitized erythrocyte suspension in each well. Then each well received 0.3 ml complete medium without (control), or with the drug at twice the desired final concentration, bringing the total volume to 0.6 ml and the hematocrit to 7%. After one day in a single large candle-jar at 37°, the media were carefully removed and replaced with 0.6 ml complete medium without drug for control or with drug at the appropriate concentration for the treated cells. After an additional period of 24 hr, the media were replaced by fresh complete medium. On day 4, the parasitemia of the control and the treated samples was monitored on blood smears using a 10% Giemsa Azure type B stain in phosphate buffer pH 7.2.

Drugs were dissolved in water and the pH was adjusted to 7.4 when necessary. Each experiment with various concentrations of drugs was performed in triplicate at least twice, each time with different infected erythrocyte preparations. The percentages of growth inhibition and/or invasion in the presence of various inhibitors were calculated by taking the control as 100.

Biochemical studies. These were carried out on simian erythrocytes parasitized by *P. knowlesi*. Splenectomized *Macaca fascicularis* (Sanofi, Montpellier, France), weighing 3–6 kg, and Washington strain, variant 1 of *P. knowlesi* (Dr G. Mitchell, Guy's Hospital, London) were used. The strain was maintained either by inoculating from animal to animal or by cryopreservation [16]. Levels of parasitemia were determined at appropriate intervals by microscopic examination of a Giemsa-stained film of tail blood. Highly infected blood was collected aseptically by venipuncture in CAD (citric acid, dextrose) after the monkey had been anesthetized by an intramuscular injection of Ketamine (Iffa-Credo, France). The monkeys were cured by an intramuscular injection of 150 mg chloroquine on three consecutive days. After 6 weeks, the monkeys could be reinfected.

Cells were collected by centrifugation at 7×10^{3} *g*.min and washed once with basic medium (RPMI 1640 supplemented with 50 mM Hepes pH 7.4). White cells were removed by passage through a cellulose powder column (CF 11 Whatman) [17]. After two washes with basic medium, the infected erythrocytes were ready for experimentation.

All incubations with radioactive precursors were carried out at a hematocrit of 10–20%, in enriched medium composed of modified RPMI 1640 (40 μM choline and inositol, 140 μM serine) supplemented with 50 mM Hepes buffer (pH 7.4), 1 mM ATP, 30 μM CoA, and 0.5 mM of the essential plasmatic fatty acids, namely palmitic, stearic, oleic and linoleic acids in a molar ratio 1.7/0.7/1.3/1.3, respectively, bound to fat-free bovine serum albumin (17–20 mg/ml). ATP and CoA facilitate the formation of the fatty acid derivatives (e.g. oleyl-CoA) involved in their incorporation into PL [5, 6]. After incubations at 37° in stoppered test tubes in a shaking water bath, reactions were stopped at +4°. The cells were then centrifuged 10^{4} *g*.min. and washed once with 5 ml basic medium and twice with 5 ml 0.9% NaCl at +4°.

Cellular lipids were extracted according to the method of Folch *et al.* [18], as modified [19], then fractionated by thin layer chromatography (TLC) as described previously [6], except that the PS and PI were separated by developing the plate in $CHCl_3$/CH_3OH/CH_3COOH/H_2O (65:43:1:3, by vol.). After visualization with iodine vapor, radioactive spots were counted with a Packard 460 CD liquid scintillation spectrometer using a scintillation cocktail (Packard No. 299). No measurable radioactivity was found apart from spots corresponding to the lipids mentioned in the table and the figures. The amounts of labeled precursors incorporated into cellular lipids (nmol/10^{7} cells/time) were computed on the basis of radioactivity incorporated into lipids and the specific activity of the precursor in the incubation medium. Values were corrected for the activity of unparasitized cells present in each preparation [6]. In experiments where [U-^{14}C] serine was incorporated into PE or PC, radioactivities were corrected for the loss of one radioactive carbon unit.

To measure labeled isoleucine or hypoxanthine incorporation, incubated cells were washed twice with NaCl 9% at 4°, then mixed with 4 ml of cold trichloroacetic acid (TCA). The precipitates were

washed twice with 4 ml 10% TCA and the final residues were solubilized in 0.8 ml NCS (Amersham) for 12 hr at 37°. 1.5 ml of decolorizing H_2O_2 (110 vol.) was added, followed by 20 ml of scintillation fluid. The samples were then counted for radioactivity.

The liberation of $^{14}CO_2$ from [U-^{14}C] glucose metabolism was determined by incubating erythrocytes in glass tubes closed with rubber stoppers allowing the passage of a syringe needle, and containing a suspended glass fiber filter (1.6 cm^2 GF-D Whatman) wetted with 2N NaOH. At the end of the incubation time, reactions were stopped by acidifying the medium with 0.5 ml of 2 N H_2SO_4 and the tubes were shaken for 2 hr to assure complete trapping of $^{14}CO_2$. Radioactivity of the filter was determined in a 15 ml scintillation cocktail.

Mass spectrum analysis. The lipid fractions were chromatographically isolated by preparative TLC on precoated silica gel plates, 0.25 mm thick ($CHCl_3$: CH_3OH: CH_3COOH: H_2O, 65:45:1:3 by vol.) and the lipids were extracted from the silica gel with $CHCl_3$–CH_3OH (2:1). Mass-spectrum analyses were performed by direct insertion of the sample material into the apparatus source (LKB 2901, ion source at 240° electron beam energy 70 eV).

RESULTS

Effects of polar head group analogs on P. falciparum *in vitro.* Control experiments showed a continuous and progressive infection with a consistent 30–60-fold increase in parasitemia (i.e. the percentage of infected erythrocytes) over two successive cycles (96 hr). The effects of the different analogs were determined by incubating infected erythrocytes in their presence for 48 hr, which corresponds to a full cycle of asexual parasite multiplication. Parasitemia was always measured after 48 hr of additional growth in normal medium to eliminate abnormally growing parasites, which were no longer found on day 4 in cultures of treated cells.

