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Inhibition of Squalene Synthase of Rat Liver by Novel 3′ Substituted Quinuclidines

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ABSTRACT. Squalene synthase (SQS) is a key enzyme in the biosynthetic pathway for cholesterol and is a target for improved agents to lower plasma levels of low-density lipoprotein (LDL). A series of novel 3′ substituted quinuclidines have been discovered as inhibitors of the rat liver microsomal enzyme. In this study, we demonstrate the inhibitory effects *in vitro* and *in vivo*, of two examples of the series. When microsomes were preincubated with compounds, before addition of substrate, both 3-(biphenyl-4-yl)quinuclidine (BPQ) and 3-(biphenyl-4-yl)-3-hydroxyquinuclidine (BPQ-OH) were found to cause biphasic inhibition of the enzyme with apparent inhibition constants (K_i') for the sensitive phases of 12 nM and 15 nM, respectively. The K_i' values for the insensitive phases were 1.8 μM and 2.9 μM, respectively. The two examples inhibited equally both steps of the SQS-catalysed reaction, as shown by parallel inhibition of $^3H^+$ release and labelled squalene formation from [1-^{3}H] farnesyl pyrophosphate (FPP). BPQ and BPQ-OH were shown to be inhibitors of hepatic sterol synthesis from mevalonate with ED_{50} values of 10.6 and 7.1 mg/kg, respectively, after acute oral administration to the rat. BPQ-OH was chosen for further study and, to determine its selectivity of effect on the mevalonate pathway *in vivo*, the effect of a dose of 70 mg/kg on the pattern of labelled mevalonate incorporation into the various lipid fractions of the rat liver was examined. As expected, the incorporation into squalene and sterol products was inhibited by about 70%. An appearance of label in fractions corresponding to farnesyl and geranylgeranylpyrophosphates, as well as the corresponding alcohols, was observed in treated but not control animals. In addition, the administration of compound resulted in the appearance of peaks of mevalonate-derived radioactivity in an acidic fraction believed to represent metabolites of farnesol. Such results are consistent with inhibition of the mevalonate pathway at, and not before, SQS. In contrast, there was a significant increase in the incorporation of labelled mevalonate into ubiquinone 10, and the synthesis of dolichols was apparently unchanged. The results suggest a specific effect of BPQ-OH on rat liver SQS. The compound is, therefore, an interesting lead for further investigation of this class of compounds. BIOCHEM PHARMACOL 51;11:1477–1487, 1996.

KEY WORDS. anticholesteremic agents; squalene synthase; enzyme inhibitors; farnesyl pyrophosphate; farnesol; ubiquinone; dolichol

Compounds that inhibit cholesterol synthesis have proven to be useful in reducing levels of LDL† in man with consequent beneficial effects in cardiovascular disease [1]. This has led to the search for additional agents with greater specificity and efficacy. An important control point in cholesterol synthesis is the formation of squalene from FPP [2]. This is catalysed by the microsomal enzyme, squalene synthase (EC 2.5.1.21), in a two-step reaction. First, two molecules of FPP are dimerised head-to-head with the loss of a proton to form the intermediate, presqualene pyrophosphate. In the second step, the intermediate is reduced with NADPH and rearranged to form squalene [3, 4].

In principle, the squalene synthase step is an ideal point at which to inhibit the pathway. Squalene and its derivatives have only the one known function, to give rise to intermediate sterols and to cholesterol. However, pathway intermediates generated from mevalonate prior to squalene synthase are also precursors of nonsterol isoprenoid products, such as ubiquinone, dolichol, the farnesyl group of heme A, and prenylated proteins, all of which are vital for normal cell function. Thus, inhibitors of enzymes prior to squalene synthase have the potential to reduce the formation of such products. Blockade of the pathway at squalene synthase may also be advantageous because the substrate, FPP, can be metabolised *via* hydrolysis to farnesol [5] which,

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† *Abbreviations:* SQS, squalene synthase; BPQ, 3-(biphenyl-4-yl)quinuclidine; BPQ-OH, 3-(biphenyl-4-yl)-3-hydroxyquinuclidine; FPP, farnesylpyrophosphate; GGPP, geranylgeranylpyrophosphate; PSPP, presqualenepyrophosphate; LDL, low-density lipoprotein; F1 and F2, aqueous and organic fractions of SQS reaction products; AP, Alderley Park; HPMC, hydroxypropyl methyl cellulose; K_i', apparent inhibition constant; $S_{0.5}$, substrate concentration to achieve half maximal velocity; V_{max}, substrate concentration at which velocity approaches the maximum; h, Hill coefficient; pet ether, petroleum ether.

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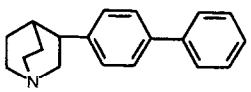
in turn, can be oxidised to carboxylic acids [6] that are water-soluble urinary excretion products. In contrast, inhibition at later steps may result in the deleterious accumulation of lipid-soluble intermediates. Thus, squalene synthase has become the target of intensive efforts to discover new classes of pharmaceutically acceptable LDL-lowering agents.

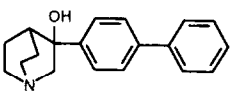
Two classes of highly potent *in vitro* and *in vivo* inhibitors of squalene synthase have been described in the recent literature; a family of fungal metabolites known as the squalestatins [7] and also as the zaragozic acids [8] and, also, a series of synthetic compounds that are lipophilic biphosphonates [9]. However, no examples of these classes of inhibitors have so far been reported to have reached clinical trial.

We report here on the *in vitro* and *in vivo* properties of two examples, BPQ and BPQ-OH, of a novel series of 3'-substituted racemic quinuclidines structurally quite different from the squalestatins or bisphosphonates. One example (BPQ-OH), in particular, was found to have marked *in vivo* effects. Detailed evaluation of the *in vivo* effects of this compound on the metabolism of labelled mevalonate indicates that the inhibition is entirely specific for squalene synthase.

BPQ



BPQ-OH



MATERIALS AND METHODS

Materials

[1-^{3}H]FPP, triammonium salt (15–30 Ci/mmol) and R,S[5-^{3}H]mevalonolactone (60 Ci/mmol) were obtained from DuPont NEN (Stevenage, U.K.) and American Radiolabelled Chemicals (St. Louis, MO), respectively. Unlabelled FPP was synthesised as the triammonium salt according to Davisson *et al.* [10]. C-18 'Bond-Elut' columns were from Varian (Harbour City, CA, U.S.A.) and Spherisorb, Kromasil, and Hypersil HPLC columns as specified were from Hichrom (Berkshire, U.K.). Solvents were obtained from Fisons (Loughborough, U.K.) and were 'Analar' grade for extractions and HPLC grade for chromatography. Samples of lipid standards used to calibrate the HPLC methods were obtained from Sigma except for *trans, trans* farnesol, which was purchased from Aldrich (Dorset, U.K.) and 2,3 oxidosqualene, which was kindly given by Dr. D R Brittain of ZENECA. PCS scintillation fluid was obtained from Amersham (Buckinghamshire, U.K.).

