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INTRACELLULAR DISTRIBUTION OF ISOPRENOID PHENOL AND QUINONE PRECURSORS OF UBIQUINONES AND UBIQUINONES(H_2) IN FUNGI

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Abstract—The intracellular distribution of 5-demethoxyubiquinone-6 and ubiquinone-6 in *Saccharomyces carlsbergensis* and of 2-decaprenyl(H_2)phenol, 6-methoxy-2-decaprenyl(H_2)phenol, 6-methoxy-2-decaprenyl (X-H_2)-1,4-benzoquinone, 5-demethoxyubiquinone-10(X-H_2) and ubiquinone-10(X-H_2) in *Aspergillus flavus* has been investigated. The results obtained showed that, except for 6-methoxy-2-decaprenyl(H_2)phenol, these compounds are localized mainly in the mitochondria with small but fairly constant amounts present in the supernatant (endoplasmic reticulum and Golgi apparatus?). 6-Methoxy-2-decaprenyl(H_2)phenol is atypical and is distributed in the reverse fashion.

INTRODUCTION

THE PATHWAYS for the biosynthesis of ubiquinones from *p*-hydroxybenzoic acid has been the subject of intensive study for the past 6 yr. The results of analytic, radioactive tracer, enzyme and mutant studies suggest that in many, if not all, ubiquinone-containing organisms the intermediates on the pathways will be isoprenoid phenols and quinones of the structural types originally detected in, and subsequently isolated from, the photosynthetic bacterium *Rhodospirillum rubrum*.[1] In a recent study, ten moulds and two yeasts were analysed for the presence of isoprenoid phenols and quinones.[2] The results showed the organisms fell into three groups: (a) those that contained only ubiquinones and ubiquinones (H_2);‡ (b) those that contained 5-demethoxyubiquinones and ubiquinones or 5-demethoxyubiquinones (H_2) and ubiquinones (H_2); (c) one that contained 2-decaprenyl(H_2)phenol, 6-methoxy-2-decaprenyl(H_2)phenol, 6-methoxy-2-decaprenyl(X-H_2)-1,4-benzoquinone,‡ 5-demethoxyubiquinone-10(X-H_2) and ubiquinone-10(X-H_2) (Scheme 1). Pulse labelling experiments provided evidence that in *Phycomyces blakesleeanus* and *Saccharomyces cerevisiae*, two of the organisms from group (b), 5-demethoxyubiquinones are precursors of ubiquinones, and in *Aspergillus flavus*, the sole organism in group (c), the partial pathway shown in Scheme 1 is operative for the biosynthesis of ubiquinone-10(X-H_2).

In the present paper we report the results of investigations to determine the intracellular distribution of 5-demethoxyubiquinone-6 and ubiquinone-6 in the yeast *S. carlsbergensis*, a group (b) organism,[3] and of 2-decaprenyl(H_2)-1,4-benzoquinone, 5-demethoxyubiquinone-10(X-H_2) and ubiquinone-10(X-H_2) in the fungus *A. flavus*.

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‡ (H_2) indicates that one of the isoprene units in the polyprenyl side chain is saturated: (X-H_2) indicates that the tenth isoprene unit from the nucleus is saturated.

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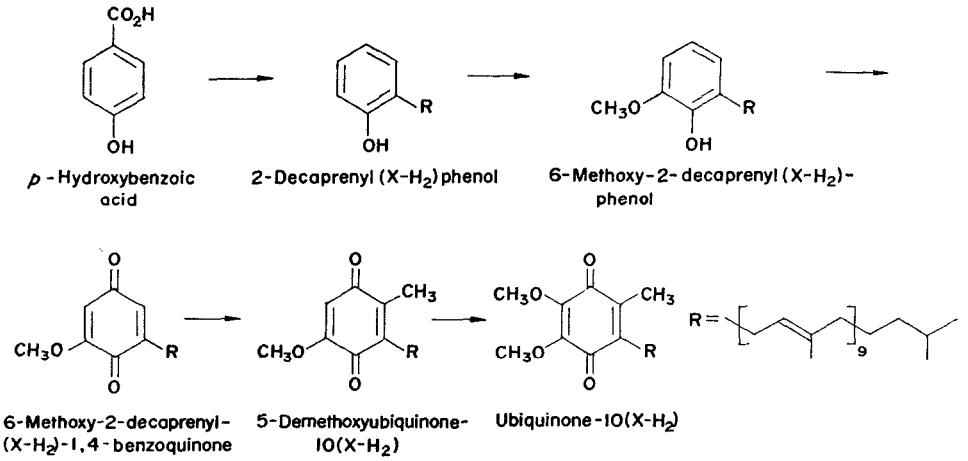
[2] AH LAW, D. R. THRELFALL and G. R. WHISTANCE, *Biochem. J.* **123**, 331 (1971).

