BIOCHIMIE, 1974, 56, 1239-1247.

Biochemical study on ubiquinone biosynthesis in *Escherichia coli* :

I. Specificity of *para* hydroxybenzoate polyprenyltransferase.

Zakia EL HACHIMI, Odile SAMUEL and Robert AZERAD◇.
Institut de Biochimie, Université de Paris XI, 91 405 - Orsay, France.
(*1-7-1974*).

Summary. — Experimental conditions to determine polyprenyl pyrophosphate *p*-hydroxybenzoate transferase activity with [^{14}C]*p*-hydroxybenzoate or [^{3}H]polyprenyl pyrophosphate have been elaborated in order to test *E. coli* strains carrying a *ubi* A$^-$ mutation in the ubiquinone biosynthetic pathway. The nature of prenylation products formed by extracts of 38-1 strain in the presence of different polyprenyl pyrophosphates indicates that solubilized 3-polyprenyl 4-hydroxybenzoate decarboxylase is highly specific for its octaprenyl substrate while *p*-hydroxybenzoate polyprenyltransferase is not.

Ubiquinones are widely distributed in nature and form a family of 2-polyprenyl 3-methyl 5,6-dimethoxy 1,4-benzoquinones differing only by the polyprenyl side chain length [1]. This long hydrocarbon side chain gives the ubiquinones their lipophilic properties appropriate to their location within membranes where the quinonic ring functions in aerobic electron transport [1, 2]. In *Escherichia coli* the main ubiquinone has a side chain consisting of eight isoprene units, beside traces of lower isoprenologs [3].

Seven classes of ubiquinone mutants have been isolated and described by Gibson *et al.* These mutants, blocked on the ubiquinone biosynthetic pathway, have been characterized : i) by the accumulation of ubiquinone precursors (3-octaprenyl 4-hydroxybenzoic acid, 2-octaprenyl phenol, 2-octaprenyl 6-methoxy 1,4-benzoquinone, 2-octaprenyl 3-methyl 6-methoxy 1,4-benzoquinone, 2-octaprenyl 3-methyl 5-hydroxy 6-methoxy 1,4-benzoquinone [4, 5, 6] and recently 2-octaprenyl 6-methoxy phenol [7], ii) by direct biochemical identification of the missing enzymic reaction. This last method has been found particularly useful for identification of mutants (*ubi A*) blocked immediately after *p*-hydroxybenzoic acid, where no significant accumulation of this acid occurs ; in these mutants no activity of *p*-hydroxybenzoate octaprenyltransferase can be detected [7]. For this purpose, Gibson and coworkers [8] have developped a quantitative enzymic test using [^{14}C]*p*-hydroxybenzoic acid and an endogenous polyprenyl precursor as substrates. The latter could be accumulated only in strains carrying a supplementary mutation in the common pathway of aromatic biosynthesis, *i.e.* unable to achieve both menaquinone and ubiquinone biosynthesis. This limitation prohibited the use of this biochemical test for mutants carrying only one mutation in the ubiquinone biosynthetic pathway. A similar test has been used by Rudney and coworkers [9] either in animal cell-free fractions or in *Rhodospirillum rubrum* extracts, where the endogenous polyprenyl pyrophosphate was replaced by a synthesizing system from *Micrococcus lysodeikticus* supplemented with isopentenylpyrophosphate.

We describe here a method involving the condensation of an *exogenous* labelled (or unlabelled) polyprenyl pyrophosphate with labelled (or unlabelled) *p*-hydroxybenzoic acid, catalyzed by a cell-free bacterial extract. This method has been controlled using a 83-1 strain of *E. coli* grown in such conditions that the endogenous polyprenyl precursor is/or is not accumulated, and a 2-45 strain, a new mutant isolated in our laboratory, and proved by other methods to be a *p*-hydroxybenzoate octaprenyltransferase-less mutant.

In the second part of this paper we examine the nature of polyprenylation products formed by extracts of 83-1 strain in the presence, either of the endogenous side chain precursor, or of exogenous polyprenyl pyrophosphates. The results obtained suggest that the respective *in vitro* specificities of polyprenylating enzyme and polyprenylhydroxybenzoatedecarboxylating enzyme towards the side chain length are very different.