Assay of Squalene Synthase in Rat Liver Microsomes

Microsomes were prepared from rat liver by homogenising in 50 mM phosphate buffer, pH 7.4, 4.0 mM $MgCl_2$, 1.0 mM EDTA, and 1.0 mM dithiothreitol using a Potter homogeniser. The homogenates were centrifuged at 5000 and 15,000 *g*, for 10 and 15 min, respectively, at 4°C, pouring the supernatants through cotton gauze after each spin. Microsomes were then isolated from the supernatant by centrifugation at 100,000 *g* for 60 min at 4°C. Microsomes were then resuspended in homogenisation buffer and stored in aliquots at −70°C for up to 2 months. For *in vitro* studies, the quinuclidine inhibitors were dissolved in DMSO. The SQS reaction system in a volume of 200 μL contained 50 mM phosphate buffer, pH 7.4, 4.95 mM $MgCl_2$, 9.9 mM KF, 0.9 mM NADPH, 19.6 μM FPP, and microsomes, 100 μg protein/mL. The compounds (in 5 μL DMSO), microsomes (in 20 μL buffer) and other reagents (155 μL) were preincubated for 15 min at 37°C. The reactions were started by the addition of 20 μL of an aqueous solution of FPP containing about 0.1 μCi of [1-^{3}H]FPP; incubations were at 37°C for 15 min. The reactions were then stopped by the addition of 50 μL of 4% KOH. The radiochemical products of the reaction were separated using 'Bond-Elut' mini columns fitted in a 'Vac-Elut' manifold. Before use, the columns were first washed with 1.0 mL of methanol followed by 1.0 mL of 0.1 M KOH. Stopped reaction mixture (200 μL) was then applied, each to a separate column, and drawn through under low vacuum into a vial followed by an additional volume of 250 μL of 0.1 M KOH to comprise fraction 1 (F1). This aqueous fraction contains labelled protons released from step 1 of the SQS reaction. Then, 1.0 mL of 10% ethyl acetate in hexane was applied to the column and eluted again under low vacuum into a second vial to comprise fraction 2 (F2). PCS (4 mL) scintillation fluid was added to the vials for counting. F2 contains squalene and its further oxidation product, oxidosqualene. Blank samples, in which microsomes were added only after stopping the reaction, contained less than 0.5% or less than 0.1% of added radioactivity in F1 and F2, respectively, indicating efficient retention of labelled substrate by the column.

Each set of incubations included control samples containing DMSO, but no compound, as well as blanks. Under the standard conditions of the assay, 10,000 dpm in F1 corresponds to a reaction velocity of 22.2 fmol H^+/sec/μg protein; 10,000 dpm in F2 corresponds to 7.41 fmol squalene/sec/μg protein. In all experiments with the *in vitro* enzyme system, substrate utilisation was less than 10%. Enzyme kinetics were analysed using the *GraFit* program as described in detail in a companion publication [11].

Analysis of Radiolabelled Products in Fraction 2 (Figure 1)

Incubations with and without the addition of 2.5 μM of inhibitor BPQ were set up as described above. Fraction 2 was collected from 'Bond-Elut' columns in the usual way, evaporated under N_2 and redissolved in 100 μL acetonitrile-isopropanol (80:20). This was then applied to a Spherisorb 3 ODS1 HPLC column and separated by a water-

acetonitrile-isopropanol gradient system; see below for details. Samples of farnesol, 2,3 oxidosqualene, squalene, and lanosterol were also applied to the column to measure their elution positions.

Animal Experiments and Administration of Compounds

Female rats of the AP strain in the weight range 125–150 g were used. They were housed under reversed lighting conditions for a minimum of 10 days before use and experiments were conducted in the mid-dark phase. To make fine suspensions of the compounds for administration, they were first dissolved in DMSO to which an aqueous solution of HPMC was added; final concentrations of the vehicles were 10% DMSO and 0.1% HPMC. The suspensions were administered by oral gavage at the stated dose.

Labelled Mevalonate Incorporation into Liver Nonsaponifiable Lipids (Figure 4)

Groups of 5 rats were administered quinuclidine inhibitors or vehicle as above. One hour after administration of inhibitors, the rats were injected i.p. with 2.5 μCi [5-^{3}H]mevalonolactone. One hour later, the animals were terminally anaesthetised with halothane and samples of about 1 g of liver were weighed into Pyrex screw capped tubes. 8% KOH in ethanol (2 mL) was added to each tube, the tubes capped, and the contents saponified by heating at 80°C for 2 hr. The tubes were agitated during this time to disintegrate the liver. After cooling, 2 mL of water was added and nonsaponifiable lipids were extracted with 2 aliquots of 5 mL hexane.

The combined extracts were evaporated under N_2 at 40–50°C, PCS scintillation fluid added, and radioactivity determined. The results were expressed as dpm per g wet weight of liver. A four-parameter logistic curve was fitted to the data using weighting proportional to the means of the replicates. From this, values of ED_{50}, together with 95% confidence limits were derived.

Labelled Mevalonate Incorporation into Sterol and Nonsterol Products (Figures 5–8)

Groups of 3 rats were administered either vehicle or 70 mg/kg of BPQ-OH as above. One hour later, the rats were injected with 500 μCi of [5-^{3}H]mevalonolactone and, after a further 1 hr, the animals were terminally anaesthetised with halothane and two separate samples of liver were removed.

(I) STEROLS, SQUALENE, FARNESOL METABOLITES AND DOLICHOLS. From the first sample of liver, 3 1-g portions were weighed out and saponified as above, except that 4 aliquots of pet. ether 40/60°C were used to extract. The extracts from the 3 samples were combined at this stage and evaporated under N_2 as before and redissolved in 5 mL of methanol. This was applied to a '3 cc' C-18 'Bond-Elut' column and eluted with methanol to provide a fraction containing sterols, farnesol, and squalene for HPLC analysis (see below), as shown in Fig. 5. The column was then eluted with chloroform-methanol (2:1) to provide a fraction containing dolichols for HPLC analysis, Fig. 8 (see below). The extraction and initial separation was based upon the methods of Elmberger *et al.* [12]. The aqueous phase from the pet. ether extractions, which contained pyrophosphates, was then acidified with HCl (pH 1), incubated at 37°C for 1 hr, and the resulting alcohols extracted with pet. ether as above. This extract was then washed 3 times with 1 mL 0.1 M NaOH and separated into aqueous and organic layers, the latter containing isoprenoid alcohols derived from originally aqueous soluble materials, presumably pyrophosphates. These extracts were then evaporated under N_2 at 45°C and redissolved to provide the sample for HPLC analysis (see below) as shown in Fig. 6. The NaOH washings that contained isoprenoid acids were pooled, acidified (pH 1), extracted 3 times with pet. ether, and the extracts evaporated under N_2 and then redissolved with chloroform-methanol (2:1) for HPLC analysis, Fig. 7 (see below). The latter parts of the separation protocol to provide fractions containing isoprenoid alcohols derived from pyrophosphates and acids were done in a similar way to those of Gonzalez-Pacanowska *et al.* [6].

