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Use of Radioactive Ethanolamine Incorporation into Phospholipids To Assess In Vitro Antimalarial Activity by the Semiautomated Microdilution Technique

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Phospholipid biosynthetic activity is intense in the erythrocytic stage of *Plasmodium falciparum* because of the parasite's own enzymatic machinery. The incorporation of various labeled phospholipid precursors in comparison with the incorporation of nucleic acid and protein precursors was tested to evaluate *P. falciparum* growth in vitro. These precursors, namely, [^{3}H]ethanolamine, [^{3}H]hypoxanthine, [^{3}H]palmitate, [^{14}C]serine, [^{3}H]choline, [^{3}H]inositol, and [^{3}H]isoleucine, were all accurate indicators of parasite growth. However, because of its high level of incorporation, [^{3}H]ethanolamine proved to be the best tool for assessing parasite viability. When culture parameters were carefully controlled, [^{3}H]ethanolamine incorporation into phospholipids was proportional to pulse time, precursor concentration, and initial parasitemia and was sensitive to the number of uninfected erythrocytes (hematocrit). It can be used for a wide range of infected erythrocytes, from 2×10^4 to 5×10^5. The use of [^{3}H]ethanolamine for in vitro antimalarial drug screening is a good alternative to the method of Desjardins et al. (R. E. Desjardins, C. J. Canfield, J. D. Haynes, and J. D. Chulay, Antimicrob. Agents. Chemother. 16:710–718, 1979). The major advantage is that the culture medium can be supplemented with hypoxanthine, which results in better parasite growth. [^{3}H]ethanolamine is also an ideal tool when compounds that interfere with DNA and/or RNA metabolism are to be investigated for their effect on *Plasmodium* growth.

The worldwide resurgence of malaria has called for new chemotherapeutic programs and drug resistance evaluation of *Plasmodium falciparum* that involve less time-consuming and tedious methods than standard morphological assessment of Giemsa-stained parasites by photonic microscopy.

Tests for in vitro screening of antimalarial drugs or for determining the susceptibilities of parasites to antimalarial drugs are based on differences between normal and infected erythrocytes. These tests generally involve intercalation of fluorescent compounds such as ethidium bromide (21) or Hoechst 33342 (6) into nucleic acids or radioisotope incorporation. In the latter case, isoleucine incorporation into proteins (9) or purine nucleotide precursor incorporation into nucleic acids (2, 3, 7, 22) has been used. These parameters can be used directly or can be used after subtraction of the low background value caused by the presence of uninfected erythrocytes in the infected suspension.

The method of Desjardins et al. (3), which measures labeled hypoxanthine incorporation into DNA and RNA, is widely used. The major disadvantage of this method is that it is impossible to supplement the culture medium with cold hypoxanthine, even though it would be quite beneficial for parasite growth (14, 23). A makeshift technique has been proposed. This technique includes a subculture step to remove hypoxanthine prior to the use of the cultured material in the [^{3}H]hypoxanthine incorporation assays (14).

We propose the measurement of incorporation of labeled precursors into phospholipids (PLs) to assess intraerythrocytic *Plasmodium* growth. Indeed, PL biosynthesis of *P. falciparum* is high because of the intense membrane biogenesis that accompanies its growth. The more than sixfold increase in the erythrocytic PL content is mainly due to the parasitic enzymatic machinery, since such biosynthetic activity is entirely absent in mammalian erythrocytes (18). To synthesize PLs, the parasite depends on exogenous fatty acids (FAs) and polar heads, namely, choline, ethanolamine, serine, and inositol. In the present study, we demonstrate that radioactive ethanolamine incorporation into PLs could be a very useful tool for the assessment of parasite viability in vitro. Moreover, this method is a good alternative to hypoxanthine incorporation into nucleic acids.

