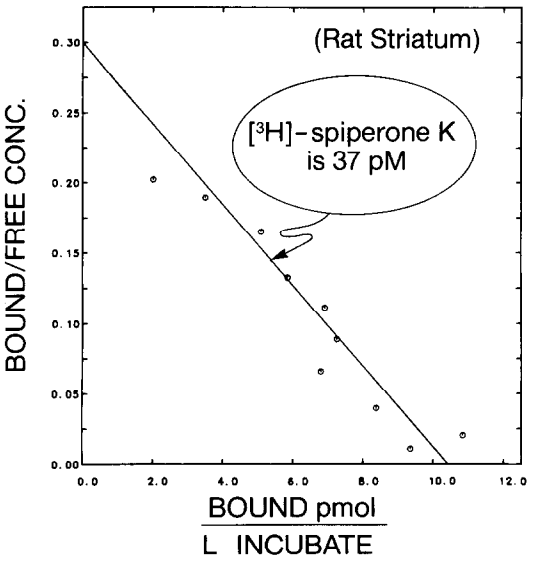

Fig. 2. Typical Scatchard analysis of data from a saturation-type experiment for [^{3}H]spiperone (10–1000 pM) binding to D_2 dopamine receptors in rat striatum. The K_D was 37 pM, as determined by the LIGAND program.

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The formation of the ultimate carcinogen of benzo(*a*)pyrene during non-enzymic lipid peroxidation

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B(*a*)P-7,8-diol, 9,10-epoxide exists in two stereoisometric forms, diol epoxide I (BPDE I) and diol epoxide II (BPDE II). These epoxides are widely believed to be the ultimate carcinogens formed from the polycyclic aromatic hydrocarbon benzo(*a*)pyrene [1]. The half-life of the diol epoxides under physiological conditions is 20–30 sec [2] and, as a consequence of rapid and spontaneous hydrolysis, each epoxide forms two distinct stereoisometric tetrols. B(*a*)P-7,8-diol, 9,10-epoxide is an obligatory intermediate in the formation of B(*a*)P tetrols from the pro-carcinogen B(*a*)P-7,8-diol [3], and analysis of the tetrols formed from the B(*a*)P-7,8-diol therefore demonstrates the intermediate formation of diol epoxides.

We have demonstrated previously that peroxidising polyunsaturated fatty acids can convert B(*a*)P to oxidised products [4] which are mutagenic [5] and in this investigation we present biochemical and biological evidence to suggest that B(*a*)P-7,8-diol is converted to this ultimate carcinogen in the presence of polyunsaturated fatty acids (PUFA) undergoing peroxidation catalysed by Fe^{2+}/ascorbate in the absence of any enzyme system.

Materials and methods

B(*a*)P-7,8-diol (ITT Research Institute, Chicago, U.S.A.) dissolved in dimethyl-sulphoxide was added at a final concentration of 20 μM and was incubated with ferrous sulphate (1 mM), ascorbate (1 mM) and arachidonic acid ($C_{20:4}$) or docosahexaenoic acid ($C_{22:6}$) (500 μM) dissolved in acetone, in phosphate buffer (0.025 M) at pH 7.4 for 30 min. Control incubations were carried out in parallel from which (a) docosahexaenoic acid ($C_{22:6}$) or (b) Fe^{2+}/ascorbate was omitted.

The oxidation products of B(*a*)P-7,8-diol were analysed by HPLC as follows: Unreacted B(*a*)P-7,8-diol and its oxidation products were extracted from the incubation mixture, twice, with 2 vol. of ethyl acetate. Extracts were pooled and 2 mg/100 ml of naphthoglucopyranoside (NG) was added as internal standard, then dried over anhydrous sodium sulphate. The samples were filtered through a 0.5 μm Millipore filter and evaporated to dryness under vacuum in a Speedvac rotary concentrator. The residue was redissolved in 250 μl methanol: H_2O (1:1) and 50 μl injected onto a DuPont Zorbax ODS column (25 cm × 4.6 mm i.d.). The DuPont model 8100 high pressure liquid chromatograph was linked to a Perkin Elmer series LS3 Fluorimeter as detector, fitted with an Apple IIe microcomputer. Products were eluted from the column using a flow rate of 1 ml/min with a linear gradient. Solvent composition was changed from 45% methanol in water to 75% methanol in water during 40 min and then increased to 100% methanol during 0.5 min. 100% methanol was pumped through the column for a further 30 min, to ensure complete elution of retentive tetrols or unreacted B(*a*)P-7,8-diol. The fluorimeter was set at excitation and emission wavelengths of 242 and 390 nm respectively. B(*a*)P tetrols were identified by comparison with authentic B(*a*)P tetrol standards (ITT Research Institute, Chicago, U.S.A.) and quantified by calculating peak areas (Chromatochart,