(II) UBIQUINONES. Ubiquinones are labile to heating under alkaline conditions. Thus, ubiquinones were extracted directly from samples of liver. Portions of 1 g were homogenised in water and then extracted with chloroform-methanol, separating the phases according to the method of Bligh and Dyer [13]. The organic layer was evaporated under N_2 at 55°C and redissolved in 5 mL of methanol. This was then applied to a 3-cc C-18 'Bond-Elut' column. The column was eluted first with methanol, which was discarded, and then with chloroform-methanol (2:1). The latter fraction was collected, evaporated again, redissolved in methanol, and centrifuged to remove solid material. This was, then, analysed by HPLC (see below). The separation of ubiquinones was based upon the methods of Elmberger *et al.* [12].

(III) HPLC ANALYSIS. Isoprenoid alcohols, sterols and squalene (Figs. 5 and 6) were separated on a C-18 reverse phase, Spherisorb 3 ODS1 (10 cm × 4.6 mm i.d.) column. The sample was redissolved and injected in acetonitrile/isopropanol (90:10). The separation was initially a gradient of acetonitrile/water (60:40) to acetonitrile/isopropanol (95:5) for 15 min followed by a gradient of isopropanol in acetonitrile varying the former from 5 to 20% over 25 min. For the separation of dolichols, (Fig. 8), the samples were redissolved and injected in acetonitrile/isopropanol (60:40) separating first with a 5-min isocratic period of acetonitrile/isopropanol (60:40), followed by a gradient of isopropanol in acetonitrile varying from 40 to 75%.

To separate isoprenoid acids, as shown in Fig. 7, a Kromasil KR100 5C18 (25 cm × 4.6 mm i.d.) column was used. The samples were redissolved and injected in acetonitrile/

water (90:10) and separated in a gradient of acetonitrile in 0.01 M triethylamine phosphate pH 7, in varying the former from 30% to 80% over 30 min.

Ubiquinones were separated on a Hypersil H5 ODS column. The sample was dissolved in methanol and eluted with a gradient of methanol/water (90:10) to methanol/isopropanol (80:20) over 20 min, then a 15-min isocratic phase with the latter solvent over a further 20 min.

HPLC was done using a Varian Vista 5500 machine. The fractions were collected into vials, PCS scintillation fluid added and counted.

The results shown in Figs. 5–8 and in Table 2 are the means of 3 determinations, each derived from a single animal.

RESULTS

The effects of the quinuclidine compounds on SQS of rat hepatic microsomes were determined by measuring the conversion of [1-^{3}H]-FPP to reaction products. As described in Materials and Methods, the reaction products were separated by a convenient reverse phase minicolumn method into an aqueous fraction (F1), which contained protons from the first step of the reaction, and an organic fraction (F2) that contained squalene and its further metabolites. The separation was done without prior saponification of the reaction mixture. Several experiments were done to validate the use of this separation method.

The recovery of labelled protons in F1 was checked in 3 ways. First, when ^3H_2O was added to a stopped reaction mixture, the recovery of radioactivity in F1 was found to be complete (101%). Second, after control incubations, the radioactivity in F1 was found to be 87–92% (range of 3 determinations) of the labelled proton release measured by the micro distillation method of Agnew and Popjack [3]. Third, when the F1 fraction derived from control incubations was extracted with hexane, greater than 80% of the radioactivity remained in the aqueous phase; 10% comigrated with squalene on TLC. F1 radioactivity is, therefore, a good approximation of the label associated with the protons released from step 1 of the SQS reaction.

F2, as demonstrated by reverse phase HPLC, shown in Fig. 1, contained both labelled squalene and its metabolic product, 2,3-oxidosqualene, representing 76% and 23%, respectively, of the radioactivity in this fraction. The recoveries of these components using the minicolumn method were compared directly with duplicate incubates that were saponified, extracted in hexane (see Materials and Methods), and applied directly to the HPLC. As compared with the saponified samples, the recoveries of labelled squalene and oxidosqualene in F2 were 81 and 83% (means of duplicate estimations), respectively. It was also determined, using unlabelled material, that lanosterol added to a stopped reaction mixture elutes in F2. However, only minor amounts (<1%) of this labelled sterol were detected in F2 as products of the incubation. This was confirmed in the saponified and hexane-extracted samples. Labelled farnesol, expected from the enzymatic degradation of FPP, was also a minor component (<1%) of F2. Thus, under the conditions of the incubations of FPP with microsomes, squalene and its enzymatic oxidation product, oxidosqualene, were the major lipid products. Moreover, the radioactivity in F2 represents a good estimate of the labelled squalene (including its products) formed in step 2 of the SQS reaction. The ratio of radioactivity in F2 and F1 in control incubations was 2.5, a figure close to the theoretical value of 3 expected from the use of FPP uniformly labelled with tritium in the C1 position.

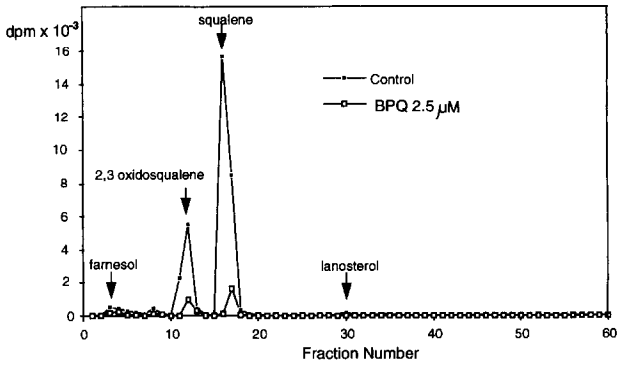


FIG. 1. Labelled products in F2 separated by reverse-phase HPLC and the inhibitory effects of BPQ. Rat hepatic microsomes were preincubated for 15 min with or without 2.5 μM BPQ and incubated with 19.6 μM [1-^{3}H]-FPP for 15 min. Reaction products were separated by a reverse-phase minicolumn and the organic phase was applied to a Spherisorb 3 ODS HPLC column and eluted with an acetonitrile/isopropanol/water gradient system. Fractions were collected and radioactivity determined. The elution positions of standards are indicated by the arrows.

Figure 1 also shows that the addition of quinuclidine inhibitor, BPQ, to the incubation system considerably reduced the formation of labelled squalene and oxidosqualene, indicative of inhibition of SQS. The appearance of radioactivity in F1 (data not shown) was also reduced in parallel.

Figure 2 shows the appearance of radioactivity in F2 as a function of substrate concentration. The reaction followed saturation kinetics with respect to substrate. The Hill equation [14] was fitted to these data to provide estimates of 1.2 μM as the substrate concentration to achieve half-maximal velocity ($S_{0.5}$) and 20 μM as the level at which the velocity approached the maximal (V_{max}). The Hill coefficient (h) was 1.26. In subsequent experiments, a level of FPP of 19.6 μM was used to determine the effects of the inhibitors. Under these conditions, in the absence of inhibitors, the reaction rate measured either by F1 or F2 radioactivity was linear with respect to time and to the concentration of microsomes.