MATERIALS AND METHODS

Chemicals. [1-^{3}H]ethan-1-ol-2-amine (18 Ci/mmol), [G-^{3}H] hypoxanthine (8.6 Ci/mmol), [*methyl*-^{3}H]choline (13 Ci/mmol), [9,10(*n*)-^{3}H]palmitic acid (54 Ci/mmol), L-[4,5-^{3}H] isoleucine (0.11 Ci/mmol), L-[U-^{14}C]serine (0.13 Ci/mmol), and myo[2-^{3}H]inositol (15 Ci/mmol) were purchased from Amersham Corp. (Les Ulis, France). Ethanolamine, hypoxanthine, and chloroquine-diphosphate (CQ) were from Sigma (St. Louis, Mo.). All reagents were of analytical grade. RPMI 1640 medium (12) was obtained from GIBCO (France); complete medium corresponded to RPMI 1640 medium supplemented with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.4) and 10% type AB$^+$ human serum.

Biological materials. Human blood and type AB$^+$ human serum were from the local blood bank (Montpellier, France). The Nigerian strain of *P. falciparum* (15) was maintained by serial passages in human erythrocytes by the petri dish-candle jar method (10) by using 7% hematocrit.

Standard procedure for assessing parasite viability by measuring the incorporation of labeled precursors into macromolecules. The standard procedure for assessing parasite viabil-

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ity involved measurement of the incorporation of labeled precursors (polar heads or FAs) into the PLs of an infected erythrocyte suspension. For comparison, radioactive isoleucine and hypoxanthine incorporations into proteins and nucleic acids, respectively, were also measured. Unless otherwise specified, the final volume in each well of the 96-well flat-bottom microculture plates was 230 μl, consisting of 50 μl of complete medium with or without the drug (control) and 150 μl of *P. falciparum*-infected erythrocyte suspension (0.1 to 3% final hematocrit and 0.05 to 1% initial parasitemia). Background values were obtained from normal human erythrocytes that were incubated under the same conditions. After 24 h of preincubation at 37°C, 30 μl of complete medium containing 1 μCi of radioactive precursors ([^{3}H]ethanolamine [^{3}H]hypoxanthine, [^{3}H]palmitate, [^{14}C]serine, [^{3}H]choline, [^{3}H]inositol, or [^{3}H]isoleucine) was added to each well, and candle jar incubations were continued for an additional 48 h. At the end of the incubation, the contents of each well were harvested onto glass fiber filters (Skatron no. 11731) by using a semiautomated cell harvester (Skatron). The cells were lysed with water, and the insoluble material (such as membranes, nucleic acids, and proteins) that was retained on the filter was thoroughly washed with water for 30 s. Filter disks were then dried for 35 s and were placed in a scintillation vial containing 2 ml of liquid scintillation fluid (Emulsifier-safe, Packard no. 299) and counted in a Beckman LS 3801 spectrometer. Parasitemia was routinely monitored on blood smears by using a 10% Giemsa azure type B stain in phosphate buffer (pH 7.2). Experiments were carried out at least twice in triplicate. Growth inhibition in the presence of drug was calculated, with the control values (without drug) being taken as 100%. The effect of drug is expressed as the inhibitory concentration that reduced parasitemia by 50% (IC_{50}).

RESULTS

Incorporations of labeled precursors into normal and *P. falciparum*-infected erythrocytes and assessment of the inhibitory effect of CQ. We measured the incorporation of labeled precursors, polar heads (i.e., ethanolamine, serine, choline, and inositol), and FAs (palmitate) into the PLs of an infected erythrocyte suspension. For comparison, radioactive hypoxanthine and isoleucine incorporations into nucleic acids and proteins, respectively, were also monitored. After incubation, cells were lysed and the water-insoluble material was collected on glass fiber paper by semiautomatic filtration; there was no appreciable contamination by the original radioactive precursors. Table 1 shows the radioactivities that were recovered on the filter paper after incubating normal or infected erythrocytes for 72 h in the presence of various radioactive precursors with the same amount of radioactivity per well (1 μCi). With control erythrocytes, except when palmitate was used as a precursor, the radioactivity recovered on filters was lower than 1,400 dpm. Since there is no synthesis of nucleic acids, proteins, or lipids in the mature erythrocyte (19), these values represent the radioactive background which must be deducted from the radioactive incorporations observed in infected erythrocytes. This was corroborated by the fact that these values were close to the background levels of radioactivity observed when incubations were carried out for 48 h at 4°C (data not shown). Only the radioactive palmitate recovered in control cells seemed to be present at a particularly high level.