[3] AH LAW and D. R. THRELFALL, unpublished observations (1971).



SCHEME 1. PARTIAL PATHWAY FOR THE BIOSYNTHESIS OF UBIQUINONE-10 (X-H_2) BY *A. flavus*.

RESULTS

Homogenates of the organisms were fractionated by differential centrifugation and the fractions obtained analysed for the presence of isoprenoid phenols and quinones. The distribution of mitochondria and mitochondrial fragments between the various fractions was determined by measuring the activities of two marker enzymes, NADH–oxidase and succinic oxidase.

Studies with A. flavus

In the first experiment, designed to test the suitability of the fractionation method, 3-day-old cells (270 g wet wt.) of *A. flavus* were fractionated by the procedure described in the Experimental, and the fractions obtained quantitatively analysed for ubiquinone-10(X-H_2) and for NADH– and succinic–oxidase activity (Table 1). The results showed that the method was perfectly satisfactory, the greater part of the ubiquinone-10(X-H_2) and of

TABLE 1. FRACTIONATION OF 3-day-old *A. flavus*

Fraction*	Protein (mg)	Succinic oxidase		NADH-oxidase		Ubiquinone-10(X-H_2)	
		Rate (μl O_2/min /mg protein)	Total O_2 uptake (% total)	Rate (μl O_2/min /mg protein)	Total O_2 uptake (% total)	(μg)	(% total)
Debris and nuclear	58	0	0	0	0	0	0
Mitochondrial	370	1·8	71	2·5	59·0	811	50·8
Microsomal	448	0·6	29	1·4	40·4	657	41·2
Supernatant	1070	0	0	0	0	127	8

* From 270 g fresh wt.

the NADH– and succinic oxidase activity appearing in the mitochondrial (23,000 *g*) fraction.

To determine the intracellular distribution of the ubiquinone intermediates it was necessary, because of the small amounts present, to label them from *p*-hydroxy[U-^{14}C] benzoic acid and to assay them by radioactive counting. Ideally, the radiosubstrate should have been added to the growth medium shortly before harvesting the cells, but under these conditions ubiquinone becomes highly labelled making the determination of radioactivity in minor components very difficult. Therefore, an incubation procedure was employed. Accordingly, 2-day-old *A. flavus* was incubated at 28° and pH 6·0 with shaking (180 rev/min) with *p*-hydroxy[U-^{14}C]benzoic acid for 4·5 hr. On fractionation of these cells it was found that, although the expected distribution between the fractions of ubiquinone-10(X-H_2) and of NADH– and succinic–oxidase was obtained (Tables 2 and 3), the debris and nuclear

TABLE 2. FRACTIONATION OF 2-day-old *A. flavus* THAT HAD BEEN INCUBATED WITH PARA-HYDROXY[U-^{14}C]-BENZOIC ACID

Fraction*	Protein (mg)	Succinic oxidase† Rate (μl O_2/min/mg protein)	Ubiquinone-10(X-H_2) Amount (μg)	Sp. radioactivity (dpm/μg)	Total radioactivity (dpm) 2-Decaprenyl(H_2)phenol‡	6-Methoxy-2-decaprenyl(H_2)-phenol‡	6-Methoxy-2-decaprenyl (X-H_2)-1,4-benzoquinone	5-Demethoxyubiquinone-10(X-H_2)
Debris and nuclear	26	3·4	69	268	796	70	Trace	6000
Mitochondrial	244	3·4	484	232	2620	372	7480	49,400§
Microsomal	157	2·5	221	160	970	132	4020	16,230
Supernatant	820	0	127	111	743	1027	1130	6350

* From 275 g fresh wt. fed with 5·5 μc of *p*-hydroxy[U-^{14}C]benzoic acid.

† Almost identical values were obtained for the NADH-oxidase.

‡ The presence of radioactivity in these compounds was confirmed by chromatographing small amounts of the impure samples on paraffin-impregnated silica gel G layers developed with aq. 95% acetone and preparing a radioautogram of the developed plate. In all cases the applied radioactivity migrated as 2-decaprenyl(H_2)phenol or 6-methoxy-2-decaprenyl(H_2)phenol.