Figure 1 shows that the different compounds tested inhibit *P. falciparum* growth in a dose-dependent fashion. The concentrations of the drugs reducing the parasitemia to 50% of the control value (IC_{50}) are shown in Table 1, as well as the structure of the different analogs.

We first measured the effect of *N*-substituted amino-alcohol. Naturally occurring 2-dimethylaminoethanol had a very weak antiplasmodial activity: the addition of 10^{-3} M to the culture medium inhibited *P. falciparum* growth by 35% whereas 5×10^{-3} M was necessary to assure no survival. When the methyl groups were substituted by bulkier ethyl groups, a significant decrease in the IC_{50} was observed, 2-diethylaminoethanol (IC_{50} = 400 μM) being three times as active as 2-dimethylaminoethanol. When nitrogen was included in a ring structure, a striking increase in the antiplasmodial activity was obtained and the IC_{50} of 1-aziridineethanol (50 μM) was 26 times lower than that of 2-dimethylaminoethanol.

We also tested ethanolamine analogs having an amine function at position 1 and different substitutes attached to the chain between the hydroxyl and amino groups. The activity of the analogs substituted on the carbon at the α position of the amino group depended on the substitutes. Thus D-2-amino-1-butanol (IC_{50} = 80 μM), which has an ethyl group, was four times as effective as DL-2-amino-1-propanol (IC_{50} = 300 μM), which was replaced by a methyl group. We also observed that the L-configuration of 2-amino-1-butanol showed the same pattern of inhibition as D-2-amino-1-butanol. However, DL-2-amino-1,3-propanediol, which has a hydroxyethyl group, had the highest antiplasmodial activity, with an IC_{50} value of 50 μM. A comparison of the IC_{50} of DL-2-amino-1-propanol (300 μM) and of DL-1-amino-2-propanol (800 μM) indicates that

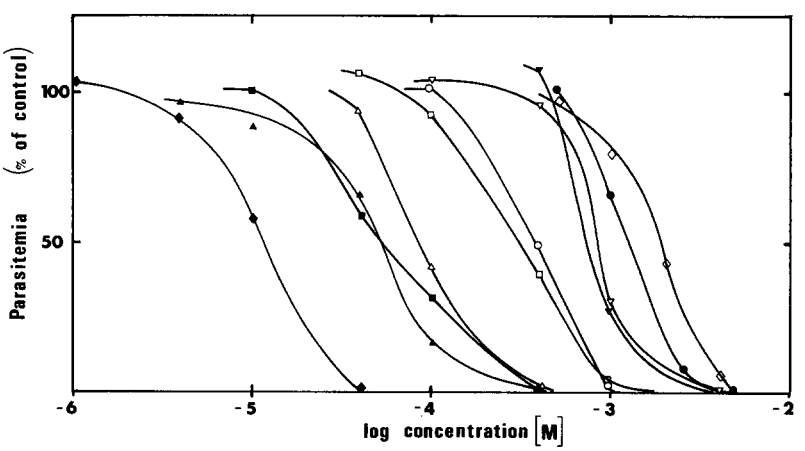
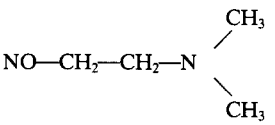
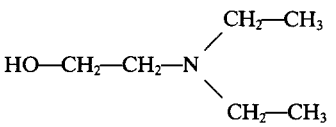
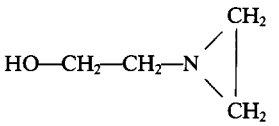
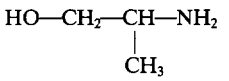
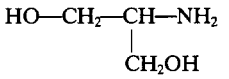
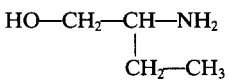
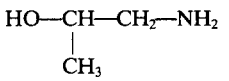
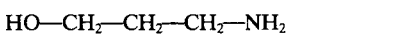
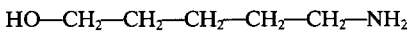
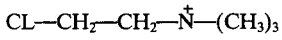


Fig. 1. Effects of different polar head group analogs on *P. falciparum* growth. Erythrocytes with an initial parasitemia of 0.2–0.3% were cultured for 4 days with daily medium changes. Parasitemias on day 4 are expressed as percentages of control (6–13% according to the experiments) after the parasites had been exposed to various concentrations of the analogs for the first 48 hr. Experiments were repeated at least once. 2-Dimethylaminoethanol (●), 2-diethylaminoethanol (○), aziridine ethanol (■), 2-amino-1-propanol (□), serinol (▲), 2-amino-1-butanol (△), 1-amino-2-propanol (▼), 3-amino-1-propanol (▽), chlorocholine (◇), 2-chloroethanolamine (◆).

Table 1. General structures of phospholipid polar head group analog bases and IC_{50} against *P. falciparum in vitro*

Analog base	Structure	IC_{50} (μM)
2-Dimethylaminoethanol		1300
2-Diethylaminoethanol		400
Aziridine ethanol		50
DL-2-Amino-1-propanol		300
DL-2-Amino-1,3-propanediol (serinol)		50
D-2-Amino-1-butanol		80
DL-1-Amino-2-propanol		800
3-Amino-1-propanol		800
5-Amino-1-pentanol		>2000
Chlorocholine		2000

substitutions on the carbon at the α position of the amino group may be more efficient for antiplasmodial activity than substitutions at the β position.

We also tested amino-alcohol with the linear structure OH-(CH_2) n-NH_2 where $n \geq 3$. 3-Amino-1-propanol with three methylene groups was found to be a poor inhibitor of *Plasmodium* growth, with an IC_{50} of 800 μM. Greater lengthening of the chain between the nitrogen and hydroxy groups, such as with 5-aminopentanol ($n = 5$) or 6-amino-1-hexanol ($n = 6$), completely abolished antimalarial properties ($IC_{50} > 2$ mM).