The radioactivities associated with the water-insoluble material of infected erythrocytes were much higher than those observed in the control erythrocytes. Particularly notable were radioactivity values obtained after incorporation of [^{3}H]ethanolamine and [^{3}H]hypoxanthine, which reached 110,000 and 86,000 dpm, respectively, i.e., 78- and 183-fold their respective backgrounds in control erythrocytes. The radioactivity incorporation values were then as follows: 18,000 dpm for palmitate, 12,300 dpm for serine, followed by about 5,000 dpm each for choline and inositol and 1,500 dpm for isoleucine, which had the lowest incorporation. Thus, all the radioactive precursors could be used to assess parasite viability by taking the incorporation into infected erythrocytes relative to that into control cells into account.

TABLE 1. Comparison of the incorporation of various radioactive precursors into water-insoluble material of parasitized or normal erythrocytes[a]

Precursors	Radioactivity (dpm)	
	Parasitized erythrocytes	Control erythrocytes
[^{3}H]ethanolamine	110,200 ± 3,660	1,400 ± 40
[^{3}H]hypoxanthine	86,000 ± 640	470 ± 30
[^{3}H]palmitate	18,000 ± 430	6,420 ± 120
[^{14}C]serine	12,320 ± 270	380 ± 10
[^{3}H]choline	5,200 ± 110	1,000 ± 50
[^{3}H]inositol	4,890 ± 20	90 ± 5
[^{3}H]isoleucine	1,480 ± 110	290 ± 30

[a] Experiments were carried out with suspensions at 2% hematocrit of control erythrocytes or infected erythrocytes (0.2% initial parasitemia) in a final volume of 230 μl. After 24 h, radioactive precursors (1 μCi) were added to each microculture. Reactions were stopped at 72 h by filtration onto glass fiber papers with a cell harvester. Results are expressed as total disintegrations per minute ± 1 standard error of the mean.

We then investigated the possibility of using each of these PL radioactive precursors in the assessment of *Plasmodium* growth inhibition by CQ, the classic antimalarial drug. The infected erythrocytes were incubated for 24 h in the presence of various CQ concentrations; the cultures were then pulsed with the different labeled precursors (1 μCi) for 48 h, which corresponded to one full asexual multiplication cycle of the parasite. Control experiments without CQ showed continuous and progressive infection, since parasitemia steadily increased 10-fold over one and a half cycles (72 h). Chloroquine showed strong antimalarial activity, which occurred in the concentration range of 4×10^{-8} to 4×10^{-7} M, as determined in Giemsa-stained smears (data not shown). This was associated with a breakdown in the incorporation of each of the radioactive precursors into their respective macromolecules (Fig. 1). The results obtained with [^{3}H]hypoxanthine were very similar to those obtained with [^{3}H]ethanolamine, [^{3}H]palmitate, [^{14}C]serine, [^{3}H]choline, [^{3}H]inositol, and [^{3}H]isoleucine. Regardless of the radioactive precursor, sigmoidal dose-response curves were obtained, with IC_{50}s in the 7×10^{-8} to 9×10^{-8} M concentration range. These results suggest that all of these radioisotope precursors could be used as an alternative to [^{3}H]hypoxanthine to measure antimalarial activity in vitro.

Hypoxanthine and ethanolamine seemed to be the most appropriate precursors, with regard to their high levels of incorporation. Further experiments were thus carried out to determine the optimal conditions for measuring parasite viability with radioactive [^{3}H]ethanolamine, i.e., incubation time, precursor concentration, parasitemia, and hematocrit.

