The effect of cholesterol on ubiquinone and tetrahymanol biosynthesis in *Tetrahymena pyriformis*

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The biosynthesis of ubiquinone-8 from radioactive mevalonate by cultures of *Tetrahymena pyriformis* is demonstrated. Under normal conditions the incorporation of this radioactive precursor into ubiquinone and the triterpenoid alcohol tetrahymanol reflects the amounts of these two compounds in the cell. Growth of *T. pyriformis* in the presence of cholesterol results in a complete inhibition of incorporation of radioactive mevalonate into tetrahymanol while there is a corresponding increase of radioactive incorporation into ubiquinone. This increased incorporation of mevalonic acid into ubiquinone must reflect a reduced level of mevalonic acid in the cell under these conditions and is not due to increased ubiquinone biosynthesis, indicating tight regulation of the pathway prior to mevalonate formation.

The eukaryotic protozoan *Tetrahymena pyriformis* is of particular interest because its external membranes contain a unique triterpenoid alcohol, tetrahymanol (Mallory *et al.*, 1963), which the organism is able to biosynthesize via the mevalonic acid pathway. Tetrahymanol appears to have a role in the membranes of *T. pyriformis* similar to that of cholesterol in the membranes of higher organisms. This proposal is strongly supported by the observation that a variety of added sterols, including cholesterol, are able to inhibit completely tetrahymanol biosynthesis and to replace quantitatively tetrahymanol in the membranes of this organism (Conner *et al.*, 1968; Beedle *et al.*, 1974; Conner *et al.*, 1978; Rogers *et al.*, 1979).

We have investigated the nature of the inhibition of tetrahymanol biosynthesis by cholesterol and have shown that the primary effect is at the level of the enzyme squalene synthetase, the synthesis of which would appear to be completely inhibited by cholesterol (Beedle *et al.*, 1974; Warburg *et al.*, 1982). Consistent with regulation at the level of squalene synthetase was the observation that *in vitro* the incubation of cholesterol-grown cells with radioactive mevalonic acid or isopentenyl pyrophosphate resulted in no incorporation into tetrahymanol but a corresponding accumulation of the radioactivity in farnesol (Warburg *et al.*, 1982). However, attempts to demonstrate a similar increase in farnesol or farnesyl pyrophosphate *in vivo* after growing cells in the presence of cholesterol and radioactive mevalonic acid have been unsuccessful. The results described in the present paper establish that the

radioactive farnesyl pyrophosphate that must initially accumulate in cholesterol-grown cells *in vivo* is almost quantitatively converted into ubiquinone without a net increase in ubiquinone biosynthesis. The regulatory implications of these observations are discussed.

Materials and methods

General conditions used for the growth of *T. pyriformis*, extraction and isolation of lipids and measurement of radioactivity have been described previously (Beedle *et al.*, 1974; Warburg *et al.*, 1982). Total lipids were extracted by the method of Bligh & Dyer (1959) and applied to silica gel G t.l.c. plates which were developed with petroleum spirit (b.p. 40–60°C)/acetone (4:1, v/v). In this system the R_F values for farnesol, tetrahymanol and ubiquinone are approx. 0.30, 0.45 and 0.55 respectively. Identification of the ubiquinone species was achieved by reverse-phase t.l.c. on hexadecane-impregnated plates, developed in propan-1-ol/methanol/hexadecane (10:25:0.7, by vol.) according to Faust *et al.* (1979). Tetrahymanol was quantified by g.l.c. using β -amyryn as an internal standard (Shepherd *et al.*, 1977).

Ubiquinone was quantified by spectrophotometric analysis using the oxidized and reduced difference spectrum at 275 nm and an ϵ of 12 700 litre·mol⁻¹·cm⁻¹ for ubiquinone (Crane & Barr, 1971). Briefly, the spectrum of the ubiquinone extract was recorded in ethanol (1 ml) and then aqueous NaBH₄ (10 μ l; 10 mg/ml) was added to

both reaction and blank cuvettes and the spectrum was retaken. Estimates were routinely carried out after t.l.c. separation of the total lipid extract and the value was corrected for loss of ubiquinone during chromatography by comparison of the ubiquinone content of control cultures before and after chromatography. It was not possible to estimate the ubiquinone content in the total lipid extract of cholesterol-grown cells because of the very high absorption at 275 nm, resulting from the desaturation of cholesterol by this organism to give the $\Delta^{5,7}$ -conjugated diene derivative. No correction was made for possible incomplete lipid extraction. However, it was repeatedly observed that the total radioactivity extracted equalled the total radioactivity taken up by the cells when radioactive mevalonic acid was used, and hence extraction of ubiquinone and tetrahymanol must be quantitative.

All radiochemicals were obtained from Amersham International and ubiquinone-6 and ubiquinone-10 were obtained from Sigma. Other materials were obtained from normal laboratory suppliers.