Abbreviations : PPHB : 3-polyprenyl 4-hydroxybenzoate ; DMK-8 : desmethylmenaquinone-8 ; MK-8 : menaquinone-8 ; Q-8 : ubiquinone-8.

◇ To whom all correspondance should be addressed.

MATERIALS AND METHODS.

Growth media, bacterial strains, cell-free extracts.

The 2-45 mutant was derived from *E. coli* K_{12} by N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis and isolated as described by Cox *et al.* [10]. It was grown on the minimal medium of Vogel and Bonner [11] supplemented with glucose 5 g/l. The 83-1 strain of *E. coli W* was grown in the same minimal medium supplemented with 0.1 mM phenylalanine, 0.05 mM tyrosine, 0.1 mM tryptophane, 1 μM 2,3-dihydroxybenzoic acid and 0.1 μM *p*-aminobenzoic acid. When a cell-free extract with no side chain precursor was needed, this medium was supplemented with 0.1 mM *p*-hydroxybenzoic acid and 0.1 mM *o*-succinylbenzoic acid.

Most cultures were grown in semi-anaerobic conditions, in 2 l flasks filled with 1 l volumes and stirred magnetically at 37°. Aerobic cultures were grown in the same volume conditions at 37° in a New Brunswick rotatory shaker.

Cells were harvested either in logarithmic phase or in stationary phase as indicated, by centrifugation at 20,000 x g at 0°C for 10 min, washed with a 0.05 M potassium phosphate buffer pH 7, then homogenized by sonication during 3 minutes at 0°C (1 g of wet cells per ml of buffer containing 0.1 mM dithiothreitol). The cell-free extract was obtained by centrifugation at 3,000 x g for 20 min.

Chemicals.

p-hydroxy-[2,3,4,5-^{14}C]-benzoic acid (1.35 μCi/μmole) was prepared from [2,3,4,5-^{14}C]-toluene (specific activity : 3.98 mCi/mmole, CEA Saclay) [12].

[1-^{3}H] solanesyl pyrophosphate (specific activity : 10 μCi/μmole), [1-^{3}H] phytyl pyrophosphate (specific activity : 1 μCi/μmole), and [1-^{3}H] farnesyl pyrophosphate (specific activity : 4 μCi/μmole) were prepared by the method of Cramer and Böhm [13] as described in [14].

2-Nonaprenyl phenol was obtained by condensation of solanesol with phenol in the presence of BF_3-etherate in dioxane [15].

Assay for p-hydroxybenzoate octaprenyltransferase.

The routine assay mixture contained : 0.5 ml of enzyme extract (26 mg proteins/ml), 10 μmoles of $MgCl_2$, 0.30 μmoles (0.4 μCi) of [^{14}C] *p*-hydroxybenzoate in potassium phosphate buffer 0.05 M, pH 7 ; final volume : 0.65 ml.

The routine assay mixture with exogenous [^{3}H] polyprenyl pyrophosphates was supplemented with 10 nmoles of each pyrophosphate ; final volume : 0.7 ml.

Each assay was done in duplicate at 37°C for 1 hour. The reaction was stopped by addition of acidified acetone (2.4 ml of acetone plus 0.1 ml of 5 N HCl) [8].

The tubes were centrifuged at 5,000 x g for 10 minutes. The pellets were washed twice with acidified acetone, the washings were combined and the acetone evaporated. Then, 1 ml of water was added and the solutions were extracted twice with hexane. The hexanic fractions were combined and dried. An aliquot was counted in a Packard Tri-Carb scintillation spectrometer and another aliquot was applied on TLC plate for a radiochromatographic analysis of the reaction products.

Chromatographic techniques.

Hexanic extracts were chromatographed on Silicagel F_{254} plates in benzene (solvent A) or in acetone-light petroleum (B.P. 40°C - 60°C), 3:7 (v/v) (solvent B) [16], and on paraffin-impregnated Whatman n° 1 paper in a paraffin-saturated acetone-water mixture, 9:1 (v/v) [17]. 5 mm (TLC) or 10 mm (paper chrotamogram) bands were counted in the usual toluene scintillation mixture.