§ This fraction contained 60 μg of 5-demethoxyubiquinone-10(X-H_2) with a sp. radioactivity of 820 dpm/μg.

and microsomal fractions had relatively high rates of oxidase activity (Table 2). This suggested that under the incubation conditions employed the intracellular structures of the *A. flavus* cells were starting to undergo marked morphological changes. However, it was felt that the results obtained for the distribution between the fractions of the radioactivity associated with the various intermediates (Tables 2 and 3), when considered in conjunction with the results from the first analysis (Table 1), could be interpreted with confidence. An important feature of this experiment from the biosynthetic point of view was the fact that the specific activity values of the ubiquinone-10(X-H_2) varied markedly from fraction to fraction (Table 2).

Studies with S. carlsbergensis

S. carlsbergensis was used in place of *S. cerevisiae* because it contained much higher levels of 5-demethoxyubiquinone-6.[3] This meant that the fractions could be analysed by chemical means. Pulse labelling experiments, of a similar nature to those carried out with *S. cerevisiae*,[2] had established that in this organism 5-demethoxyubiquinone-6 is a precursor of ubiquinone-6 and no other intermediates are present in radiochemically detectable amounts.[3]

TABLE 3. FRACTIONATION OF 2-day-old *A. flavus* THAT HAD BEEN INCUBATED WITH PARA-HYDROXY[U-^{14}C] BENZOIC ACID

Fraction*	Succinic oxidase	Distribution (% total radioactivity)				
	Total O_2 uptake (% total)	2-Decaprenyl(H_2)-phenol	6-Methoxy-2-decaprenyl(H_2)-phenol	6-Methoxy-2-decaprenyl(X-H_2)-1,4-benzoquinone	5-Demethoxy-ubiquinone-10(X-H_2)	Ubiquinone-10(X-H_2)
Debris and nuclear	6·8	15·5	4·4	0	7·7	10·3 (7·7)†
Mitochondrial	63·3	51·1	23·2	59·2	63·3	62·3 (53·7)
Microsomal	29·9	18·9	8·3	31·9	20·9	19·6 (24·5)
Supernatant	0	14·5	64·1	8·9	8·1	7·8 (14·1)

* See Table 2.

† The distribution as % total weight is given by the figures in parenthesis.

Three-day-old cells were fractionated by a modification of the procedure described by Tzogoloff.[4] Analysis of the fractions showed that the procedure was efficient, the greater part of the ubiquinone-6 and of the NADH- and succinic oxidase being present in the mitochondrial (20,000 *g*) fraction (Table 4). 5-Demethoxyubiquinone-6, as expected, had the same distribution pattern as ubiquinone-6 (Table 4).

TABLE 4. FRACTIONATION OF 3-day-old *S. carlsbergensis*

Fraction*	Protein (mg)	Succinic oxidase		Ubiquinone-6		5-Demethoxyubiquinone-6	
		Rate (μl O_2/min/mg protein)	Total O_2 uptake (% total)	(μg)	(% total)	(μg)	(% total)
Debris and nuclear	90	0·9	6·6	64	6·4	13	4·5
Mitochondrial	340	2·7	72·2	651	65·1	178	61·2
Microsomal	300	0·9	21·2	179	17·9	62	21·3
Supernatant	1500	0	0	106	10·6	38	13

* From 228 g fresh wt.

Prompted by the finding that in the radiochemical experiment with *A. flavus* the ubiquinones isolated from different fractions had different specific radioactivity values (Table 2), it was decided to carry out a similar experiment using *S. carlsbergensis*. The opportunity was also taken to examine the morphological changes which appear to take place under these conditions, by increasing the incubation time from the normal 3 hr. Three-day-old cells were suspended in buffer, pH 5·7, containing 0·7 g of glucose and *p*-hydroxy[U-^{14}C]benzoic acid and incubated at 30° with shaking (250 rev/min) for 10 hr. The results showed that, (a) the specific radioactivity of the ubiquinone-6 in the supernatant fraction differed appreciably from those of the ubiquinone-6 in the other fractions, and (b) the changes with regard to the distribution between the fractions of ubiquinone-6 and of succinic oxidase were even more marked than in *A. flavus*: 29% of the ubiquinone-6 and 39% of the succinic oxidase activity sedimenting in the debris and nuclear fraction (Table 5).