Figure 3 shows the concentration-dependent effect of inhibitor BPQ-OH on SQS reaction rate measured by F2 radioactivity. The compound was preincubated with microsomes for 15 min before addition of substrate. BPQ-OH

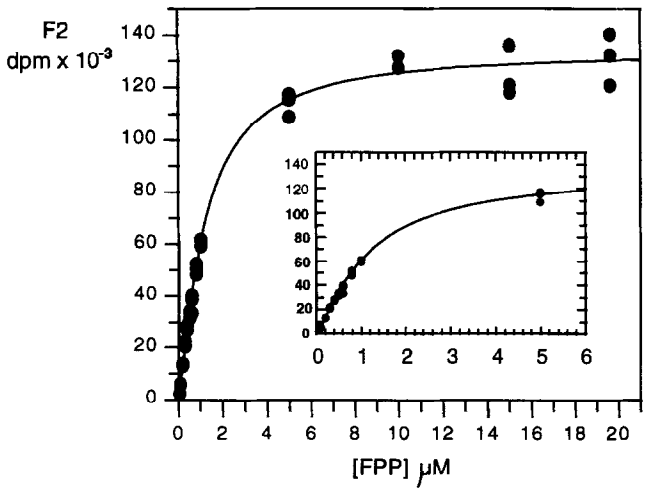


FIG. 2. Appearance of radioactivity in F2 as a function of substrate concentration. The insert shows an expanded scale for the lower levels of substrate. Rat liver microsomes were incubated for 15 min with a range of concentrations of [1-^{3}H]-FPP and the conversion to F2 reaction products measured as described in Materials and Methods. The data was fitted to the Hill equation to derive estimates of h, $S_{0.5}$ and V_{max}.

caused dose-dependent inhibition of SQS, but the interpretation was complicated by the finding that the data fitted best to biphasic kinetics. The apparent inhibition constants (K_i') values for the sensitive and insensitive phases were estimated as 15 nM and 2.9 μM, respectively, and the relative contributions of sensitive and insensitive phases to the reaction velocity were 81% and 19%, respectively. Very similar data were obtained when F1 radioactivity was measured; see Table 1. BPQ was also found to cause dose-dependent biphasic inhibition with similar K_i' values, as shown in Table 1. In this case, the sensitive kinetic phase

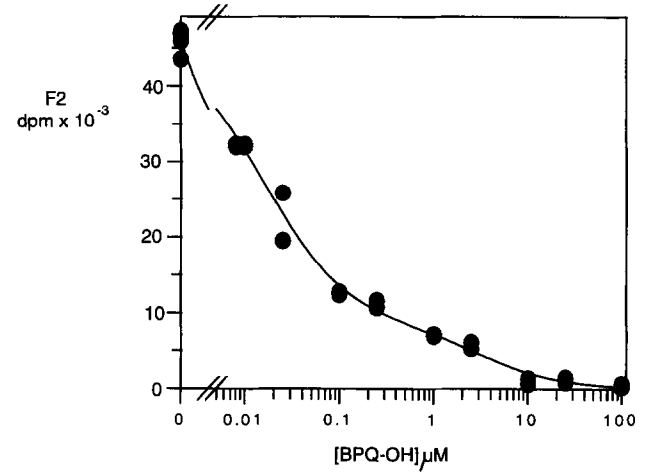


FIG. 3. Inhibition of the SQS reaction by BPQ-OH. Rat liver microsomes were preincubated for 15 min with the indicated concentrations of inhibitor and incubated with 19.6 μM [1-^{3}H]-FPP and the radioactivity in F2 determined. The data was fitted using the *GraFit* program.

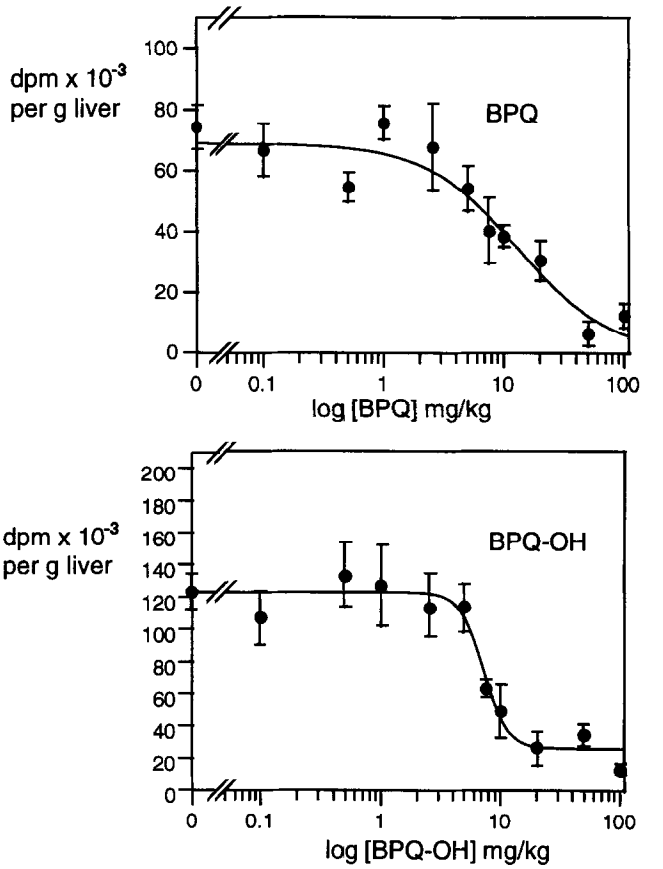


FIG. 4. Dose-response inhibition of incorporation of labelled mevalonate into hepatic nonsaponifiable lipids *in vivo*. Groups of 5 rats were administered compounds or vehicle alone by oral gavage. One hour after administration of inhibitors, animals were injected i.p. with 2.5 μCi [5-^{3}H]mevalonolactone and, after a further 1 hr, the animals were terminally anaesthetised with halothane and a liver section removed. Liver samples were saponified and extracted with hexane to determine incorporation into total nonsaponifiable lipids. A four-parameter logistic curve was fitted to the data using weighting proportional to the means of the replicates. From this, values of ED_{50}, together with 95% confidence limits, were derived.

represented about 52% of the total enzyme activity. These experiments were carried out with the 15-min preincubation of inhibitors with microsomes because, under this condition, the products, F1 or F2, accumulated linearly with time for the sensitive phase [11]. Without preincubation, it was found that there were downward curvatures of the time-courses, a finding indicative of slow binding of the inhibitors. Thus, quantitative analysis of dose-responses were best done in experiments under preincubation conditions.