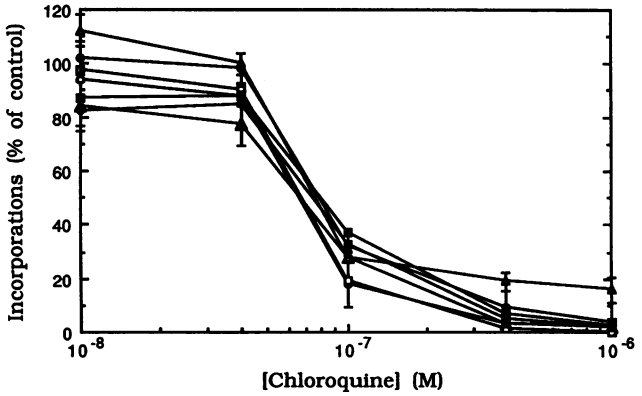

FIG. 1. Inhibitory effect of CQ on *P. falciparum* growth measured with various radioactive precursors. Suspensions (200 μl) of infected erythrocytes at 2% hematocrit and 0.6% initial parasitemia were incubated at 37°C in the absence (control) or the presence of CQ at the indicated concentrations. After 24 h, the cultures were pulsed for 48 h with 1 μCi of one radioactive precursor, namely, [^{3}H]ethanolamine (■), [^{3}H]hypoxanthine (□), [^{3}H]palmitate (△), [^{14}C]serine (●), [^{3}H]choline (○), [^{3}H]inositol (▲), or [^{3}H]isoleucine (◆) in 30 μl of medium. At the end of the incubation period, water-insoluble products were harvested on glass fiber filters. Radioactive incorporations on day 3 are expressed as percentages of their respective controls (2,000 to 110,000 dpm, as shown in Table 1). Each value is the mean ± 1 standard error of the mean of triplicate determinations.

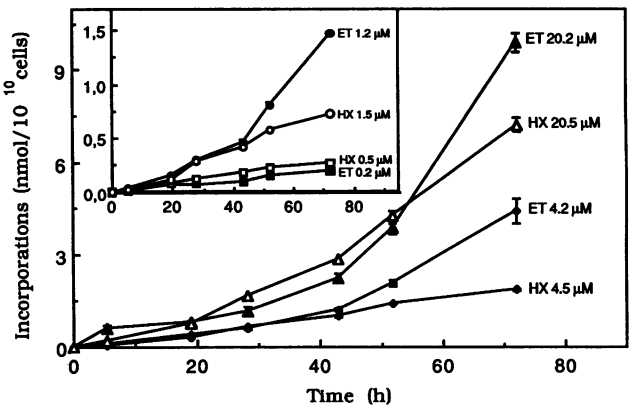

FIG. 2. Effect of ethanolamine or hypoxanthine concentration on the time course of their incorporation into macromolecules of *P. falciparum*-infected erythrocytes. Infected erythrocyte suspensions, at 0.55% initial parasitemia, were incubated at 37°C for the indicated times in the presence of [^{3}H]ethanolamine (ET) at 0.2 μM (18 Ci/mmol) (■), 1.2 μM (3.2 Ci/mmol) (●), 4.2 μM (0.93 Ci/mmol) (◆), and 20.2 μM (0.19 Ci/mmol) (▲) or [^{3}H]hypoxanthine (HX) at 0.52 μM (11.2 Ci/mmol) (□), 1.5 μM (3.8 Ci/mmol) (○), 4.5 μM (1.3 Ci/mmol) (◇), and 20.5 μM (0.29 Ci/mmol) (△). In all cases, the amount of label was kept constant (1 μCi). Results are expressed as nanomoles incorporated per 10^{10} cells ± 1 standard error of the mean (n = 3). For clarity, the curves corresponding to [^{3}H]hypoxanthine and [^{3}H]ethanolamine at low concentrations are magnified in the inset.

For comparison, hypoxanthine incorporation (hypoxanthine was used at trace doses) was monitored under the same conditions.