DISCUSSION

The most thorough investigations into the intracellular distribution of ubiquinone in the eucaryotic cell have been made using the soft tissues of higher animals.[5] The results obtained

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TABLE 5. FRACTIONATION OF 3-day-old *S. carlsbergensis* THAT HAD BEEN INCUBATED WITH PARA-HYDROXY-[U-^{14}C]BENZOIC ACID

Fraction*	Protein (mg)	Succinic oxidase		Ubiquinone-6		5-Demethoxyubiquinone-6	
		Rate (μl O_2/min/mg protein)	Total O_2 uptake (% total)	Amount (μg)	Sp. radioactivity (dpm/μg)	Amount (μg)	Sp. radioactivity (dpm/μg)
Debris and nuclear	415	1·5	39	212	27	40	342
Mitochondrial	248	2·6	40·2	254	24	35	383
Microsomal	208	1·6	20·8	112	23	28	377
Supernatant	1250	0	0	158	42	36	518

* From 206 g fresh wt. fed with 1·86 μc *p*-hydroxy[U-^{14}C]benzoaic acid.

show that the greater part of the total cell content of ubiquinone is present in the mitochondria,[5] whilst the remainder is found associated with the endoplasmic reticulum (microsomes)[6–8] and Golgi apparatus.[9] Reports[10,11] of its presence in the nucleus have not been confirmed.[12,13] In plants ubiquinone has been shown to be localized in the mitochondria of higher plants,[14] algae,[15] yeasts[16] and fungi[17] and to be associated with higher plant cell walls.[14] There is general agreement that in the mitochondria of all eucaryotic cells it functions in the electron transport chain at a site between the NADH– flavoprotein– and succinic dehydrogenase–flavoprotein-spurs and the cytochrome chain.[5]

The results of our studies show that, as expected, most of the ubiquinone present in *A. flavus* and *S. carlsbergensis* is distributed in a similar manner to the two mitochondrial marker enzymes, NADH– and succinic oxidase (Tables 1–5). They also show that 5-demethoxyubiquinone-6 in *S. carlsbergensis* (Tables 4 and 5) and, on a radioactivity basis, 2-decaprenyl(H_2)phenol, 6-methoxy-2-decaprenyl(X-H_2)-1,4-benzoquinone and 5-demethoxyubiquinone-10(X-H_2) in *A. flavus* are distributed in much the same way (Tables 2 and 3). 6-Methoxy-2-decaprenyl(H_2)phenol is atypical, since it is nearly all present in the supernatant fraction (Tables 2 and 3). In all the analyses fairly constant proportions (8–14%) of the ubiquinone and, with the exception of 6-methoxy-2-decaprenyl(H_2)phenol, its intermediates were recovered from the supernatant fraction. On the basis of the work carried out with animals, these small amounts are probably associated with the Golgi apparatus and, since the microsomal fraction was prepared by using a sedimenting force of 72,000 *g* instead of the more usual 100,000 *g*, endoplasmic reticulum. Some support for the belief that most of the microsomes were present in the supernatant fractions is the finding that after correction of the microsomal fractions for mitochondrial contamination (Table 6) only in one experiment was the presence of ubiquinone in this fraction not attributable to mitochondria or mitochondrial fragments.

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TABLE 6. CORRECTED VALUES FOR THE DISTRIBUTION OF UBIQUINONE-10(X-H_2) IN *A. flavus* AND 5-DEMETHOXYUBIQUINONE-6 AND UBIQUINONE-6 IN *S. carlsbergensis*

Fraction	*A. flavus*		*S. carlsbergensis*			
	Ubiquinone-10(X-H_2)		Ubiquinone-6		5-Demethoxyubiquinone-6	
	Non-radioactive expt. (μg)	Radioactive expt. (μg)	Non-radioactive expt. (μg)	Radioactive expt. (μg)	Non-radioactive expt. (μg)	Radioactive expt. (μg)
Debris and nuclei	0	17	4	0	0	6
Mitochondria	1141	757	890	578	243	93
Microsomes	327	0	0	0	10	4
Supernatant	127	127	106	158	38	36

The amount of ubiquinone or 5-demethoxyubiquinone to be transferred to the mitochondrial fraction was calculated by using the expression: (Wt. of ubiquinone or 5-demethoxyubiquinone in mitochondrial fraction/% of total succinic oxidase activity in mitochondrial fraction) X % of total succinic oxidase activity in fraction. A negative value was taken to mean that all the ubiquinone in a fraction was of mitochondrial origin.

