were motile and fluorochromasia-positive after a 3-hour incubation with EB and FDA at 5°C.

Ethidium bromide or FDA has been used in viability assays of free-living protozoa and other cells (*13, 17, 21–25*), and parasitic protozoa of the genera *Acanthamoeba, Babesia, Crithidia, Entamoeba, Giardia, Plasmodium*, and *Trypanosoma* exhibit fluorescent substrate–induced fluorochromasia (*26*). We believe, therefore, that the EB-FDA procedure may be of value in immunologic, biochemical, and pharmacological research with protozoan pathogens responsible for many human and animal diseases.

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15. Fluorescein diacetate (5 mg/ml in acetone) was kept at −20°C until use. For each experiment FDA (30 μg/ml) was prepared in PBS. Ethidium bromide (20 μg/ml in PBS) was kept at −20°C before use. Promastigotes were stained with EB and FDA by mixing 50 μl of the cell suspension in PBS with 25 μl of FDA and 25 μl of EB. After 2 minutes at 25°C, 50 μl of this was mixed with 50 μl of Protoslo or 1 percent (weight to volume) Polyox in PBS [D. M. Spoon, C. O. Feise, R. S. Youn, *J. Protozool.* **24**, 471 (1977)]. When extracellular amastigotes or infected macrophages were stained with EB and FDA, PBS was substituted for Protoslo or Polyox.
16. Yellow-green fluorescence due to FDA metabolism and red fluorescence due to EB–nucleic acid complexes were detected by epifluorescence microscopy of cells on glass slides under cover glass or in a phase hemocytometer. An American Optical Fluorostar microscope with a mercury-vapor lamp was used with various filter combinations. No cells showed autofluorescence. Viable promastigotes, macrophages, and intracellular or extracellular amastigotes showed FDA-induced fluorochromasia. Dead promastigotes, macrophages, and extracellular amastigotes exhibited red fluorescence. Slow penetration of EB into living cells at the concentrations used prevented detection of dead amastigotes in living macrophages.
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4 April 1984; accepted 23 August 1984

## Ascorbic Acid and the Behavioral Response to Haloperidol: Implications for the Action of Antipsychotic Drugs

Abstract. *Haloperidol, a widely used antipsychotic drug, was tested for its ability to block the behavioral response to amphetamine and to elicit catalepsy in rats treated with saline or ascorbic acid (1000 milligrams per kilogram of body weight). By itself, ascorbic acid failed to exert significant behavioral effects, but it enhanced the antiamphetamine and cataleptogenic effects of haloperidol (0.1 or 0.5 milligrams per kilogram). These results, combined with a growing body of biochemical evidence, suggest that ascorbic acid plays an important role in modulating the behavioral effects of haloperidol and related antipsychotic drugs.*

Haloperidol is one of the most widely prescribed antipsychotic drugs in the United States today. It is often the drug of choice in the treatment of schizophrenia and the paranoid psychosis produced by chronic amphetamine abuse (*1*). Apart from its therapeutic benefits, haloperidol also causes muscular rigidity and other extrapyramidal side effects (*2*). An overwhelming body of evidence suggests that both the antipsychotic and the motoric effects of haloperidol are mediated, in large part, by a blockade of forebrain dopamine receptors. In animal tests of antipsychotic efficacy, for example, haloperidol blocks the open-field behavior produced by amphetamine and other dopamine agonists in rats (*3*). The clinical potency of haloperidol is closely related to its ability to bind and presumably block dopamine receptors in vitro (*4*). Moreover, when administered alone, haloperidol elicits catalepsy, an animal analog of muscular rigidity that is believed to reflect a blockade of dopamine receptors in the neostriatum (*5*).

The dopamine agonists that have been tested with haloperidol either release ascorbic acid (AA) from brain tissue (*6, 7*) or are prepared with AA before injection (*8*). Furthermore, AA appears to be an essential ingredient of assays in vitro that have demonstrated a high affinity of haloperidol for the dopamine receptor (*9*). When combined with evidence that both AA and haloperidol accelerate the firing rate of neostriatal neurons (*6, 10*), these data suggest that an AA-induced interaction with the dopamine receptor modulates at least some components of the behavioral response to haloperidol. We now report results consistent with this hypothesis that, in standard animal tests, AA enhances the antipsychotic and extrapyramidal actions of haloperidol.