Results

In order to investigate the effect of cholesterol on the metabolic fate of radioactive mevalonic acid *in vivo*, cultures of *T. pyriformis* cells were grown initially for 3 h in the presence or absence of cholesterol. We have previously shown that after 3 h there is over 90% inhibition of mevalonic acid incorporation into tetrahymanol *in vitro* and a corresponding decrease in squalene synthetase activity (Warburg *et al.*, 1982). After 3 h tracer amounts of radioactive mevalonic acid were added to the growing culture and the incubation continued for a further 21 h. The cells were harvested and the total lipids were isolated and separated by t.l.c. Comparison of the distribution of radioactivity after chromatography between control and cholesterol-grown cells revealed over 90% inhibition of the incorporation of radioactive mevalonic acid into tetrahymanol and an almost quantitative (values varied from 75 to 100% in different experiments) redirection of this radioactivity into a less polar yellow band corresponding to ubiquinone (Fig. 1). This radioactivity remained associated with the yellow ubiquinone band on t.l.c. after either exhaustive acetylation or methylation. That this new radioactive material was indeed ubiquinone was confirmed by reverse phase t.l.c., after which all the radioactivity was associated with a yellow band running between the available standards ubiquinone-6 and ubiquinone-10 (Fig. 2). This is consistent with the fact that the ubiquinone found in *T. pyriformis* is ubiquinone-8 (Vakirtzi-Lemonias *et al.*, 1963). It is noteworthy that the relative distribution of radioactivity between tetrahymanol (containing

Fig. 1. Thin-layer radiochromatogram of lipids biosynthesized from $[2-^{14}\text{C}]$ mevalonate by *T. pyriformis* and separated on silica gel

Incubations (600 ml) were carried out for 21 h in the presence of $[2-^{14}\text{C}]$ mevalonic acid lactone ($2\ \mu\text{Ci}$, $53\ \text{mCi/mmol}$). Lipids were extracted by the method of Bligh & Dyer (1959). (a) Control incubation; (b) incubation in the presence of cholesterol (15 mg). A, ubiquinone; B, tetrahymanol; C, farnesol.

six mevalonic acid units) and ubiquinone-8 (containing eight mevalonic acid units) was about 5:1 (Fig. 1; Table 1) and this ratio, after correction for the number of mevalonic acid units, corresponds approximately to the relative amounts of tetrahymanol ($3.6\ \mu\text{mol/g}$ dry wt.) and ubiquinone-8 ($0.6\ \mu\text{mol/g}$ dry wt.) found in this organism after these 24 h incubations (Table 2).

Although for cells grown in the presence of cholesterol there is about a 5-fold increase in the incorporation of mevalonic acid into ubiquinone-8, the total amount of ubiquinone-8 in these cultures was found to be identical with that from control cultures (Table 2), indicating that cholesterol had no effect on the rate of synthesis of ubiquinone-8. This was confirmed using $[\text{methyl-}^3\text{H}]$ methionine, which is the source of the methyl groups for the quinone ring. The incorporation of radioactivity from this substrate into ubiquinone-8 was identical for both control and cholesterol-grown cells (Table 1).

Fig. 2. Thin-layer radiochromatogram of ubiquinone biosynthesized from $[2\text{-}^{14}\text{C}]$ mevalonate by *T. pyriformis* and separated on silica gel by reverse phase chromatography

The radioactive band isolated by normal t.l.c. from *T. pyriformis* cultures grown in the presence of cholesterol (Fig. 1) was chromatographed on hexadecane-impregnated silica gel plates. UQ_6 and UQ_{10} refer to ubiquinone-6 and -10 standards respectively.

The quantitative redirection of radioactive mevalonic acid from tetrahymanol into ubiquinone-8 in the absence of a change of synthesis of ubiquinone-8 can be explained by the fact that *T. pyriformis* is very impermeable to mevalonic acid and hence pool size effects become significant. This permeability was quantified in an experiment where radioactive mevalonic acid was added with or without carrier and uptake into the cells was monitored. In the presence or absence of carrier only about 0.4% of the radioactivity was incorporated over 24 h in this experiment and it is noteworthy that all of this radioactivity is extractable into chloroform/methanol (2:1, v/v). Since essentially all this extracted radioactivity was associated with either tetrahymanol or ubiquinone-8 after t.l.c. it implies a very tight control of biosynthesis with very low levels of intermediates of the mevalonic acid pathway within the cell. The molar incorporation of radioactive mevalonic acid into ubiquinone may be calculated from the specific radioactivity of the added mevalonic acid and the isolated ubiquinone. In the presence of carrier mevalonic acid the radioactivity in the ubiquinone represented less than

Table 1. Effect of cholesterol on the incorporation of $[2\text{-}^{14}\text{C}]$ mevalonic acid and $[\text{methyl-}^3\text{H}]$ methionine into tetrahymanol and ubiquinone by *T. pyriformis*

(a) Cultures of *T. pyriformis* (600 ml) were grown for 3 h in the presence or absence of cholesterol (15 mg/l). $[2\text{-}^{14}\text{C}]$ Mevalonic acid *NN'*-dibenzylethylenediamine salt ($2\text{ }\mu\text{Ci}$; 10 mCi/mmol) and $[\text{methyl-}^3\text{H}]$ methionine ($10\text{ }\mu\text{Ci}$, 90 Ci/mmol) were added and the incubation was continued for a further 21 h. (b) Cultures were grown for 18 h followed by 3 h with cholesterol and then for 3 h with the labelled substrates as in (a).