The *in vivo* effect of inhibitors BPQ and BPQ-OH on the conversion of mevalonate into sterols was determined by measuring the incorporation of [5-^{3}H]mevalonolactone into hepatic nonsaponifiable lipids one hr after administration by oral gavage. These results are shown in Fig. 4. The estimates of the ED_{50} values for the two compounds are 10.6 (5.1–20.2) mg/kg and 7.1 (3.6–13.6) mg/kg, respec-

TABLE 1. K_i' values for quinuclidine SQS inhibitors determined from the products of Step 1 (F1) and Step 2 (F2)

		F1 (labelled proton release)	F2 (labelled squalene formation)
BPQ	(sensitive phase)	0.013 (0.005–0.038)	0.012 (0.007–0.20)*
	(insensitive phase)	0.90 (0.14–4.98)	1.85 (0.89–3.86)
BPQ-OH	(sensitive phase)	0.017 (0.008–0.036)	0.015 (0.012–0.020)*
	(insensitive phase)	2.8 (0.14–55.0)	2.9 (1.10–7.60)

* Contributions of the sensitive phase to total enzyme activity based on F2 data were estimated as 52% and 81% for BPQ and BPQ-OH, respectively. Inhibitors were preincubated for 15 min with rat liver microsomes and the reaction started with the addition of 19.6 μM [1-^{3}H]-FPP. Reaction products F1 and F2 were separated and K_i values determined by *GraFit* analysis of the concentration dependence data. The units for K_i' are μM with 95% confidence limits in parenthesis.

tively (95% confidence limits in parenthesis). Because BPQ-OH was the more effective *in vivo*, further investigations into the selectivity of the inhibition of the pathway was carried out with this compound. In a time-course experiment, BPQ-OH was administered at 7 and 70 mg/kg, and the labelled mevalonate given at various times thereafter. At the higher dose, inhibition was maximal between 1 and 4 hr and significant inhibition was maintained up to at least 12 hr (data not shown).

Fig. 5 shows the results of an experiment in which a dose equivalent to 10 times the ED_{50} was administered and the incorporation of [5-^{3}H]mevalonolactone into the various components of the nonsaponifiable lipid extract determined after separation by reverse-phase HPLC. As expected of a selective inhibitor of SQS, BPQ-OH decreased the incorporation of mevalonate into squalene and sterols and caused increases of 9 and 3-fold in the label appearing in fractions coincident with standards of farnesol and geranylgeraniol, the likely *in vivo* degradation products of FPP and GGPP. It was determined that FPP is completely resistant to the alkaline saponification conditions used to extract the isoprenoid samples for chromatography and, thus, the farnesol and geranylgeraniol seen in Fig. 5 are mostly unlikely to be an *in vitro* artefact. In a separate experiment, (data not shown) in which animals were dosed with 7 mg/kg of BPQ-OH, increases of mevalonate-derived radioactivity in peaks coincident with farnesol and geranylgeraniol were 4 and 1.3-fold respectively.

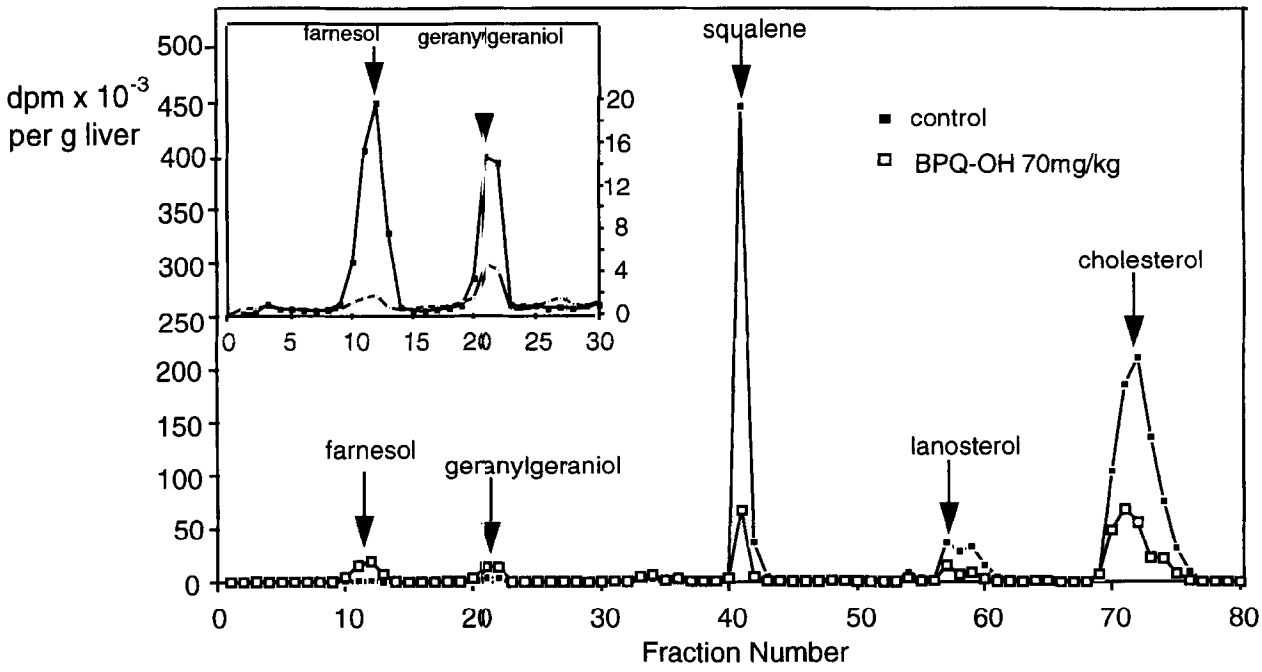


FIG. 5. The effect of the administration of BPQ-OH on the incorporation of labelled mevalonate into the components of rat hepatic nonsaponifiable lipids separated by reverse-phase HPLC. Groups of 3 rats were administered BPQ-OH, 70 mg/kg by oral gavage or vehicle alone (controls). One hour later, the animals were injected i.p. with 500 μCi [5-^{3}H]mevalonolactone and, after a further 1 hr, the animals were terminally anaesthetised with halothane and a liver section removed. Liver samples were saponified and extracted with pet. ether and the solvent phase applied to a C-18 'Bond-Elut' column. This was eluted with methanol and applied to a Spherisorb 3 ODS column. The HPLC column was eluted with an acetonitrile/isopropanol/water gradient system and fractions collected to determine the distribution of radioactivity. The results are the mean of the 3 animals. The elution positions of standards are indicated by the arrows. The insert shows an expanded scale for the first 30 fractions.

Next, the appearance of mevalonate-derived radioactivity in the fractions containing the isoprenoid pyrophosphates was determined. The aqueous phase derived from the first solvent extraction of the saponified liver samples was acidified (pH 1) to convert the pyrophosphates to the corresponding alcohols. These were extracted, washed with alkali (this phase was retained; see below), and applied to the reverse-phase HPLC column with the results shown in Fig. 6. The appearance, compared to controls, of a large peak of radioactivity coincident with the elution position of farnesol is evident, as well as a second peak in the position of geranylgeraniol. This indicated that the treated rat livers contained mevalonate-derived radioactivity in farnesyl and geranylgeranyl pyrophosphates to a considerably greater extent than controls. This is entirely consistent with an inhibition of the pathway at, and not before, squalene synthase. Note that the labelled material in Fig. 6 is quite different from the labelled farnesol and geranylgeraniol seen in Fig. 5 because the latter probably represents isoprenoid alcohols present in the liver *in vivo*.