The behavioral response to *d*-amphetamine (1.0 mg per kilogram of body weight) was recorded from male Sprague-Dawley rats (approximately 300 g) housed individually under standard laboratory conditions (*11*). Each rat had been treated 10 minutes earlier with an intraperitoneal injection of haloperidol (0.1 mg/kg), AA (1000 mg/kg), haloperidol (0.1 mg/kg) mixed with AA (1000 mg/kg), or a comparable volume of saline (*12*). The *d*-amphetamine was adminis-

Table 1. Mean (± standard error of the mean) total scores for individual items of behavior produced by *d*-amphetamine. Behavioral scores were totaled and analyzed as described in the text. A planned comparison in conjunction with an analysis of variance indicated that the difference between the haloperidol and AA treatment groups was statistically significant ($P < 0.01$). The overall analysis of variance for preliminary treatment was also significant ($P < 0.01$).

| Preliminary treatment | *n* | Amphetamine-induced behaviors | | | | |
|---|---|---|---|---|---|---|
| | | Locomotion | Rearing | Sniffing | Forelimb shuffling | Repetitive head movements |
| Saline | 10 | 3.10 ± 0.95 | 5.40 ± 1.11 | 16.7 ± 1.60 | 6.90 ± 1.00 | 16.5 ± 1.63 |
| AA | 8 | 1.13 ± 0.55 | 2.50 ± 0.83 | 13.4 ± 0.97 | 4.63 ± 1.31 | 13.0 ± 0.95 |
| Haloperidol | 7 | 0.71 ± 0.41 | 1.29 ± 0.70 | 9.43 ± 1.05 | 2.29 ± 0.87 | 7.29 ± 1.88 |
| AA-Haloperidol | 8 | 0.00 ± 0.00 | 0.00 ± 0.00 | 7.00 ± 0.00 | 0.13 ± 0.13 | 1.00 ± 0.45 |

tered subcutaneously as the free base in a volume of 1.0 ml/kg. The response to amphetamine was recorded for 1-minute periods beginning 5 minutes after the injection; successive observations were made 10 minutes later and at regular 15-minute intervals thereafter for the duration of the response (approximately 90 minutes). An observer, who was unaware of the treatment conditions for each animal, monitored individual components of behavior, including locomotion, rearing, sniffing, forelimb shuffling, and repetitive head movements (*13*). Each behavior was rated according to its duration and intensity during each observation period, and the scores were summed across the entire response (*14*). Scores for each behavior for different groups of animals (at least seven animals per group) were expressed as the mean and examined by an analysis of variance.

Amphetamine produced all the listed behaviors in both saline- and AA-treated rats. Although the response to AA was slightly less than that to saline, the differences were not statistically significant. Haloperidol, on the other hand, dramatically reduced each response. Even more dramatic was the effect of the combined AA-haloperidol treatment. In this group, locomotion and rearing were completely blocked and the scores for all the other behaviors were significantly less than the scores recorded from rats treated with haloperidol alone (Table 1).

An AA-induced potentiation of the haloperidol response also was observed in tests of catalepsy. Rats comparable to those described above received an intraperitoneal injection of either saline or AA (1000 mg/kg). The animals were challenged 15 minutes later with an intraperitoneal injection of either saline or haloperidol (0.5 mg/kg). At 15 and 60 minutes after the last injection, each rat was tested for catalepsy, which was defined as the amount of time that the animal maintained both forepaws on a platform elevated 12 cm above a flat surface. Mean catalepsy time was calculated for each group and analyzed by a repeated-measures analysis of variance (*15*). Although neither saline nor AA alone or in combination produced catalepsy, haloperidol elicited this response and AA significantly enhanced it (Fig. 1). In fact, AA potentiated the haloperidol response at both test intervals. When examined at 90 and 120 minutes, catalepsy was comparable in saline- and AA-treated rats, indicating that AA did not prolong the action of this antipsychotic drug.