The pattern for the inhibition of *Plasmodium* growth was completely different with ethanolamine and choline analogs containing halogen instead of the hydroxyl group. 2-Chloroethylamine was the most efficient inhibitor of *Plasmodium* growth with an IC_{50} of 12 μM (results not reported in Table 1), whereas chlorocholine, with an IC_{50} of 2 mM, was a very poor inhibitor. Lastly, compounds bearing a carboxylic function, such as 2-amino-4-hydroxybutyric acid (homoserine), or DL-2-hydroxy-3-aminopropionic acid (isoserine), did not show any appreciable activity at 10^{-3} M. Also, 2-amino-ethylphosphonic acid, which enters the composition of phosphonolipids [20], did not show antiplasmodial activity at 2×10^{-3} M (results not shown).

Mechanism of the effect of D-2-*amino*-1-*butanol.* Our objective was to determine if the antiplasmodial effect of head group analogs is the result of specific effect on PL metabolism rather than, for example, the result of a generalized and non-specific toxic effect on the infected cells. To this end, we evaluated their action on PL metabolism as well as on DNA, protein and glucose metabolism. These time-consuming experiments were conducted with D-2-amino-1-butanol, one of the most efficient analogs (IC_{50}: 80 μM) among those preserving a free hydroxyl group, and also partly with 2-chloroethylamine (IC_{50}: 12 μM), which has no hydroxyl group. These experiments were carried out with *P. knowlesi*-infected erythrocytes, since they can be obtained in large amounts from infected monkeys.

Examination of lipid fractions from infected cells incubated for 3 hr in the presence of 2×10^{-3} M 2-amino-1-butanol showed that the incorporation of [^{3}H]-glycerol into the PC, PS and PE fractions was substantially inhibited, compared to the control cells. But in the presence of 2-aminobutanol, a significant portion of the radioactivity appeared in an unidentified spot migrating between the PE and neutral lipid fractions (Fig. 2).

Since the incorporation of unnatural base analogs, containing a free hydroxyl group, into the PLs of cells has been reported [8–13], one would expect a

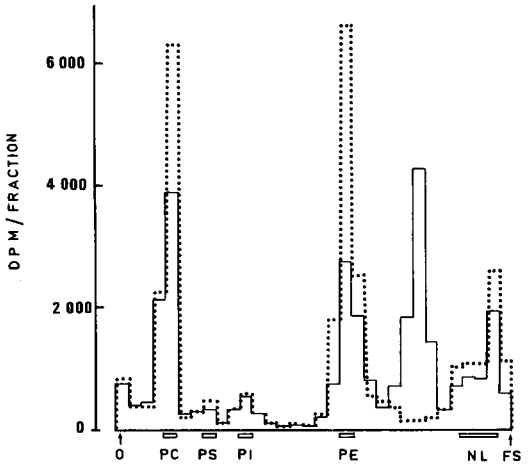
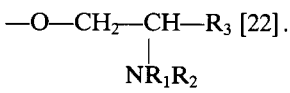


Fig. 2. Radiochromatograms of [^{3}H]glycerol-labelel lipid fractions of *P. knowlesi*-infected erythrocytes incubated in the presence of 2-amino-1-butanol. 2.1×10^9 *P. knowlesi*-infected erythrocytes with 8% parasitemia were incubated for 3 hr in 1.5 ml of enriched basic medium containing 0.5 mM [^{3}H] glycerol (7 Ci/mol). The lipid fractions were isolated and chromatographed on TLC plates in $CHCl_3$/CH_3OH/CH_3 COOH/H_2O (65:43:1:3 by vol). After visualization with iodine vapor, radioactivity was counted on 0.5 cm silica fractions O: chromatographic origin; PC, PS, PI, PE, NL: location of phosphatidylcholine, -serine, -inositol, -ethanolamine and neutral lipids respectively; FS: front solvent ----- control incubation; ——— incubation in the presence of 2×10^{-3} M amino butanol.

new PL with 2-amino-1-butanol as the polar head. The R_f value is slightly higher than that for PE and corresponds to the expected chromatographic mobility for phosphatidyl-2-amino-1-butanol [11]. Unfortunately, enzymatic synthesis of this new PL by transphosphatidylation of egg yolk PC with phospholipase D from cabbage (Sigma P 8398) [21] was not successful, probably because 2-amino-1-butanol could not act as a substrate for the enzyme.

Mass spectrometry provided a substantial amount of structural information about the phospholipid nature of the new radioactive spot. The only phospholipid structure for which the typical diacyl glycerol ion (Fig. 3) can occur involves an ethanolamine polar head



The other principal characteristic ions result from successive decompositions of the diacylglycerol ion. The ions of naturally biosynthesized phospholipids are accompanied by homologous ions that vary by 28 or 56 atomic mass units and each case can additionally vary by two atomic mass units reflecting the homologous patterns of fatty acid composition. By comparing the mass spectra of natural phospholipids, biosynthesized by parasitized erythrocytes, with authentic samples of compounds, it was possible to