Time course of [^{3}H]ethanolamine and [^{3}H]hypoxanthine incorporation as a function of their external concentrations. Microcultures of *P. falciparum* were incubated with increasing concentrations of ethanolamine or hypoxanthine, ranging from 0.2 to 20.5 μM (without taking their low concentrations in serum [11, 17] into consideration). Figure 2 shows their incorporation into *P. falciparum* macromolecules as a function of incubation time at various external concentrations. At all concentrations, incorporations of both radioactive precursors were significant after only 20 h of incubation; thus, parasite growth could be assessed after short incubation times. Incorporation of both radioactive precursors generally increased linearly with time up to 42 h of incubation. Thereafter, the incorporations were enhanced, particularly with [^{3}H]ethanolamine, which probably corresponded to the increase in parasitemia.

Effect of parasitemia and hematocrit on [^{3}H]ethanolamine and [^{3}H]hypoxanthine incorporation. We first studied the effect of a number of infected erythrocytes on [^{3}H]hypoxanthine or [^{3}H]ethanolamine incorporation, with the total cell number being kept constant while varying the initial parasitemia. Regardless of the radioactive precursor, radioisotope incorporation increased linearly as the initial parasitemia increased (from 0.1 to about 1%) (Fig. 3). At higher levels of parasitemia, radioisotope incorporations remained slightly below the expected values and then reached a plateau. Examination of smears of every microculture at different levels of parasitemia showed progressive infection, with a steady 10-fold increase in parasitemia after 72 h. Hence, by using [^{3}H]ethanolamine as an index, parasite viability can be assessed over a wide range of initial levels of parasitemia and even at an initial level as low as 0.1%, which correspond to only 4.5 × 10^4 infected cells per assay.

Figure 4 shows the results that were obtained when both infected and uninfected cells were increased with the final volume remaining constant. After 72 h of incubation, [^{3}H]hypoxanthine and [^{3}H]ethanolamine incorporation increased almost linearly with hematocrit, from 0.1 to 3%. Above 3% hematocrit, [^{3}H]hypoxanthine incorporation decreased as the number of total erythrocytes increased, while [^{3}H]ethanolamine incorporation reached a plateau and appeared to be less affected by the increase in the number of uninfected erythrocytes. This slight deviation in radioactivity incorporation could be explained by a probable decrease in parasite viability after long incubation times at high hematocrit levels without any change of medium. When the effect of hematocrit was investigated for a shorter period (48 instead of 72 h),

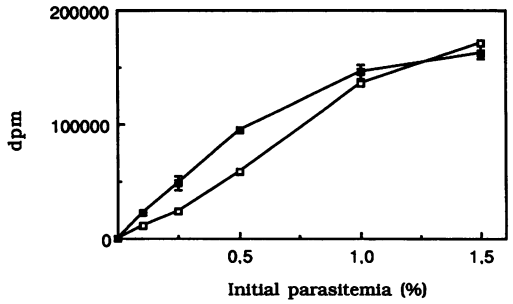

FIG. 3. Effect of initial parasitemia on [^{3}H]ethanolamine (■) or [^{3}H]hypoxanthine (□) incorporation into macromolecules of *P. falciparum*-infected erythrocytes. Incubations were carried out for 72 h with the erythrocyte suspension at 2% hematocrit, at the indicated initial parasitemia levels (from 0.1 to 1.5%), and in a final volume of 230 μl. Radioactive precursors (1 μCi) were added after 24 h in 30 μl of complete medium. Results are expressed as total disintegrations per minute ± 1 standard error of the mean.

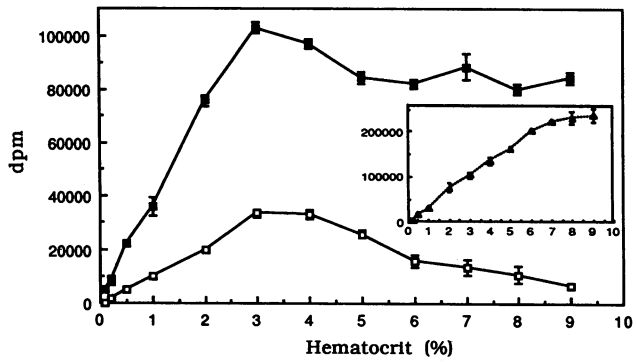

FIG. 4. Effect of hematocrit on [^{3}H]ethanolamine (■) or [^{3}H]hypoxanthine (□) incorporation into macromolecules of *P. falciparum*-infected cells. A total of 200 μl of infected erythrocyte suspension at a constant initial 0.6% parasitemia and the indicated hematocrit was added to each well, and after 24 h, 30 μl of complete medium containing 1 μCi of [^{3}H]ethanolamine or [^{3}H]hypoxanthine was added. Reactions were stopped at 72 h by filtration. The inset shows a similar experiment but in which the microcultures were pulsed immediately with [^{3}H]ethanolamine (without preincubation) for a shorter period (48 h).