The alkaline aqueous phase from the above extraction contained acidic isoprenoids. This phase was acidified, extracted, and subjected to HPLC as shown in Fig. 7. The samples from controls contained little radioactivity. However, those from BPQ-OH treated animals were found to contain several peaks of mevalonate-derived radioactivity. The least polar peak was coincident with a standard of

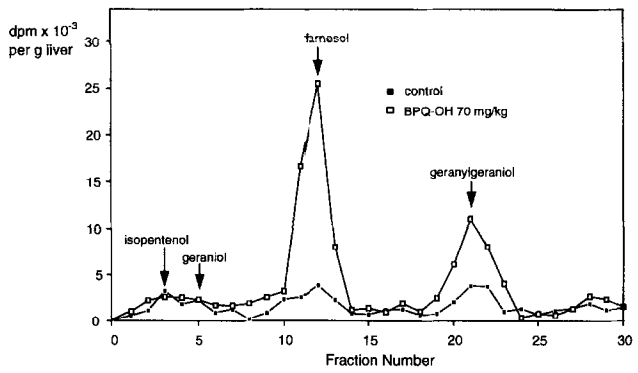


FIG. 6. The effect of the administration of BPQ-OH on the incorporation of labelled mevalonate into fractions derived from isoprenoid pyrophosphates. Groups of 3 rats were administered BPQ-OH, 70 mg/kg by oral gavage or vehicle alone (controls). One hour later, the animals were injected i.p. with 500 μCi [5-^{3}H]mevalonolactone and, after a further 1 hr, the animals were terminally anaesthetised with halothane and a liver section removed. Liver samples were saponified and extracted with pet. ether. The aqueous phase was acidified (pH 1), extracted again with pet. ether, the extract washed with NaOH, and applied to a Spherisorb 3 ODS column. The HPLC column was eluted with an acetonitrile/isopropanol/water gradient system and fractions collected to determine the distribution of radioactivity. The results are the mean of the 3 animals. The elution positions of standards are indicated by the arrows. The radioactivity in the isoprenoid alcohol fractions are assumed to be derived from the corresponding pyrophosphates in the saponified liver sample.

farnesoic acid, but the two major peaks (I and II) had shorter retention times. Because their extraction and chromatography characteristics are those of acids, the major peaks of radioactivity are assumed to be various acidic derivatives of farnesol and farnesoic acid.

As well as being a precursor of squalene, FPP is also the substrate for the *cis*- and *trans*prenyltransferases, the starting steps in the synthesis of dolichols and ubiquinones, respectively. It was important to establish whether or not BPQ-OH exerted any effect on the synthesis of these two components. Thus, dolichol fractions were prepared from control and compound-treated animals given labelled mevalonate as before. The separations by reverse-phase HPLC into the dolichol components is shown in Fig. 8. Although incorporation into these fractions was comparatively very low (only about 0.01% of that in cholesterol), it can be seen that the administration of BPQ-OH had little or no effect on the appearance of labelled peaks in these positions. Note that the earlier fractions 1–20 contain radioactivity associated with squalene, cholesterol, and other sterols, these being incompletely separated from the dolichol fractions by the initial Bond-Elut step. Similarly, ubiquinone-containing fractions were prepared and also subjected to HPLC. At least 2 ubiquinone fractions (ubiquinones 9 and 10) could be tentatively identified by coelution with standards. The counts in the ubiquinone 10 fraction were significantly ($P < 0.01$) elevated (3–4-fold) in the treated compared to the controls. However, the label in ubiquinone 9 was not significantly different between treated and control animals. These results suggest that BPQ-OH, given at 70 mg/kg, did not inhibit the synthesis of dolichols or ubiquinones in the rat liver.

Table 2 summarises the quantitative recovery of mevalonate-derived radioactivity in the various HPLC fractions prepared from the hepatic lipids of control and BPQ-OH (70 mg/kg)-treated animals.

DISCUSSION

The two examples of the 3′ substituted quinuclidines are here demonstrated to be effective inhibitors of the rat liver SQS both in the *in vitro* microsomal assay and in the intact organ *in vivo* after oral administration of the compounds. The results of the detailed mevalonate-incorporation experiments with BPQ-OH suggests that the inhibition exerted by this compound is specific for the target enzyme.

In the recent literature, two other groups of compounds are reported as having both *in vitro* and *in vivo* action on SQS; these are the substituted bisphosphonates [9] and the squalestatins or zaragozic acids [7, 8]. In a rat liver microsomal enzyme assay, under conditions very similar to those used here, the geranyl and biphenyl bisphosphonates were reported as having IC_{50}s of about 1 nM, with the zaragozic acids having even greater potency with K_i values in the picomolar range. It was proposed that the bisphosphonates are inhibitors by virtue of their interaction with the FPP