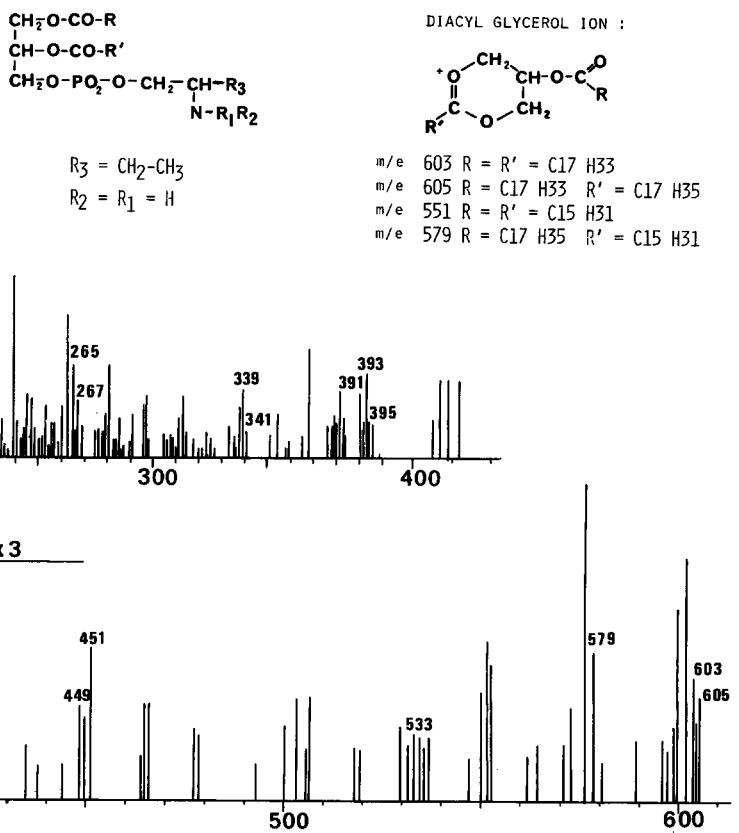


Fig. 3. Mass spectrum of the new phospholipid. Only characteristic diacyl-glycerol ions and the resulting decomposition fragments are reported. The lack of an *m/e* 551 peak showed the absence of the dipalmitoyl-glycerophosphorylaminobutanol derivative.

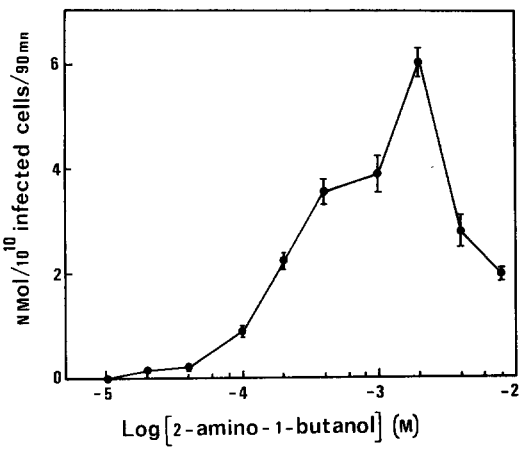


Fig. 4. Effect of increasing concentrations of 2-amino-1-butanol on [^{3}H]glycerol incorporation in the new radioactive spot. *P. knowlesi*-infected erythrocytes were incubated at hematocrit of 10% in basic medium containing the indicated concentrations of 2-amino-1-butanol for 1 hr at room temperature. Then cells were pelleted, supernatant removed, and 2.6×10^9 cells (17.6% parasitized) were further incubated for 90 min at 37° in 1.2 ml of RPMI-enriched medium containing 25 μM [^{3}H]glycerol (350 Ci/mol) and the various concentrations of aminobutanol. Each point represents the mean of triplicate experiments. Vertical bars correspond to ± S.E.

identify the following structures: stearoyl–palmitoyl and dipalmitoyl glycero-3 phosphatidylethanolamine; oleyl–stearyl, stearoyl–palmitoyl and dipalmitoyl glycero-3 phosphatidylcholine.

The same type of study was performed on the new lipid. The mass spectrum showed ion fragments corresponding to a typical diacylglycerol ion. This reflects the presence of an ethanolamine skeleton as the polar head of the new lipids which can be assumed to be 2-amino-1-butanol. The presence of ions *m/e* 605, 603 and 579 showed that the new PL was mainly a mixture of stearoyl-oleyl, dioleyl and stearoyl-palmitoyl glycero-3-phosphatidylaminobutanol.

In the following experiments, the infected cells were preincubated in the basic medium for 60 min at room temperature with different concentrations of 2-aminobutanol. Then the medium was changed and radioactive precursors were added to the reaction mixture with or without the analogs, as described in Fig. 4. This figure shows [^{3}H]glycerol incorporation in the new radioactive spots, when infected erythrocytes were incubated with various concentrations of the analog. Significant incorporation occurred in the presence of 2-aminobutanol concentrations as low as 2×10^{-5} M, and increased gradually up to 2×10^{-3} M. At higher concentrations, radioactivity recovered in the new lipid decreased sharply. This result may signify that this concentration was required to alter the viability of the parasite and its whole metabolism after 150 min contact with the drug.

These results suggest that 1-amino-2-butanol is incorporated as a base into the PLs of *P. knowlesi*-infected erythrocytes, presumably by the base activation pathway as seen in other tissues [8–11].

Figure 5 shows that, in the same experiment, [^{3}H] glycerol incorporation into PS and PI of treated erythrocytes was not affected at 2×10^{-3} M 2-aminobutanol but was inhibited by more than 40% at 4×10^{-3} M. *De novo* PC biosynthesis was already significantly decreased by 2×10^{-3} M 2-aminobutanol (−30%) and dropped rapidly at higher concentrations. Incorporation into PE was much more affected, exhibiting significant inhibition with analog concentrations as low as 10^{-4} M (− 16%) and inhibition quickly increased at higher concentrations (− 40% at 10^{-3} M aminobutanol).

Incorporation of [^{3}H]glycerol into diacylglycerol and triacylglycerol (data not shown) was slightly inhibited at 10^{-3} M, but inhibitions reached 68 and 71% respectively for the highest concentrations of the analog, and resembled the incorporation into PC in many respects.

The predominant effect of 2-aminobutanol on PE metabolism prompted us to examine how this analog affects the incorporation of the various natural polar heads as well as the incorporation of free fatty acids and lysopalmitoylphosphatidylcholine into the different PLs. Figure 6 shows that incorporation of labeled choline and inositol into PC and PI respectively was affected only at concentrations of 2-aminobutanol equal to or higher than 4×10^{-3} M. In contrast, [^{14}C]ethanolamine incorporations, which labeled both PE and PC as previously demonstrated [6], were fairly inhibited at a concentration of 10^{-4} M aminobutanol. Both syntheses were similarly and progressively inhibited by increasing concentrations of aminobutanol, to the extent that only 20–30% of the PL radioactivities were recovered at 2×10^{-3} M.