In biosynthetic terms these results would seem to provide good evidence that mitochondria and the ubiquinone-containing organelles in the supernatant (endoplasmic reticulum and Golgi bodies?) can synthesize ubiquinone from *p*-hydroxybenzoic acid. The fact that all the intermediates involved in this synthesis are extremely lipophilic suggests that within the organelles the enzymes involved must be grouped together to form ubiquinone-synthetases. Indeed, with the isolation of a sterol carrier protein from rat liver,[18,19] it is not unreasonable to assume that such a synthetase would consist of an isoprenoid phenol/quinone carrier protein (ubiquinone carrier protein) surrounded by the appropriate enzymes. In yeast mitochondria,[16] and presumably all other mitochondria, most of the ubiquinone is present in the inner membrane and it is probable that this is the membrane that will contain the isoprenoid phenol and quinone precursors. If this is the case then the question is raised: Is ubiquinone synthesized at its functional site or is it made elsewhere and then transported to (using ubiquinone carrier protein?), and laid down in, the electron transport chain? Similar questions can also be asked about the biosynthetic situation in the other ubiquinone containing structures of the cell. The finding that the specific radioactivity values of the ubiquinones labelled from *p*-hydroxy[U-^{14}C]benzoic acid varied from fraction to fraction in *A. flavus* and in *S. calsbergensis* (Tables 2 and 5), suggests that the rate of ubiquinone biosynthesis must differ in (a) the various intracellular organelles and (b) mitochondria at different stages of development and degeneration.

EXPERIMENTAL

Biological material. A. flavus Herb I.M.I. 16,145 and *S. carlsbergensis* Herb I.M.I. 80,178 were obtained from the Commonwealth Mycological Institute, Kew, Surrey, U.K. *A. flavus* was grown for 2–3 days under conditions identical to those previously described.[2] *S. carlsbergensis* was grown for 3 days at 30° in shake culture (300 rev/min; Gallenkamp orbital shaker) on the medium of Ohnishi, Kawaguchi and Hagihara:[20] the medium was dispensed as 1-l. vol. in 2-l. conical flasks.

Incubation with p-hydroxy[U-^{14}C]benzoic acid. The organisms were harvested, washed twice by resuspension in cold distilled water and then incubated with *p*-hydroxy[U-^{14}C]benzoic acid (7·8 mc/m-mole), prepared by alkali fusion of L-[U-^{14}C]tyrosine.[21]

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Fractionation of the fungi. A. flavus. The procedure used was similar to those that have been used for the fractionation of *A. niger*,[22] *Neurospora crassa* and *Polystiticus versicolor*.[23] The fungus from 5 l. of growth medium was harvested by straining through cheese cloth, washed twice by resuspension in ice-cold H_2O and suspended in 500 ml of ice-cold 0·03 M potassium phosphate–0·01 M EDTA buffer, pH 7·4, which was 0·5 M with respect to sucrose by homogenization at top speed for three periods of 2 min in a Waring Blendor. The homogenate was ground in a mortar with 250 g of fine acid-washed sand (prepared by washing acid-washed sand with 0·1 M EDTA followed by H_2O) for 10 min at 0° and then filtered through four layers of cheese cloth. The residue was resuspended in 500 ml of 0·5 M sucrose buffer and the above procedure repeated. The supernatants from the grinding procedure were bulked and fractionated by centrifuging twice at 500 *g* for 10 min in a refrigerated centrifuge, twice at 23,000 *g* for 20 min and twice at 72,000 *g* for 100. The precipitates were then bulked according to their sedimentation values, washed by resuspension in 0·5 M sucrose buffer, and resedimented at the appropriate value. The 500 *g*, 25,000 *g* and 72,000 *g* precipitates were designated cell debris and nuclear fraction, mitochondrial fraction and microsomal fraction respectively. The supernatant from the pellet sedimenting at 72,000 *g* was designated supernatant fraction.