Quinone extraction.

Whole cells were harvested and stirred in acetone, then centrifuged at 20,000 x g. The acetonic supernatant was evaporated to dryness and the residue extracted several times with hexane. Ubiquinone and menaquinone fractions were separated by TLC of the hexanic extract in a benzene-chloroform mixture, 1:1 (MK-8, Rf = 0.65 ; Q-8, Rf = 0.35), and each of them purified again by TLC in benzene.

Menaquinone and desmethylmenaquinone estimation.

The menaquinone fraction was resolved into menaquinone-8 and desmethylmenaquinone-8 by descending reverse phase paper chromatography during 16 h (MK-8, Rf = 0.1 ; DMK-8, Rf = 0.13). In most cases, the MK:DMK ratio was obtained without preliminary separation using a dual-wavelength spectrophotometer (Perkin Elmer model 356).

RESULTS.

Characterization of the 2-45 mutant.

This mutant has been shown to carry at least two mutations :

i) it is unable to carry out ubiquinone biosynthesis, though not accumulating any phenolic or

quinonic precursor of ubiquinone ; addition of *p*-hydroxybenzoic acid in the culture medium has no effect on ubiquinone production and it was verified that [^{14}C] *p*-hydroxybenzoic acid is not used to produce any labelled ubiquinone precursor. These features were taken as an indication of the absence of polyprenyl pyrophosphate-*p*-hydroxybenzoate polyprenyltransferase activity in this mutant.

ii) the menaquinonic fraction of this mutant was constituted exclusively of desmethylmenaquinone-8 (table I) despite the anaerobic growth conditions resulting from ubiquinone deficiency. It has been shown in various bacteria, that in such conditions only menaquinones are found ; desmethylmenaquinones are formed only in aerobic conditions [18]. Similar results (table I) have been obtained with the *E. coli* parent strain. No modification of the quinone distribution pattern was observed when the growth medium was supplemented with 1 mM L-methionine. This mutant was thus considered to be also defective for S-adenosylmethionine-desmethylmenaquinone methyltransferase activity.

Biosynthesis of ubiquinone-8 and menaquinone-8 in 83-1 strain :

Strain 83-1, blocked between dehydroquinic acid and dehydroshikimic acid [19], is unable to synthesize quinones when grown on a minimal medium supplemented as described in Materials and Methods. In these conditions, a stationary phase was obtained after 40 hours. When the medium was supplemented with 0.1 mM *p*-hydroxybenzoate, full growth was obtained after 20 hours and ubiquinone-8 was formed. When 0.1 mM *o*-succinylbenzoate, a menaquinone precursor [20], was added, menaquinone-8 was formed at the same rate as in the wild-type strain (table II). By using increasing amounts of *p*-hydroxybenzoate in the presence of 0.1 mM *o*-succinylbenzoate, it was possible to demonstrate a competitive effect between ubiquinone and (menaquinone + desmethylmenaquinone) production.

E. coli 83-1 cells grown without *p*-hydroxybenzoate did not yield ubiquinone but were supposed to accumulate the side chain precursor [21]. They were harvested in the late logarithmic phase and a cell extract was prepared and incubated with

TABLE I.

Formation of ubiquinone, menaquinone and desmethylmenaquinone in aerobic and anaerobic cultures of E. coli K_{12} *and 2-45 strain.*

Strain	Growth conditions	Quinones formed (µmoles/g dry weight)		
		Q 8	MK-8	DMK-8
E. coli K_{12}	aerobic	0.63	0 12	0.29
	semi-anaerobic	0.32	0.44	$\leqslant$ 0.01
E. coli 2–45	aerobic or semi-anaerobic	0.00	0.00	0.36–0.40

TABLE II.