The metabolism of [^{14}C] serine, which was incorporated simultaneously into PS, PE and PC [6, 7], was also severely affected. Although the radioactivity recovered in PS only decreased by 14% with

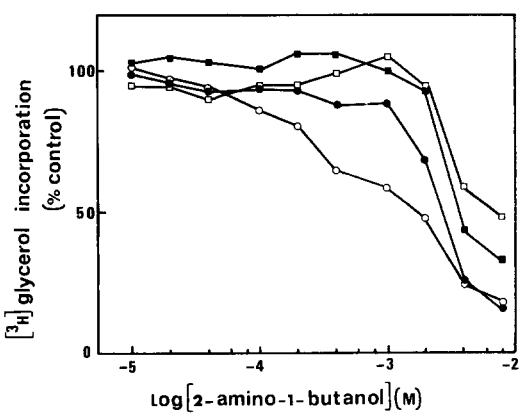


Fig. 5. Effects of different concentrations of 2-amino-1-butanol on [^{3}H]glycerol incorporation into the PL of *P. knowlesi*-infected erythrocytes. Cells were processed as described in Fig. 4. Data are means of three independent experiments. The incorporations are expressed as percentages of control represented by cells incubated in the absence of aminobutanol. The 100% values for [^{3}H] glycerol incorporation into PC (●), PS (■), PI (□) and PE (○) were 15.4 ± 0.4, 1.02 ± 0.03, 1.2 ± 0.1 and 11.6 ± 0.7 nmole/10^{10} infected cells/90 min ± S.E. respectively.

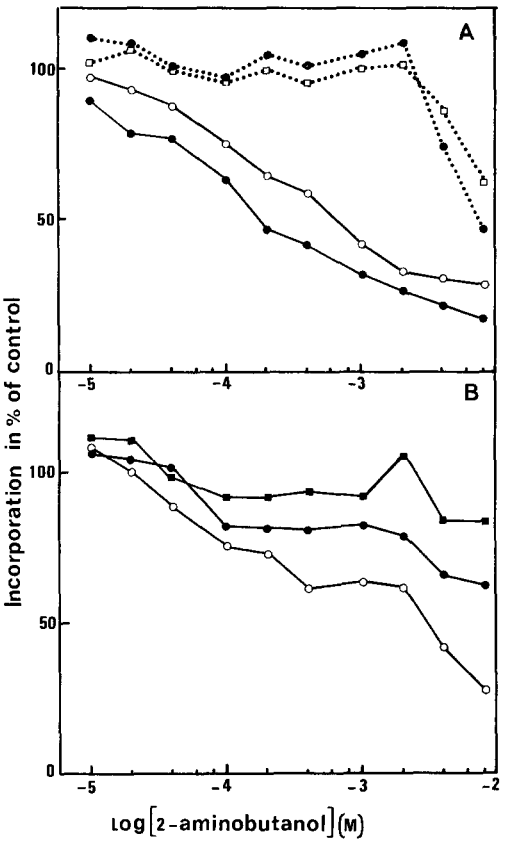


Fig. 6. Incorporation of the natural polar head groups into the phospholipids of *P. knowlesi*-infected erythrocytes as a function of 2-amino-1-butanol concentration. Incorporations were measured as in Fig. 4 except that the radioactive precursors were: (A) 40 μM [^{3}H]choline (65 Ci/mole), 40 μM [^{3}H]inositol (45 Ci/mole) or 40 μM [^{14}C]ethanolamine (4 Ci/mole; (B) 140 μM [^{14}C]serine (6 Ci/mole). Data are means of three independent experiments and are expressed as percentages of the incorporations observed in the absence of aminobutanol. Media contained 2.6×10^9 cells, 17.6% parasitized, for choline, inositol and serine incorporation, and 1.2×10^9 cells, 54% parasitized for ethanolamine incorporation. In control cells, the incorporation (nmol/10^{10} infected cells/90 min ± S.E.) were: [^{3}H] choline into PC (●– – –●) 18 ± 0.7; [^{3}H]inositol into PI (□– – –□) 2.7 ± 0.2; [^{14}C]ethanolamine into PE (○——○) and PC (●——●) 49 ± 4 and 35.7 ± 1.3 respectively; [^{14}C]serine into PS (■——■), PE (○——○) and PC (●——●) 10.2 ± 0.6, 30.5 ± 3.2. 7.4 ± 0.3 respectively.

4×10^{-3} M aminobutanol, concentrations of the analog as low as 10^{-4} M caused significant reductions in the labeling of PE (−25%) and of PC (−18%). Higher concentrations of aminobutanol decreased the radioactivities of the two PLs more substantially. Thus, these results confirm that this analog selectively alters the biosynthesis of PE when it is added at low concentrations to the incubation medium of infected erythrocytes.

In experiments with labeled choline, ethanolamine and serine, we counted intracellular water-soluble radioactivities. With [^{3}H] choline, this radioactivity was represented essentially by free choline and phosphorylcholine; radioactive CDP-choline, which is immediately converted into PC, was not detectable (Ancelin *et al.*, in preparation). A similar distribution of [^{14}C]ethanolamine, i.e. free ethanolamine and phosphorylethanolamine, probably occurs. Figure 7 shows that 4×10^{-5} M 2-aminobutanol significantly decreased the water-soluble radioactivity of cells incubated with [^{14}C]ethanolamine, whereas 10^{-3} M was required to reduce the intracellular radioactivity with [^{3}H]choline. In contrast, 2-aminobutanol did not affect the recovery of [^{14}C]serine by infected erythrocytes below 8×10^{-3} M.