Table 1. Retention times and relative retention times (compared to 7,10/8,9 B(*a*)P tetrol) of Benzo(*a*)pyrene tetrols separated by HPLC on a Zorbax ODS column (25 cm × 4.6 mm i.d.). Flow rate 1 ml/min linear gradient solvent system: 45–75% methanol in water in 40 min, 75–100% methanol in 0.5 min, then 100% methanol

Benzo(*a*)pyrene metabolite	Derived from Benzo(*a*)pyrene diol epoxide	Retention time (sec)	Retention time relative to 7,10/8,9 tetrol
7,10/8,9 tetrol	(I)	1154 ± 28	1.00
7,9/8,10 tetrol	(II)	1266 ± 20	1.10
7/8,9,10 tetrol	(I)	1362 ± 20	1.18
7,9,10/8 tetrol	(II)	1605 ± 19	1.39
7,8 diol	–	2721 ± 44	2.36
NGA Standard	–	664 ± 19	0.58

Values represent the mean ± S.E.M. of three experiments.

Table 2. Formation of benzo(*a*)pyrene tetrols from B(a)P-7,8-diol and peroxidising docosahexaenoic acid (500 μM) in the presence of Fe^{2+}/ascorbate in 30 min

	nmoles tetrol produced/30 min	
Tetrol	Derived from B(*a*)P diol epoxide I	Derived from B(*a*)P diol epoxide II
7,10/8,9	4.9	–
7,9/8,10	–	1.4
7/8,9,10	1.1	–
7,9,10/8	–	2.1
Total tetrol	6.0	3.5
% Total tetrol derived from BPDE I and BPDE II	63%	37%

Hyden Data Systems). Recovery was assessed using NG as internal standard. The retention times of four authentic tetrol standards calculated using the HPLC separation described are shown in Table 1.

The *Salmonella typhimurium* mutagenicity assay was conducted in the absence of an S9 metabolising system [6] using strains TA98 and TA100. B(*a*)P-7,8-diol (20 μM) incubated in the presence of actively peroxidising fatty acids, $C_{22:6}$ and $C_{20:4}$ (500 μM), Fe^{2+}/ascorbate and phosphate buffer (pH 7.4) as previously described, were added to bacterial cultures (a) immediately after preparation and (b) after a 30-min pre-incubation period at 37°. Results were expressed as mean number of revertants per plate ± S.E.M. Positive results were considered to be at least twice the number of spontaneous revertants in controls.

Results and discussion

Peroxidising $C_{22:6}$ in the presence of Fe^{2+}/ascorbate was shown to oxidise B(*a*)P-7,8-diol to a variety of products which were found to co-elute with authentic standards of B(*a*)P-tetrols. The total conversion of B(*a*)P-7,8-diol in 30 min was 47.5%. The combined data from two such experiments are shown in Table 2 and indicate the amounts of each of the four tetrols identified. Tetrol 7,10/8,9 was found to be the predominant species (52%) and products derived from B(*a*)P-diol epoxide I (BPDE I) were found to constitute 63% of the total products, while tetrols derived from B(*a*)P-diol epoxide II (BPDE II) made up 37% of the total. The formation of B(*a*)P-tetrols was not detected in control incubations.

Mutagenic products of the oxidation of B(*a*)P-7,8-diol mediated by peroxidising fatty acids were detected in strains TA98 and TA100 of *S. typhimurium* (Table 3). Mutagenicity was only demonstrated in samples incubated with bacterial cultures immediately after preparation and pre-incubation for 30 min at 37° rendered samples inactive in the Ames test. No mutagenic activity was detected in control samples.

The oxidation of the pro-carcinogen B(*a*)P-7,8-diol to the highly reactive diol epoxide intermediate is a critical step in the expression of carcinogenic and mutagenic activity of the parent compound B(*a*)P. We have demonstrated that this reaction can occur non-enzymically, mediated only by PUFA with ascorbate-iron, a system known to undergo peroxidation and to generate free radical intermediates. This work may further implicate dietary polyunsaturated fatty acids in their role in carcinogenesis.

Table 3. Mutagenicity of the oxidation products of benzo(*a*)pyrene-7,8-diol formed by peroxidising arachidonic acid ($C_{20:4}$) and docosahexaenoic acid ($C_{22:6}$)

	No. revertants/plate	
Sample	TA98	TA100
$C_{20:4}$ + B(*a*)P-7,8-diol	25 ± 1	148 ± 7 (+)
$C_{22:6}$ + B(*a*)P-7,8-diol	43 ± 2 (+)	173 ± 23 (+)
Control (phosphate buffer + B(*a*)P-7,8-diol)	18 ± 3	76 ± 3

Results are expressed as the mean no. revertants/plate ± S.E.M. of four plates per test.