radioactive ethanolamine incorporation into PLs increased linearly with hematocrit up to 7% and then plateaued (see inset of Fig. 4).

In this context, we investigated the possible effect of hematocrit on the measured IC_{50} of CQ (Fig. 5). At 1.7% hematocrit, the IC_{50} was about 2×10^{-8} M with both [^{3}H]ethanolamine and [^{3}H]hypoxanthine. With a fourfold increase in the hematocrit, there was a significant fourfold increase in the IC_{50} (8×10^{-8}M), irrespective of the type of precursor.

DISCUSSION

The development of *P. falciparum*, the malaria-causing parasite, depends on nutrients obtained from the host erythrocyte or external plasma to synthesize macromolecules such as nucleic acids, proteins, or lipids. *Plasmodium* lipids are particularly abundant because of the large increase in the total amount of membranes that accompanies its growth

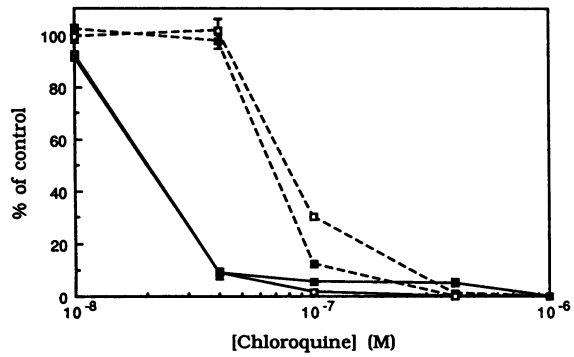

FIG. 5. Effect of hematocrit on inhibition of *Plasmodium* growth by CQ as assessed with [^{3}H]ethanolamine (■) or [^{3}H]hypoxanthine (□). A total of 200 μl of infected cell suspension (0.6% parasitemia) at 1.7% (dashed lines) or 8.1% (dotted lines) hematocrit was incubated for 24 h with CQ at the indicated concentrations, and then 30 μl of complete medium containing 1 μCi of labeled precursor was added. Reactions were stopped at 72 h.

inside the host erythrocyte. The major component of these new lipid molecules is PL. Consequently, *P. falciparum* is able to biosynthesize PLs intensively through its own enzymatic machinery. This intensive de novo biosynthesis of PLs is carried out during the erythrocyte stage by using exogenous building blocks (FAs and polar heads). This activity may be used to assess parasite growth, since there is a complete absence of any lipid synthesis pathway in the mature mammalian host erythrocyte (18). Plasma FAs and lysoPLs are both sources of FAs, whereas exogenous polar heads such as choline, ethanolamine, serine, and inositol are readily incorporated into *Plasmodium* PLs. Phosphatidylcholine is synthesized by a de novo pathway and also by methylation of phosphatidylethanolamine, which itself is derived from de novo biosynthesis or decarboxylation of phosphatidylserine (19). Thus, we used incorporation of the radiolabeled precursors mentioned above into parasitic PLs to assess *Plasmodium* viability. As a reference, we also measured hypoxanthine incorporation into parasite nucleic acids (this precursor was used by Desjardins et al. [3] to assess *P. falciparum* growth).