The effects of the analog on the utilization of [^{3}H]oleate and [^{14}C-palmitoyl] LPC which supply parasitized cells with fatty acids [5, 6] are presented in Fig. 8. In the presence of 2×10^{-4} M or 2×10^{-3} M 2-aminobutanol, whole incorporations of both precursors into the different lipids (i.e. conventional PLs + new PL + neutral lipids) were not changed. At 2×10^{-4} M the new PL was essentially formed at the expense of PE, since incorporations of radioactive oleate and LPC were significantly decreased only in PE (−24 and −32% respectively). With 2×10^{-3} M, reductions of the incorporations into PE were even more marked, and significant reductions of oleate incorporation into PC (−24%) and diacylglycerol (−32%) were also registered.

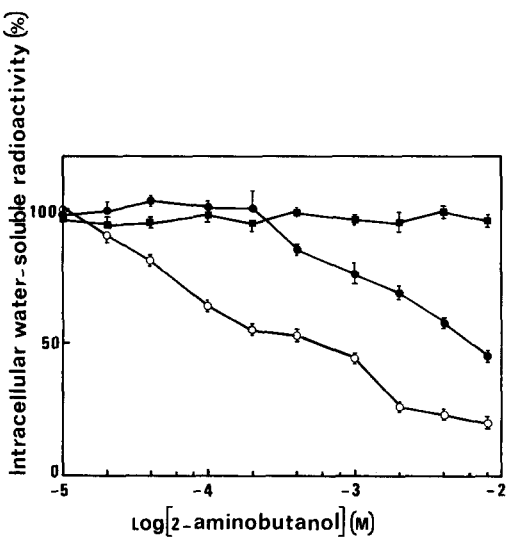


Fig. 7. Effects of 2-amino-butanol on intracellular water soluble radioactivities from labeled choline, ethanolamine and serine. 10^9 infected erythrocytes with a 15% parasitemia were preincubated for 1 hr at 37° in 400 μl modified RPMI 1640 (20 μM choline and ethanolamine, 50 μM serine) containing 50 μM Hepes, pH 7.4 and 2-aminobutanol at the indicated concentrations. Then 200 μl of the same medium with one radioactive precursor was added and incubations were continued for 30 min. After centrifugation at +4°, the cells were washed three times. Aqueous phases of the Folck's extraction procedure were pooled and counted for radioactivity. The incubations are expressed as percentages of control cells incubated in the absence of the analog and are means of three separate experiments. The 100% values ± S.E. for incubations in the presence of choline (3.5 Ci/mole) (●——●), ethanolamine (6 Ci/mole) (○——○) and serine (11 Ci/mole) (■——■) were 10.3 ± 0.7, 21.9 ± 1.1 and 6.5 ± 0.4 nmoles/10^{10} total cells/30 min. Vertical bars correspond to ± S.E.

Similar experiments were carried out with 2-chloroethylamine. *P. knowlesi*-infected erythrocytes were incubated for 3 hr in the presence of 2×10^{-4} M or 2×10^{-3} M 2-chloroethylamine and with one of the following labeled precursors: glycerol, choline, inositol, serine, oleic acid or lysopalmitoylphosphatidylcholine. However, this analog never modified the incorporation of the radioactive precursors into the different phospholipids and neutral lipids (results not shown).

For comparative purposes, the effects of 2-aminobutanol on protein, DNA and glucose metabolism, as a function of its concentration in the incubation medium, were also investigated. Hypoxanthine, which is the preferred purine for intraerythrocytic plasmodia [4], and isoleucine, which undergoes high levels of incorporation into parasite protein and is absent from Rhesus monkey hemoglobin [23], were chosen to label, respectively, the nucleic acids and proteins of *P. knowlesi*-infected erythrocytes. The output of radioactive $^{14}CO_2$ from [U-^{14}C] glucose served as an index of glucose metabolism [24].

Figure 9 shows that, below 2×10^{-3} M, no variation in the trichloroacetic acid-insoluble (TCA) fraction was observed between the control and the aminobutanol-treated cells, whatever the precursor; however, higher concentrations of the analog substantially inhibited the incorporation of hypoxanthine and isoleucine into their respective macromolecules. In contrast, similar experiments carried out with [U^{14}C] glucose (1 μCi/ml incubation medium) showed no change in the output of $^{14}CO_2$ at any of the analog concentrations.

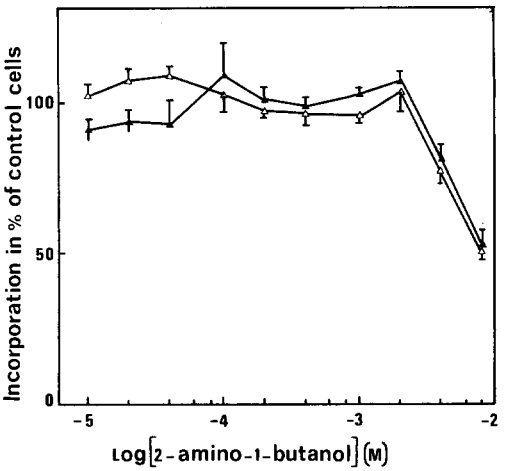


Fig. 9. Effect of 2-amino-1-butanol on the incorporation of labeled hypoxanthine (△) and isoleucine (▲) into the TCA-insoluble material of *P. knowlesi*-infected erythrocytes. [^{3}H] Hypoxanthine (5.3 μCi/ml medium) was added to 1.2 ml suspension containing 2.5×10^9 infected cells with a 17.6% parasitemia. [^{14}C] Isoleucine (1.6 μCi/ml medium) incubate contained only 0.51×10^9 cells, 54% parasitized. The incubations and processing of the cells were carried out as described in Fig. 4 and the text. Results are means of three separate experiments and are expressed as percent of the radioactivity in control cells, which contained 1,530,000 ± 137,000 dpm for [^{3}H]hypoxanthine and 195,000 ± 5000 dpm for [^{14}C]serine.