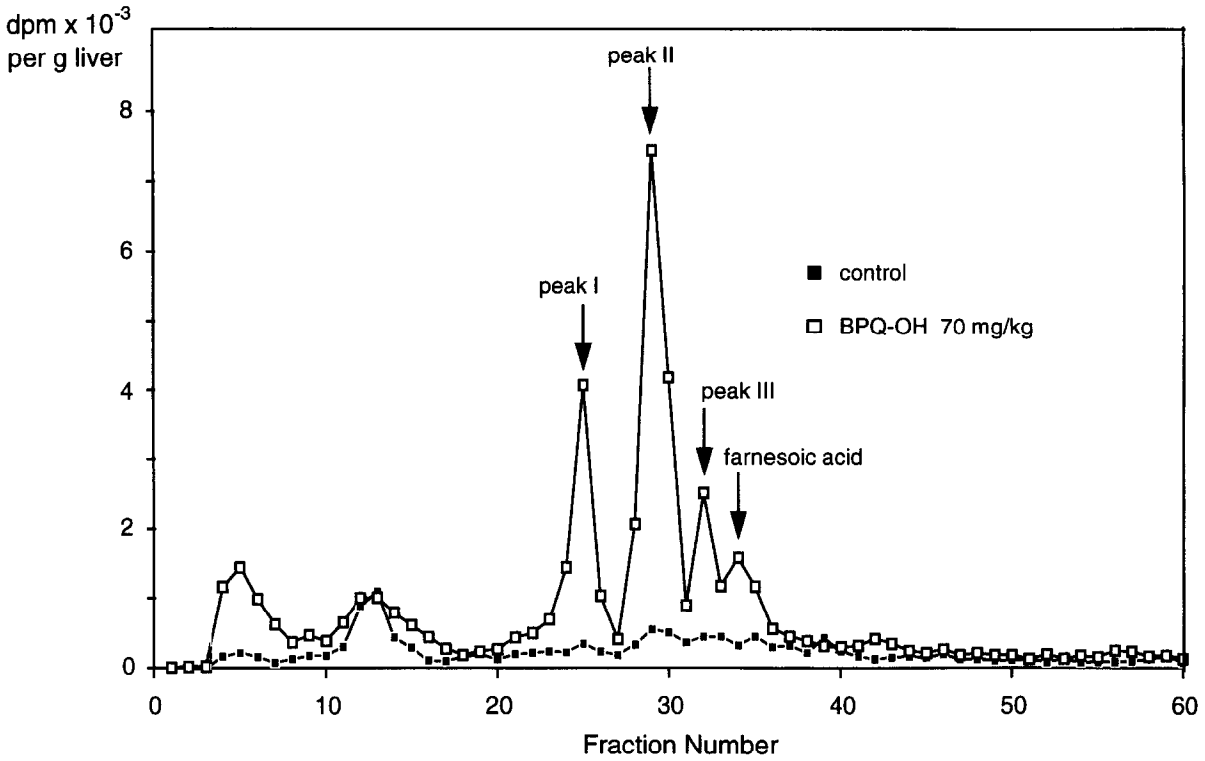


FIG. 7. The effect of the administration of BPQ-OH on the incorporation of labelled mevalonate into fractions of isoprenoid acids. Groups of 3 rats were administered BPQ-OH, 70 mg/kg by oral gavage, or vehicle alone (controls). One hour later, the animals were injected i.p. with 500 μCi [5-^{3}H]mevalonolactone and, after a further 1 hr, the animals were terminally anaesthetised with halothane and a liver section removed. Liver samples were saponified and extracted with pet. ether as described in Materials and Methods and in the legend to Fig. 6. The alkaline aqueous phases after washing the pet. ether extract was reacidified (pH 1) and extracted again with pet. ether. After evaporation and redissolving in chloroform-methanol (2:1), the sample was applied to a Kromasil KR100 5C18 HPLC column and eluted using a gradient system comprising 0.01 M triethylamine phosphate (pH 7), acetonitrile, and water. Fractions were collected to determine the distribution of radioactivity. The results are the mean of the 3 animals. The elution position of the farnesoic acid standard is indicated by the arrows. The labelling of peaks I–III is used to cross-reference the results to those shown in Table 2.

binding site(s), whereas the zaragozic acids were thought more likely to mimic the intermediate, presqualene pyrophosphate. Examples of both types of compound were also found to be extremely potent inhibitors of cholesterol synthesis after i.v. administration, with ED_{50} values of 0.03 and 0.2 mg/kg for the biphenyl bisphosphonate and zaragozic acid A, respectively. However, both of these inhibitor types have been reported to have poor oral availability, presumably as a result of the di- and tri-anionic chemical structures.

The 3′ substituted quinuclidines offer a different pharmaceutical approach. As lipophilic cations, protonated at physiological pH, they are unlikely to be direct mimics of the pyrophosphate substrate or intermediate. Both steps in the SQS reaction have been hypothesised to involve several putative carbocationic intermediates [15] and it is possible that the quinuclidines mimic the lipophilic and electrostatic properties of one or more intermediates of this type. Such a mechanism has been postulated for other enzymes in the sterol synthesis pathway; for example, the 8-azadecalins as inhibitors of oxidosqualene cyclase [16].

The inhibitory potency of the quinuclidines were determined in a rat liver microsomal assay. This was run under conditions which are very similar to those described in the recent literature to demonstrate the *in vitro* efficacy of SQS inhibitors [8, 9, 17]. Various methods, such as column chromatography [3], TLC [9], and spot wash [18] have been used to separate the reaction products. However, for convenience we adopted a simple reverse-phase separation to measure aqueous radioactivity (F1) and squalene formation (F2). For convenience we did not degas the incubation mixture (a step usually taken to minimise squalene epoxidase activity) and, as can be seen in Fig. 1, the F2 radioactivity of controls is a combination of label in squalene (77%) and in oxidosqualene (23%). Because the latter is a direct product of the former, they can both be taken together as squalene formation. In reaction products, saponified and then separated by HPLC, we determined that lanosterol comprised less than 1% of total radioactivity. In any case, lanosterol also elutes with 70% recovery in F2. The column efficiently retained the substrate, FPP. In control incubations, about 80% of F1 radioactivity remained in the aqueous phase after hexane extractions and 10% comigrated with squalene. Thus, F2 is a reliable estimate of squalene formation and F1 at least a reasonable approximation to proton release. The ratio between radioactivity in

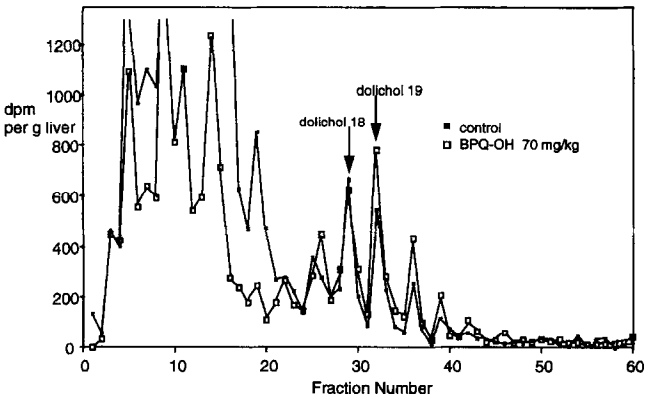


FIG. 8. HPLC separation of mevalonate-derived radioactivity in dolichol-containing fractions prepared from livers of control or BPQ-OH treated rats. Groups of 3 rats were administered BPQ-OH, 70 mg/kg by oral gavage, or vehicle alone (controls). One hour later, the animals were injected i.p. with 500 μCi [5-^{3}H]mevalonolactone and, after a further 1 hr, the animals were terminally anaesthetised with halothane and a liver section removed. Liver samples were saponified and extracted with pet. ether and the extract applied to a C-18 'Bond-Elut' column. This was eluted first with methanol and then with chloroform-methanol (2:1). The latter eluate was applied to a Spherisorb 3 ODS column. The HPLC column was eluted with an acetonitrile/isopropanol/water gradient system and fractions collected to determine the distribution of radioactivity. The results are the means of the data from 3 different animals. The elution positions of standards are indicated by the arrows.

TABLE 2. Summary of the effects of administration of BPQ-OH (70 mg/kg) on the recovery of mevalonate-derived radioactivity in various HPLC fractions of rat hepatic lipids

HPLC fraction	Control	BPQ-OH
	10^{-3} × dpm	
Cholesterol	757 ± 24	230 ± 74*
Squalene	484 ± 236	72 ± 9
	10^{-2} × dpm	
Isopentenyl pyophosphate	33 ± 11	26 ± 4
Geranyl pyrophosphate	22 ± 7	23 ± 2
Farnesyl pyrophosphate	111 ± 22	535 ± 49*
Geranylgeranyl pyrophosphate	115 ± 44	304 ± 7*
Farnesol	50 ± 7	472 ± 64*
Geranylgeraniol	100 ± 4	319 ± 43†
Farnesoic acid	8 ± 1	27 ± 2*
Acid Peak I	8 ± 1	67 ± 8*
Acid peak II	15 ± 2	137 ± 23†
Acid peak III	5 ± 1	25 ± 5†
	10^{-1} × dpm	
Dolichol 18	110 ± 6	124 ± 10
Dolichol 19	78 ± 5	106 ± 13
Ubiquinone 9	119 ± 10	151 ± 17
Ubiquinone 10	33 ± 2	122 ± 22†

Significance of differences from controls: *$P < 0.001$, †$P < 0.01$.