From the same amount of radioactive label precursor, very high levels of incorporation were obtained for ethanolamine and hypoxanthine (78- and 183-fold the respective backgrounds in controls). A significant amount of incorporation also occurred for palmitate and serine, whereas choline, inositol, and isoleucine showed the lowest amount of incorporation. The amount of radioactive incorporation of precursors into their corresponding macromolecules followed their specific activity but also depended on the intensity of each metabolic pathway. Indeed, regardless of the precursor, the radioactive incorporations were roughly parallel to their concentrations in complete medium. [^{3}H]inositol incorporation into phosphatidylinositol was low in infected cells. This low radioactivity may be explained by the low level of synthesis of phosphatidylinositol in infected erythrocytes (19), but also by the high concentration of cold inositol (194 μM) in RPMI 1640 medium (12). Despite the high serine concentration (285 μM) of RPMI 1640 medium (12), [^{3}H]serine was highly incorporated into infected erythrocytes. This corresponded to a high level of incorporation of serine into PLs (20) and also into proteins (16). The incorporation of [^{3}H]isoleucine, which is specifically incorporated into the protein synthesized by infected cells (9, 16), was quite low, probably because of the high isotopic dilution with the cold isoleucine present in RPMI 1640 medium at 381 μM (12). The FAs in plasma are strongly bound to albumin, and their incorporation into infected erythrocytes is a function of the FA/albumin molar ratio (4, 13). The total FA concentration in complete medium containing 10% serum is estimated to be 50 μM, and it is slightly less for palmitate, i.e., in the same concentration range as choline (21 μM in RPMI 1640 medium [12]). The incorporation of palmitate is higher than that of choline, perhaps because this FA is involved in the synthesis of every PL, whereas de novo biosynthesis of phosphatidylcholine from choline is one of two metabolic pathways that provide phosphatidylcholine to the parasite (20).

Nevertheless, the radioactive palmitate recovered in control cells seemed to be particularly high. Slow acylation of lysoPLs from the surrounding plasma and possibly from the erythrocyte membrane reportedly contributes to the relatively slow turnover of the mature erythrocyte PLs (18). However, this is probably not the cause of this high level, since short incubation or incubation at 4°C also leads to high background levels (data not shown). FA absorption on the

filter and the facts that FAs can be adsorbed onto the erythrocyte membrane and can bind many proteins (4, 13) probably explain this background value. It thus appears to be difficult to use this precursor to follow parasitemia.

Finally, each PL precursor could be efficiently used in assessing parasite growth inhibition, as shown with CQ, and could constitute an alternative to [^{3}H]hypoxanthine to measure antimalarial activity in vitro. Nevertheless, hypoxanthine, which is incorporated into nucleic acids, and ethanolamine, which is incorporated into PLs, seem to be the most appropriate precursors, since they are highly incorporated by the parasite. However, like hypoxanthine (11), ethanolamine is absent from RPMI 1640 medium and is present at less than 20 μM in plasma (17), which was used at 10% in complete medium. This could lead to its rapid depletion in the incubation medium, as reported previously (20).

We show that incorporation into macromolecules was continuous for at least 72 h when the precursors were present in the incubation medium from trace doses to 20 μM. This indicates that the direct addition of commercially available [^{3}H]ethanolamine, without any cold ethanolamine supplement, does not result in any significant depletion of the precursor because of its high level of incorporation into PLs, although this occurred under conditions with high levels of hematocrit and parasitemia (20). Hence, under our standard conditions with 72-h incubations, we were able to assess parasite viability after more than one parasite cycle using trace doses of precursor, thus allowing ready utilization of RPMI 1640 medium without any supplementation.

The use of trace doses of [^{3}H]ethanolamine as a *Plasmodium* growth index did not result in any incorporation variability in independent experiments (unpublished data). On the basis of the fact that 5,000 dpm was incorporated, ethanolamine can be used to detect as few as 2×10^4 infected erythrocytes (Fig. 3). Moreover, it was striking that regardless of the precursor, radioisotope incorporation increased linearly with initial parasitemia (to 1%) or with hematocrit (to 3%), which corresponds to cell numbers in both cases of 5×10^5 initial infected erythrocytes. Thus, ethanolamine incorporation can be used over a wide hematocrit range (0.1 to 3%) and with parasitemia levels of 0.1 to 1%, as long as the number of infected erythrocytes ranges from 2×10^4 to 5×10^5.