Experiment type	Incubation conditions	Total incorporation (d.p.m.) from $[2\text{-}^{14}\text{C}]$ mevalonic acid into:		Total incorporation from $[\text{methyl-}^3\text{H}]$ methionine into ubiquinone (d.p.m.)
		Tetrahymanol	Ubiquinone	
(a)	Control	56 160	9740	51 880
(a)	+ Cholesterol	2980	47 720	56 660
(b)	Control	13 080	3460	20 500
(b)	+ Cholesterol	1580	14 160	18 020

Table 2. Effect of cholesterol on ubiquinone-8 and tetrahymanol biosynthesis in *T. pyriformis* grown in the presence or absence of mevalonic acid together with $[2\text{-}^{14}\text{C}]$ mevalonic acid

Cultures of *T. pyriformis* (600 ml) were grown for 3 h in the presence or absence of cholesterol (15 mg/l). $[2\text{-}^{14}\text{C}]$ -Mevalonic acid lactone ($2\text{ }\mu\text{Ci}$; 53 mCi/mmol) was added together with $150\text{ }\mu\text{mol}$ of mevalonic acid lactone as indicated. Incubations were continued for a further 21 h. Uptake of radioactivity into cells and incorporation into total lipids were identical with and without carrier mevalonic acid lactone. Values in parentheses are % of theoretical maximum mevalonate incorporation into product.

Incubation conditions	Ubiquinone-8 content ($\mu\text{mol/g}$ dry wt.)	Tetrahymanol content ($\mu\text{mol/g}$ dry wt.)	Uptake of radioactivity into cells (%)	Radioactive incorporation into ubiquinone-8 (μmol of mevalonate/ μmol of UQ-8)	Radioactive incorporation into tetrahymanol (μmol of mevalonate/ μmol of UQ-8)
Control	0.61	3.7	0.38	1.15×10^{-4}	7.75×10^{-5}
Control + mevalonate ($2.5 \times 10^{-4}\text{ M}$)	0.55	3.4	0.39	0.46 (5.75)	0.31 (5.17)
Cholesterol	0.59	<0.1	0.39	3.78×10^{-4}	—
Cholesterol + mevalonate ($2.5 \times 10^{-4}\text{ M}$)	0.62	<0.1	0.38	1.51 (18.9)	—

6% of the total biosynthesis in the control incubations, rising to 19% for cells grown in the presence of cholesterol (Table 2). Hence, considerable dilution of the radioactivity by internally biosynthesized mevalonic acid was occurring even in the presence of 2.5×10^{-4} M-mevalonic acid in the medium. Thus, under these conditions it was still the rate of mevalonate formation within the cell that dictated ubiquinone biosynthesis.

The results do not allow interpretation of the nature of the control, but must imply that, as squalene synthetase is inhibited in the presence of cholesterol, the rate of synthesis of farnesyl pyrophosphate must be reduced in a co-ordinated fashion and that the small amounts of mevalonic acid that are taken up by the cell are simply switched to other available pathways, of which quantitatively the most significant is ubiquinone biosynthesis.

To date we have no direct experimental information either on the levels of mevalonic acid and its products or on the regulation of mevalonic acid formation within the cell.

Discussion

The results described in this paper show many similarities to those of Faust *et al.* (1979) when they investigated the regulation of ubiquinone biosynthesis in fibroblasts by cholesterol. In both systems there is an apparent very tight control of intermediates to allow the appropriate flux for both polyisoprene and triterpene biosynthesis. In the case of *T. pyriformis* this flux may be quantified by comparison with the actual amounts of ubiquinone-8 and tetrahymanol that are biosynthesized during growth of the culture through between five and six division cycles over 21 h. As a result of this tight control, externally added radioactivity as mevalonic acid is redirected from the quantitatively more significant triterpenoid pathway to ubiquinone biosynthesis as the requirement for the former is reduced in the presence of cholesterol. In the case of the mammalian systems the higher permeability to mevalonic acids allows this switch to be saturated at high mevalonic acid concentrations, whereas the low permeability of *T. pyriformis* to mevalonic acid, coupled with the high requirement for ubiquinone biosynthesis during growth of this organism, does not readily allow this saturation of the incorporation of radioactivity from mevalonate into

ubiquinone to be demonstrated. Similar observations to those of Faust *et al.* (1979) have been described by Nambudiri *et al.* (1980), while recently a more analogous situation to that in *T. pyriformis* has been observed in cultured cells of neural origin. Volpe & Obert (1982) looked at the interrelationship between ubiquinone and squalene synthesis in the presence of U18666A, an inhibitor of squalene synthetase. They observed that this inhibitor caused an apparent stimulation of ubiquinone biosynthesis from both radioactive acetate and mevalonate, due to an almost quantitative switch in the incorporation of radioactivity from squalene into ubiquinone.

A more detailed understanding of the method of regulation existing in *T. pyriformis* will require both an investigation of control points prior to mevalonic acid and also the possible regulation of ubiquinone-8 biosynthesis from farnesyl pyrophosphate or its precursors.

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