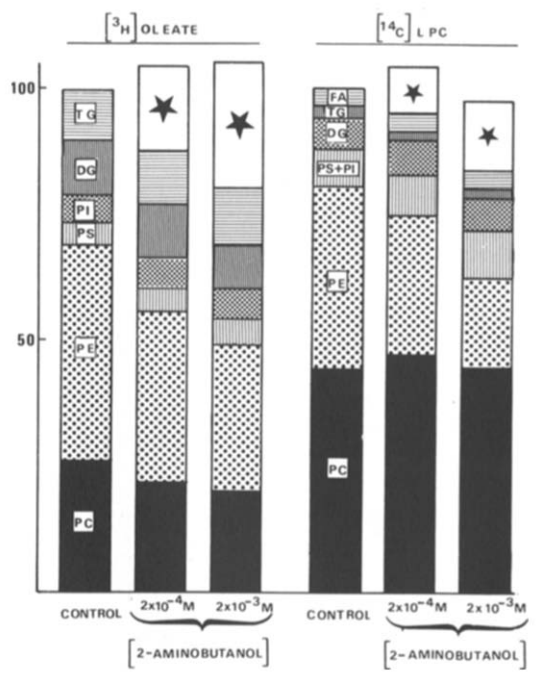


Fig. 8. Effect of 2-amino-1-butanol on the synthesis of lipids from [^{3}H]oleate and [^{14}C]lysophosphatidylcholine. 1.6×10^9 erythrocytes, 13% parasitized, were incubated as described in Fig. 4, except that the radioactive precursors were 130 μM [^{3}H]oleate (14 Ci/mole) or 70 μM [1-^{14}C-palmitoyl]-lysophosphatidylcholine (LPC) (1.4 Ci/mole) and only two concentrations of 2-amino-1-butanol were studied. Results are expressed as percentages of the sum of the radioactivities of the lipids listed in the control cells (absence of the analog) and are the mean values from three separate experiments. PC, PE, PS, PI: Phosphatidylcholine, -ethanolamine, -serine, inositol; DG: diacylglycerol; TG.; triacyglycerol; ★ correspond to the radioactivities recovered in the new lipid spot in the presence of the analog.

DISCUSSION

PL metabolism is crucial for parasite development since the PL content of infected red blood cells increases by about 500% during a 24 hr *P. knowlesi* cycle ([3, 4], Vial *et al.*, submitted). Therefore it is obvious that any alteration in its development would involve a drastic disturbance of parasite maturation. We choose to study the effects of analogs of choline and ethanolamine because PC and PE are the main PLs of the parasite and some of these analogs are known to be incorporated into biological membranes [8–13]. Our aim was to define the optimal relationships between structure and antimalarial activity by discriminating between commercially available compounds in an attempt to model new therapeutic molecules. The present results provide evidence that several of these analogs have antiplasmodial properties *in vitro* and that one of them, taken as model, 2-amino-1-butanol, does in fact exert its toxic effect through an alteration of the PL metabolism of the infected erythrocytes.

In the first part of this work, we sought the analog structures which provide the greatest inhibition of *P. falciparum* growth *in vitro*. Preliminary experiments

showed that 2 mM choline did not inhibit *Plasmodium* growth whereas ethanolamine produced a slight inhibition at 2 mM (−35%). Among the tested compounds, 1-aziridineethanol and DL-2-amino-1,3-propanediol (serinol), both with an IC_{50} of 50 μM, and D- or L-2-amino-1-butanol (IC_{50} = 80 μM) were the most efficient inhibitors of parasite multiplication. All other compounds with a free hydroxyl group showed a substantially lower potency. The above-mentioned relative capacities of these analogs for inhibition of parasite growth may be compared with data reported in studies of the structure–activity relationships of choline and ethanolamine analogs used as inhibitors in three other models: (a) the biosynthetic pathways of PLs in tissues [11], (b) the choline transport system in erythrocytes [25, 26], (c) the high affinity choline transport system in the central nervous system [27]. For example, in the three models, the spatial distance between the N and OH functions is a crucial factor and cannot be longer than three methylene groups (i.e. 3.3 Å). Analogs with a greater N–O distance become poor inhibitors of choline uptake in the central nervous system [27, 28], of the choline carrier in erythrocytes [29], and of the incorporation of the natural polar head groups into PLs [11]. We observed that 3-amino-1-propanol and 5-amino-1-pentanol are also poor inhibitors of *Plasmodium* growth. Moreover, *N*-alkyl substitutions are possible, and diethylaminoethanol is known to be a better inhibitor than dimethylaminoethanol, whereas a rigid two-carbon-ring structure on the nitrogen, as on 1 aziridine-ethanol, yields a much higher activity [25, 27]. Also substitution of the N–O aliphatic chain at α of the nitrogen were efficient for analog activities whereas substitution on carbon at β was very deleterious [11, 30].

Thus, the few analogs we have tested show the same order of inhibitory potency as in the above systems. It is obvious that the IC_{50} values do not yet fulfil the requirements for any therapeutic use against malaria. Nevertheless, this first attempt to define the relationships between certain structural features of the polar head groups of PLs and their inhibitory effect on the growth of *P. falciparum in vitro* should be extremely useful for the design of future structural analogs.

In the second part of this work, we attempted to determine whether the antiplasmodial properties of the analogs result from a generalized disturbance of parasite metabolism or from a specific alteration of PL metabolism.

2-Chloroethylamine appears to be an unusually good antiplasmodial compound, with an IC_{50} value of 12 μM *in vitro*. The reason for this property is unclear. The analog did not affect PL metabolism at concentrations 170 times higher than the IC_{50} against *P. falciparum*. This suggests that the antiplasmodial effect of the compound probably did not involve an interference with PL metabolism. On the other hand, DNA-alkylating properties of 2-chloroethylamine have been reported [31]. These potential alkylating properties of 2-chloroethylamine could be involved in the effect observed here.