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Inhibitors of choline transport into *Plasmodium*-infected erythrocytes are effective antiplasmodial compounds *in vitro*

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The increasing resurgence of *Plasmodium falciparum* malaria, linked to the resistance both of mosquitoes and parasites to various pesticides and conventional drugs, calls for new therapeutic approaches to this endemic disease [1]. Interfering with parasite-specific metabolic pathways could lead to a new range of antimalarial drugs whose structure and mode of action are different from those currently used.

Phospholipid (PL) metabolism was chosen as a target because of its magnitude and specificity. Although little or no enzymatic machinery devoted to PL biosynthesis exists in mature mammalian erythrocytes [2–6], an increase amounting to as much as 500% of the host erythrocyte PL content occurs after infection. New molecules of PL are manufactured by the parasite itself, from precursors such as fatty acids and polar heads constantly drawn from the plasma [6]. Any compounds that can curtail the availability of these precursors should be deleterious to the parasite.

We have previously shown that D-2-amino-1-butanol which is incorporated as a polar head into unnatural PL, has a deleterious effect on *Plasmodium* growth, suggesting that PL metabolism is essential to the parasite [7]. Since phosphatidylcholine (PC) is the most abundant PL in infected erythrocytes [2], in the present work, we tested compounds likely to interfere with choline incorporation. Based on their analogy with choline and on previous results obtained in other cellular systems, such as erythrocytes and the nervous system [8–10], it was probable that quaternary-ammonium-containing compounds would affect choline utilization by infected erythrocytes, and consequently block parasite development *in vitro*.

Materials and methods

Bromide salts of decyltrimethylammonium, decamethonium and hemicholinium 3 were from Sigma, St. Louis, MO, U.S.A. Modified RPMI 1640 containing 40 μM choline and inositol, and 140 μM serine, was provided by Eurobio, France.

The Nigerian strain of *P. falciparum* (Dr W. H. Richard, Wellcome Research Laboratory, Beckenham, U.K. [11]) was maintained in continuous culture according to the Petri-dish candle-jar method [12].

Splenectomized *Macaca fascicularis* monkeys (Sanofi, Montpellier, France), were infected by cryopreserved [13] *P. knowlesi* (Washington strain, variant 1, from Dr G. Mitchell, Guy's Hospital, London, U.K.), and infected erythrocytes were supplied as described previously [7].

Biochemical studies. P. knowlesi-infected erythrocytes were preincubated for various times at 37° in the presence of the different drugs and in medium composed of modified RPMI 1640 with 25 mM Hepes (pH 7.4). They were then incubated with radioactive precursors in the same medium enriched with 1 mM ATP, 30 μM CoA and 0.5 mM of the essential plasmatic unesterified fatty acids, i.e. palmitic, stearic, oleic and linoleic acids in a molar ratio of 1.7/0.7/1.3/1.3, respectively, bound to fat-free bovine serum albumin (17–20 mg/ml). Reactions were stopped at +4°. The cells were then pelleted at 10^4 g/min and washed at 4°, once with 4 ml modified RPMI and twice with 4 ml 0.9% NaCl containing 40 μM cold choline. Cellular lipids were extracted according to the method of Folch *et al.* [14] as modified [15], and then fractionated as described previously [7]. The aqueous extracts of the Folch procedure were harvested and evaporated at 37° under an N_2 stream. Residues were resuspended in ethanol–water (1–1); choline and its metabolites were then separated by silica gel TLC [16]. After visualization of appropriate standards with iodine vapor, radioactive spots were counted with a Packard 460 CD liquid scintillation spectrometer.

When [^{3}H(G)]hypoxanthine (NEN) or [U-^{14}C]isoleucine (Amersham International, Amersham, U.K.) incorporation into macromolecules was monitored, 4 ml of 10% cold trichloroacetic acid (TCA) was added to washed cells. The precipitates were washed twice with 4 ml 10% TCA, then solubilized in 0.8 ml NCS (Amersham) and counted for radioactivity.

Results and discussion

The present results demonstrate that analogs of choline, containing one or two quaternary ammoniums, i.e. decyltrimethylammonium (DTMA), or decamethonium (DM) and hemicholinium 3 (HC3), are lethal to *Plasmodium falciparum in vitro* in a dose-dependant manner, with very