The success of haloperidol in reducing the symptoms of schizophrenia has focused considerable attention on dopamine receptors in the pathophysiology of this disease. In receptor-binding studies, a current controversial issue is whether AA inhibits binding at the agonist site of the dopamine receptor (*8*). If this is the case, a high concentration of AA should reduce the action of dopamine and, at the same time, potentiate the effect of drugs that act at an antagonist site. Our results with haloperidol support this view. Thus, an AA-induced regulation of the dopamine receptor may have functional consequences.

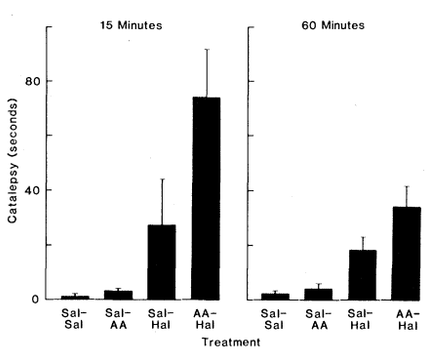


Fig. 1. The effect of prior treatment with saline (Sal) or AA (1000 mg/kg) on the mean (± standard error of the mean) time that rats remain cataleptic when tested 15 and 60 minutes after a challenge injection of saline or haloperidol (0.5 mg/kg) (Hal). The catalepsy produced by the AA-Hal combination was significantly longer than that produced by Sal-Hal at both test intervals ($P < 0.05$ in each case). Between seven and ten different animals constituted each group. A repeated-measures analysis of variance revealed a significant effect of initial treatment ($P < 0.05$) and a significant difference between the Sal-Hal and AA-Hal groups ($P < 0.05$).

It seems unlikely that our results can be explained by a general depressant effect of AA on behavior, because a 1000 mg/kg dose by itself did not significantly attentuate any of the components of the amphetamine response nor did it elicit catalepsy. Yet this dose does produce a significant increase in the extracellular concentration of AA in the neostriatum, and this effect is accompanied by an increase in neostriatal firing rate (*6*). Higher doses of AA block the amphetamine response directly (*16*), suggesting that at sufficiently high concentrations AA alone can attenuate dopamine-mediated behaviors. In fact, megavitamin therapy, which includes pharmacological doses of AA, has been successful in treating certain forms of schizophrenia (*17*).

Our results do not rule out a metabolic or pharmacokinetic interaction between AA and haloperidol, however. It is possible, for example, that the antioxidant action of AA alters the enzymatic destruction or the uptake and distribution of haloperidol in various tissues. If so, a change in the amount of haloperidol reaching the relevant dopamine receptors rather than a direct effect of AA on these receptors may explain our results.

In either case, our results point to AA as critical in modulating the behavioral effects of haloperidol. As AA concentrations increase, the response to haloperidol is enhanced. Because humans lack the liver enzyme necessary to synthesize AA, treatments that increase AA concentration may be effective in potentiating the clinical response to haloperidol and related antipsychotic drugs.

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12. The AA, freshly prepared each day and adjusted to *p*H 7.0 with 1*M* NaOH, was maintained under a steady stream of nitrogen until administration.
13. Amphetamine produces a wide variety of behavioral responses in the open field that are collectively called stereotyped because of their repetitive, invariant nature [A. Randrup and I. Munkvad, *J. Psychiatr. Res.* **11**, 1 (1974)]. Because each of these behaviors may be differentially affected by pharmacological and other manipulations, it is important to characterize each behavior individually over the entire time course of the amphetamine response to obtain an accurate behavioral description [G. V. Rebec and T. R. Bashore, *Neurosci. Biobehav. Rev.* **8**, 153 (1984)].
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18. Supported by the National Institute on Drug Abuse (grant DA 02451 to G.V.R.). We also acknowledge the assistance of Glenn D. Anderson with our statistical analysis. Smith Kline & French Laboratories provided a generous supply of *d*-amphetamine sulfate.