The results obtained with 2-amino-1-butanol confirm what can be expected from analogs containing a free hydroxyl group: they are incorporated by the infected cells when they can be associated with the formation of a false metabolite. Thus, the appearance of a new radioactive spot in lipid analysis by TLC (Fig. 2), the dose–response curves of its formation (Fig. 4), its chromatographic properties (Fig. 2), as well as its mass spectrum characteristics (Fig. 3), support the hypothesis that phosphatidyl-2-amino-1-butanol is biosynthesized by infected cells in the presence of 2-aminobutanol.

The formation of this new PL occurred without a reduction in the total lipid synthesis from labeled glycerol (Figs 4 and 5), oleate, and LPC (Fig. 8), and was maximal at 2×10^{-3} M 2-amino-1-butanol (Fig. 4). Higher concentrations inhibited the formation of the new PL and simultaneously inhibited the biosynthesis of proteins and nucleic acids (Fig. 9), which can be considered a severe alteration of the whole parasite metabolism, consequently lowering the viability of the parasite after 2.5 hr contact. As expected, this concentration was far higher than the IC_{50} for the *in vitro* antiplasmodial effect (80 μM), which was measured after contact with the analog during a full parasite cycle, i.e. 48 hr.

The dose–response study demonstrated an inhibitory effect on PE biosynthesis at concentrations of 2-aminobutanol as low as 10^{-4} M, regardless of the precursor (Figs 5 and 6). On the other hand, PS and PI biosyntheses (Figs 5 and 6) were not affected as long as the DNA and protein synthesis were not altered, i.e. below 4×10^{-3} M 2-aminobutanol. PC biosynthesis from [^{3}H]glycerol and [^{3}H]oleate were slightly affected at 2×10^{-3} M. Thus, these results suggest that the inhibitory activity was primarily at the PE biosynthesis level and that the formation of the new PL was essentially at the expense of PE which constitutes 40% of the PLs in infected erythrocytes and is, along with PC, the major structural PL present in *Plasmodia* [3]. Its biosynthesis is not clear, since ethanolamine is present in plasma only at very low concentrations [32] and is practically absent from the culture medium sustaining the growth of *P. falciparum in vitro* [15]. Nevertheless, we know that the parasite can metabolize ethanolamine into PE, although it has been shown that PS decarboxylation can provide most of the PE requirement of infected cells [6, 7].

The high incorporation of 2-aminobutanol into the new lipid (Fig. 4) makes it probable that, as in other cells [8–11], the analog was incorporated by the base-activation pathway (Kennedy pathway). The decrease in intracellular water-soluble radioactivity in the presence of labeled ethanolamine observed from 4×10^{-5} M 2-aminobutanol indicates that the analog strongly interferes with the entry of this precursor into the cells. On the other hand, the strong bond between the carrier site and the cationic substrate in the choline transport system of erythrocytes [33] accounts for the fact that much higher concentrations of 2-aminobutanol were required to decrease the entry of choline (Fig. 7). The decline of PC biosynthesis which was observed slightly before the alteration of the parasite viability (Figs 5 and 6) may result from the decreased availability of choline in the presence of high concentrations of 2-aminobutanol (Fig. 7) or from the analog's utilization of the Kennedy pathway for its own incorporation.

Furthermore, since the entry of [^{14}C] serine into the cells (Fig. 7) and the biosynthesis of PS (Figs 5 and 6) were not affected by any concentration of 2-aminobutanol, the strong inhibition of PS decarboxylation by the analog (Fig. 6B) suggests that the newly formed PL usurps the role of PE, inducing a reduction of its biosynthesis from [^{14}C] serine. Thus, a short-term regulation of PE formation by PS decarboxylase seems to occur.

These results demonstrate that 2-aminobutanol can cause two major modifications in the membranous PLs of *Plasmodium*-infected erythrocytes: first, the formation of an unnatural PL via the incorporation of this analog as a polar head-group, and secondly a blockage of PE biosynthesis. At present, it is not possible to ascertain which of these two modifications is the most toxic for parasite viability. The parasite membranes play a major role in maintaining frontiers and in providing routes for the supply of nutrients and electrolytes, and the means for disposing of the catabolite produced by the developing parasite. It is known that modifications of the polar head-groups of PLs affect various membrane properties such as PL asymmetry, membrane fluidity, or the activity of membrane-bound enzymes [8, 12, 13, 34, 35]. Therefore, the antiplasmodial effect of 2-aminobutanol may be related to a deep modification of the physicochemical properties of parasite membranes, or to its disorganization following the marked reduction in PE biosynthesis, or to the presence of new polar heads.

The sharp recrudescence of malaria in the world gives a new sense of urgency to the search for vaccines and new chemotherapeutic treatments for this disease. The highly developed PL metabolism of *Plasmodium*-infected erythrocytes [3, 4, 6, 7] appears to be a good target for chemotherapy, and we have investigated the possible anti-*Plasmodium* effect of inhibitors of this metabolism. Our results show that parasite PL composition can easily be modified in its polar head groups by the incorporation of unnatural analogs containing a free hydroxyl group, which in turn affects the synthesis of natural PL. This may be related to the regulating mechanism of PL metabolism in these parasitized cells. Our results also show that PL metabolism is essential for erythrocytic schizogony, since many analogs of the polar head groups are potent inhibitors of parasite multiplication. Hence, PL metabolism can be considered as an attractive target for a new chemotherapeutic approach to malaria, especially since these agents should exhibit a selective toxicity to the malarial parasite and be innocuous for the host cells, which lack this metabolism [3, 6]. Further research is now necessary to develop structural analogs with higher antiplasmodial activity. The results presented in this paper should provide an important basis for this research.

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