Nevertheless, at a high level of parasitemia or hematocrit, reduced labeled precursor incorporation occurred for both precursors. A probable decrease in parasite viability after long incubation periods without any change of medium could occur. The accumulation of catabolites and/or depletion of nutrients could lead to a general upset of parasite metabolism. Indeed, the exhaustion of substrates like glucose and the release of toxic metabolites like lactic acid after long incubation periods have been demonstrated previously (24). At high hematocrit (higher than 3%), a plateau for ethanolamine incorporation was observed, whereas the decreased incorporation which occurred with hypoxanthine was more intriguing. The additional effects of this precursor could be related to the high intracellular concentration of many purines in the erythrocytes, particularly hypoxanthine (11), which could be released in the medium and which could then interfere with hypoxanthine incorporation. Indeed, incubation of *P. falciparum* in the presence of a variety of purines has been shown to influence [^{3}H]hypoxanthine incorporation (2). When the final volume of each incubation was constant (230 μl), the observed effect was all the more marked when the hematocrit was high. The decreased incorporation of radioactivity could therefore be due to a decrease in hypoxanthine specific activity or to the use of other purines by the parasite. Conversely, [^{3}H]ethanolamine incorporation is not of concern, since it is present at trace levels in the plasma (17) and is probably also present in the erythrocytes.

Lastly, we showed that an increase in the hematocrit led to a significant concomitant increase in the IC_{50} of CQ when it was measured with either labeled hypoxanthine or ethanolamine. CQ is known to accumulate in *P. falciparum*-infected erythrocytes, in which the concentration of the drug can be as much as 1,000-fold higher than that in the plasma, thus leading to a high level of drug depletion in the medium after cell incubation. CQ uptake can also occur in uninfected erythrocytes, but to a lesser extent (30- to 40-fold less) (5, 8). This variability in the IC_{50} of the drug as a function of hematocrit is an important parameter when studying antimalarial drugs under physiological conditions, considering that human blood hematocrit is 50%.

The major finding of this study is that [^{3}H]ethanolamine is a useful tool for assessing *P. falciparum* growth in vitro. Because of the presence of very low levels of ethanolamine in plasma and, hence, its quasiabsence from the culture medium, high levels of radioactivity can be recovered in PLs. Since this incorporation reflects the activity of the parasitic machinery, it could be a useful tool for assessing the number of parasites in the erythrocyte suspension.

The results obtained with the [^{3}H]ethanolamine incorporation testing procedure presented here were superior to those obtained with the time-consuming morphological method. It also overcomes the disadvantages involved in visually counting parasites, since parasite viability cannot be assessed by microscopy. In this context, radioactive incorporations could avoid the detection of parasites that are visible in the microscopic smear examination but that are metabolically injured or dead.

Radioactive incorporation of ethanolamine was found to be as sensitive and reproducible as hypoxanthine incorporation for parasite growth assessment. Both radioactive precursors are stable and are used at the same radioactive concentrations. They are both readily available and at roughly the same price (differing by no more than 25%). However, the present [^{3}H]ethanolamine drug screening method showed some clear advantages over that used by Desjardins et al. (3). First, ethanolamine incorporation seems to be much less affected by long incubation periods without the daily change of medium generally required for drug screening. This is particularly true when harsh conditions such as high levels of hematocrit are used (Fig. 4). Furthermore, the culture medium can be supplemented with hypoxanthine (usually 50 mg/liter), which is widely known to improve in vitro parasite growth (23) without any interference with the incorporation of [^{3}H]ethanolamine into infected erythrocytes (data not shown). Thus, no makeshift subculture step is required to remove hypoxanthine prior to the use of the cultured material in [^{3}H]hypoxanthine incorporation assays (14). Finally, the use of [^{3}H]ethanolamine as a precursor is an indispensable tool that is required when compounds that interfere with purine nucleotide biosynthesis, or with the whole DNA and/or RNA metabolism, in general, are to be investigated for their effects on *Plasmodium* growth (1).

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