S. carlsbergensis. The procedure used was based on one described by Tzogoloff.[4] Three-day-old cells from 12 l. of growth medium were harvested by centrifugation and then suspended in 1 l. of liquid N_2. After 15 min the frozen cells were transferred to 1 l. of 0·05 M tris HCl 1 mM EDTA buffer, pH 8·2, which was 0·3 M with respect to sucrose and homogenized for three 1-min periods using an Ultra-Turrax homogenizer. The homogenate was then centrifuged at 500 *g* for 5 min and the residue discarded. The supernatant was fractionated by a procedure that was very similar to the one used for *A. flavus*. The modifications made were that, (a) the first two precipitates were sedimented at 1000 *g* and 20,000 *g* instead of 500 *g* and 23,000 *g*, and (b) the 1000 *g*, 20,000 *g* and 72,000 *g* precipitates were not washed before use.

Enzyme assay and chemical analysis of intracellular fractions. The precipitates from the differential centrifugation procedure were each suspended in a known volume (15–20 ml) of the sucrose buffer used for their isolation. A 0·2–0·5 ml sample was then taken from each suspension and diluted with 0·2 M potassium phosphate buffer, pH 7·4, to give a final protein concentration of about 0·5 mg/ml. This diluted sample was used for the determination of protein content, succinic oxidase activity and NADH–oxidase activity. The remaining undiluted samples were analysed for isoprenoid phenol and quinone content.

The supernatant fraction was used without dilution for the determination of NADH– and succinic–oxidase activities: however, to measure protein content a small sample was taken and diluted. The remainder of the supernatant fraction was lyophilized and the lyophilized powder analysed for isoprenoid phenols and quinones.

Protein content. This was determined by treating the sample with Folin-Ciocalteu reagent and measuring the absorbance at 500 nm.[24] Crystalline bovine serum albumin (Koch light) was used to prepare the standard curve.

NADH- and succinic-oxidase activity. These were determined by use of an oxygen polarograph (YSI Model 53 Biological Oxygen Monitor.)[25] The complete assay system consisted of 1 ml of 0·02 M potassium phosphate buffer, pH 7·4, 2 ml of enzyme preparation containing 0·5 mg of protein/ml, 0·05 ml of 1% (w/v) cytochrome c(horse heart) and 0·1 ml of either 0·07 M $NADH_2$ or 1·5 M sodium succinate, pH 7·4. Endogenous respiration was determined by measuring the O_2-uptake for 10 min at 30° in the absence of substrate. The appropriate substrate was then added and the O_2-uptake monitored for a further 10 min at 30°. The fractions all had negligible endogenous rates of O_2-uptake and, as judged by the addition of 0·1 ml of 0·01 M ADP to the complete assay system, were completely uncoupled.

Isolation of isoprenoid phenols and quinones. The remainder of the undiluted resuspended fractions and the lyophilized supernatant powder (after resuspension in 20 ml H_2O) were each saponified by refluxing for 45 min with 40 ml of 5% (w/v) methanolic pyrogallol and 5 g of KOH. The unsaponifiable lipids were recovered from the saponification mixture by a routine procedure.[26] After precipitation of the sterols in cold (−20°) light petroleum (b.p. 40–60°) the isoprenoid phenols and quinones were isolated and purified by a combination of adsorptive and reversed phase TLC.[2] The 2-decaprenyl(H_2)phenols, 6-methoxy-2-decaprenyl(H_2) phenols, 6-methoxy-2-decaprenyl(X-H_2)-1,4-benzoquinones and 5-demethoxyubiquinones (X-H_2) were present in such small amounts in the fractions from *A. flavus* that they could only be determined by measuring their radioactivity contents in an experiment in which the fungus was allowed to metabolise *p*-hydroxy-[U-^{14}C]benzoic acid before it was fractionated. To help in the purification of such small amounts of material the appropriate marker compounds were added to the unsaponifiable lipids prior to their purification by TLC.

The purified ubiquinone-10(X-H_2), 5-demethoxyubiquinone-10(X-H_2) (from the mitochondrial fraction),

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ubiquinone-6 and 5-demethoxyubiquinone-6 were determined spectrophotometrically by measuring the fall in extinction at their wavelength of maximum UV absorption when an ethanolic solution of the quinone was treated with $NaBH_4$.[15] In all cases the $\epsilon_{ox}-\epsilon_{red}$ value for ubiquinone, 12,250, was used for the calculations.[27]

Radioassay. The procedures used have been described elsewhere.[21] All counts were corrected for background and instrument efficiency.

Acknowledgement—A.L. was in receipt of financial support from the British Council.

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Key Word Index—Saccharomyces carlsbergensis; *Aspergillus niger*; Fungi; ubiquinones; ubiquinone precursor; intracellular distribution.