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5 March 1984; accepted 4 October 1984


# Complete Development of Hepatic Stages of *Plasmodium falciparum* in Vitro


Abstract. *An in vitro model was developed to study the hepatic phase of* Plasmodium falciparum, *the only malaria parasite lethal to man. Primary cultures of human hepatocytes were inoculated with sporozoites of Brazilian and African strains of* P. falciparum. *On days 1 through 7 after inoculation examination of fluorescence-labeled and Giemsa-stained preparations demonstrated the presence of many intracellular parasites. In three separate sets of experiments all cultures were found to be infected with as many as 650 liver schizonts measuring up to 40 micrometers. After the addition of red blood cells, intraerythrocytic forms of* P. falciparum *were detected on days 12 and 13 by an immunofluorescence assay, indicating that the hepatic cycle had been completed in vitro.*


Among the developmental stages of plasmodia occurring in man, we know the least about the intrahepatic multiplication that follows inoculation of sporozoites by mosquitoes. This is not surprising, considering the inherent difficulty of studying these stages in humans and the scarcity of suitable hosts susceptible to the human parasites.

The alternative approach of reproducing the liver cycle in vitro is therefore an attractive one. Cultures can be easily manipulated and are essential in immunologic studies and studies of antiparasite drugs designed to act against the liver stage. This approach was recently successful in studies of *Plasmodium vivax* in which sporozoites introduced into cultures of functional human hepatocytes invaded the liver cells and developed to maturity, releasing merozoites infective for red blood cells (*1*, *2*). We report here a similar approach designed to produce the complete exoerythrocytic cycle of *Plasmodium falciparum*. in vitro.

Host cells were prepared by microperfusion with collagenase of a human liver fragment removed by biopsy (*2*). The perfused fragment was minced and the freed cells were suspended in medium and seeded in monoculture at a concentration of $5 \times 10^5$ cells per 35-mm petri dish. The cells were maintained for 24 to 48 hours before inoculation of sporozoites in supplemented minimal essential medium (MEM) (*3*).

The parasites used were an African isolate and a Brazilian clone of *P. falciparum*. Infective gametocytes were produced in vitro by static culture techniques slightly modified from those previously described (*4*). Eighteen-day cultures containing mature stage 5 gametocytes were fed to female *Anopheles stephensi* or *Anopheles freeborni* mosquitoes with a water-jacketed membrane feeder maintained at 37°C.

Sixteen days later, when sporozoites were present in the mosquito salivary glands, hepatocyte cultures were prepared for infection by removing spent medium and adding 0.5 ml of fresh medium. Five to ten pairs of aseptically dissected salivary glands were then introduced into each culture. Three hours later 1 ml of medium was added, after which the medium was changed daily. At various intervals after the infected salivary glands were introduced, the cultures were examined by the indirect fluorescent antibody test (IFAT) and by visible light microscopy before and after staining with Giemsa. The IFAT was performed on cultures that had been washed in phosphate-buffered saline (PBS) (*p*H 7.4) with gentle agitation for 30 minutes. Cultures were then fixed in methanol at 4°C, rinsed in PBS, and incubated with a diluted (1:50) hyperimmune serum from an African adult (titer was 1:50,000 against the erythrocytic stages). After three washes in PBS a fluorescein-conjugated antiserum to human immunoglobulins G, M, and A (Nordic) was used at a 1:40 dilution in 0.5 percent Evans blue.

In two sets of replicate experiments in which two groups of infected mosquitoes and hepatocytes from three different patients were used, liver schizonts of *P. falciparum* were observed in all cultures. Each culture contained dozens to hundreds of schizonts, with a maximum of approximately 650 schizonts per 35-mm petri dish (Fig. 1h). The schizonts were more numerous in proximity to the salivary glands and less numerous along the edge of the culture dish.

In cultures fixed at 20 hours, extracellular sporozoites were found attached to the surface of hepatocytes; a smaller number of rounded fluorescing bodies measuring 2 to 4 μm were observed inside the cells close to the nucleus (Fig. 1, a and b). These are interpreted as being early exoerythrocytic parasites, since corresponding forms were never observed in noninfected control cultures. At 64 hours after sporozoite inoculation, schizonts measuring 5 to 10 μm were often observed in juxtaposition to the hepatocyte nucleus, as shown in fluorescent and Giemsa-stained preparations (Figs. 1c and 2a). By day 5 schiz-