The results are expressed as the mean and SE of 3 determinations (separate animals).

Groups of 3 rats were administered BPQ-OH, 70 mg/kg by oral gavage or vehicle alone (controls). One hour later, the animals were injected i.p. with 500 μCi [5-^{3}H] mevalonolactone and, after a further 1 hr, the animals were terminally anaesthetised with halothane and a liver section removed. Liver samples were saponified, extracted, and separated by HPLC as described in Materials and Methods, and the radioactivity in the relevant peaks was determined.

F2 and in F1 from control incubations was 2.5, a figure close to the theoretical value of 3 expected from the use of FPP uniformly labelled with tritium in the C1 position.

For both F2 and F1 endpoints (latter data not shown), the reaction followed saturation kinetics. However, as detailed in a companion paper [11], it is possible that this represents the summation of the activity of two different kinetic phases differing in the affinity for FPP. As can be seen in Fig. 3, such biphasic behaviour of the enzyme is revealed with the inhibitor, BPQ-OH under conditions in which inhibitor and microsomes were preincubated. The finding of two kinetic phases, one sensitive to inhibitors and the other insensitive, was consistent in repeated experiments with this compound. BPQ also demonstrated biphasic inhibition under the same conditions. The compounds differed in respect to the proportions of total enzyme activity sensitive or insensitive to inhibition. The finding of slow binding and biphasic kinetics with a number of the quinuclidine inhibitors is treated in more detail in the separate report. The relevance of such kinetic properties to the expression of inhibitory activity *in vivo* is not known.

The SQS reaction proceeds in 2 steps with a PSPP intermediate that is then reduced *via* NADPH to squalene. There are different views in the literature as to whether these might proceed on the same or distinct sites on the enzyme, see [3] and [19]. However, it is possible that some types of inhibitors might block the second step of the reaction but not the first. With the quinuclidine inhibitors, parallel effects on both proton release and squalene formation were always seen. Thus, the K_i' values for both measures of reaction velocity were in good agreement (Table 1). From this, it may be concluded that the quinuclidines are not selectively acting on step 2 and, moreover, it is unlikely that they are inhibiting the binding of NADPH because this is only used in the last step and its omission uncouples the two reactions [3].

The intracellular metabolism of FPP is complex and multicompartmental [20]. The hypothetical advantage of an SQS inhibitor in sparing the branch pathways of mevalonate metabolism presupposes that the inhibitor is truly specific and does not block FPP production nor any of the enzymes comprising the other routes of FPP metabolism. Moreover, because the quantitative utilisation of FPP by SQS is much greater than that of the branch pathways, there must also be a safe alternative for the disposal of excess FPP, a process that should not be blocked by the inhibitor. It was, therefore, important to determine whether or not the effect of BPQ-OH on the fate of labelled mevalonate *in vivo* was indicative of truly specific action on SQS.

In a study of the effect of zaragozic acid A on the fate of labelled mevalonate in HepG2 cells and in the mouse liver, Bergstrom *et al.* [8] found an increase in radioactivity in fractions corresponding to FPP and farnesol as well as in organic acids. It was hypothesised that upon inhibition of

SQS, FPP was metabolised to farnesol and, thence, to farnesoic acids and dibasic derivatives of farnesoic acid. This is a metabolic pathway first demonstrated by Gonzalez-Pacanowska *et al.* [6] in *Drosophila* cells and in rat liver homogenates. A knowledge of the fate of excess FPP is of key importance to the therapeutic utility of SQS inhibitors. In a recent article, Bansal and Vaidya [5] have identified allyl pyrophosphatases with specificities for FPP and GGPP in rat liver microsomes. Consistent with this in the present study, BPQ-OH caused an increase in the peaks of mevalonate-derived radioactivity corresponding to farnesol (10-fold) and in geranylgeraniol (3-fold over controls). The presence of increased amounts of label in FPP and GGPP *in vivo* can also be strongly inferred from the experiments in which the basic aqueous phase containing these components was acidified and the resultant alcohols separated by HPLC. In this way, increases of 5- and 3-fold for FPP and GGPP, respectively, are indicated. Also consistent with Bergstrom *et al.* [8], increases in the labelling of an organic acid fraction were also found with BPQ-OH. Separation revealed at least 3 distinct peaks, a minor one of which cochromatographed with *trans, trans* farnesoic acid. The nature of the other peaks was not investigated, but their elution positions suggest that they may well correspond to the polar organic acids observed as metabolites of farnesol in rat liver homogenates by Gonzalez-Pacanowska *et al.* [6] and believed to be C12 and C15 α, ω dibasic acids. The results of the *in vivo* studies with BPQ-OH are entirely consistent with the suggested metabolic pathway by which excess FPP is converted to farnesol, farnesoic acids, and dibasic acid derivatives.

Although the incorporation of mevalonate into ubiquinones and dolichols was low, no inhibition of the synthesis of these lipid components was observed in BPQ-OH-treated rats. Indeed, a significant increase in the labelling of ubiquinone 10 was found, although this need not indicate an elevation in the absolute rate of synthesis of this lipid. We did not attempt to measure protein prenylation *in vivo*. However, BPQ-OH, at a level of 10^{-4} M, did not inhibit the farnesylation of *ras* protein in an *in vitro* incubation system using the human placental farnesyl transferase.*

In the *in vivo* studies reported here, there has been no attempt to determine the recovery of the mevalonate derivatives in the various fractions and no quantitative accounting of the label. However, samples from control and treated livers have been treated in a strictly identical fashion to determine the changes in the pattern of metabolites due to BPQ-OH. Overall, the *in vitro* and *in vivo* studies with BPQ-OH suggest a specific inhibition of SQS in rat liver.

The ability of SQS inhibitors to block cholesterol synthesis at a point after FPP and to spare the branch pathways may be more than just a theoretical advantage. In culture systems, high concentrations of HMGCoA reductase inhibitors are known to disrupt aspects of cell function as a result of reduced prenylation of proteins [21–23], although no *in vivo* correlates are known. High doses of such reductase inhibitors in the rat, however, have been shown to inhibit ubiquinone synthesis [9] and to lower levels in liver, heart, and skeletal muscle after prolonged administration to the rat [24, 25]. The reduced form, ubiquinol, is thought to play a vital function in cellular antioxidant defences [26, 27].

It is, therefore, encouraging that specific SQS inhibitors have been discovered that demonstrate the required inhibition of sterol synthesis *in vivo*, without inhibition of protein farnesylation or of dolichol or ubiquinone synthesis. Moreover, inhibition of SQS *in vivo* leads to an alternative metabolism of FPP to water-soluble products. BPQ-OH is of interest as a lead in a new pharmacological series of orally-active SQS inhibitors.

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