Restoration of menaquinone and ubiquinone synthesis in E. coli *83-1 grown on* p-*hydroxybenzoate and on* o-*succinylbenzoate.*

Experiment number	*p*-hydroxybenzoate µM	*o*-succinylbenzoate µM	MK-8 + DMK-8 µmole/g dry weight	Q-8 µmole/g dry weight
1	0	0	$\leqslant$ 0.01	$\leqslant$ 0.01
2	0	100	0.65	$\leqslant$ 0.01
3	0	100	0.70	$\leqslant$ 0.01
4	0.01	100	0.58	0.33
5	1	100	0.39	0.36
6	10	100	0.35	0.40
7	100	100	0.23	0.42

[^{14}C]*p*-hydroxybenzoate according to the method of Young *et al.* [8]. A TLC plate in benzene of the hexane extract of the incubation mixture exhibited three radioactive peaks : a *p*-hydroxybenzoate trace which did not migrate ; a second band (Rf = 0.2) agreeing with the expected migration for a 3-polyprenyl 4-hydroxybenzoate [21] ; a third band (Rf = 0.6) corresponding to an authentic specimen of 2-nonaprenyl phenol (fig. 1A) ; total incorporation in both ubiquinone precursors was about 1.9 p. cent. A parallel experiment with boiled extract showed only the *p*-hydroxybenzoate peak. When the same extract was chromatographed on paraffin-impregnated paper (fig. 1B), two peaks were obtained, one (Rf = 0.4) migrating just ahead of the 2-nonaprenyl phenol specimen (Rf = 0.3) was identified to the 2-octaprenyl phenol ; the second one (Rf = 0.8) was a mixture of *p*-hydroxybenzoate (Rf = 0.75) and 3-octaprenyl 4-hydroxybenzoate. Table III shows the relative incorporation of [^{14}C]*p*-hydroxybenzoate in these precursors for incubation times ranging from 20 to 60 minutes.

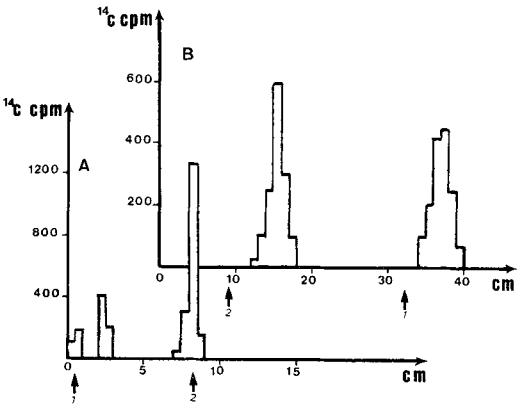

FIG. 1. — A) *TLC radiochromatogram of 30 min. incubation products of a 83-1* E. coli *extract with* [^{14}C] p-*hydroxybenzoate.*

B) *Paraffin-impregnated paper chromatogram of the same experiment.*

The arrows indicate the position of authentic specimens of : *1*, *p*-hydroxybenzoate ; 2, 2-nonaprenyl phenol.

These results corroborate those of Young *et al.* [8, 21] concerning the accumulation of a C_{40}-ubiquinone side chain precursor in mutants blocked in the common pathway of aromatic biosynthesis. In one of these mutants, Hamilton *et al.* [21] have described the formation of 3-octaprenyl 4-aminobenzoate, resulting from polyprenylation of *p*-aminobenzoate, in the absence of *p*-hydroxybenzoate, and shown that the polyprenyl pyrophosphate *p*-hydroxybenzoate polyprenyltransferase was able to use *p*-aminobenzoate as substrate instead of *p*-hydroxybenzoate. Thus the effect of *p*-aminobenzoate concentration in cultures of 83-1 was investigated, by incubating cell-free extracts of these cultures with [^{14}C]*p*-hydroxybenzoic acid, in order to detect any depletion of the endogenous polyprenyl precursor pool. It was found (fig. 2) that the maximal rate of [^{14}C]*p*-hydroxybenzoate prenylation was obtained with cells grown with 0.1 µM *p*-aminobenzoate ; this rate decreased rapidly for cells grown with higher *p*-aminobenzoate concentrations, indicating the

TABLE III.

Kinetics of ^{14}C incorporation into ubiquinone precursors during incubation of 83-1 extracts with [^{14}C] p-*hydroxybenzoate.*

	^{14}C incorporation in	
	PPHB cpm	2-octaprenyl phenol cpm
0	0	0
20 min	820	50
40 min	1200	100
60 min	2930	200

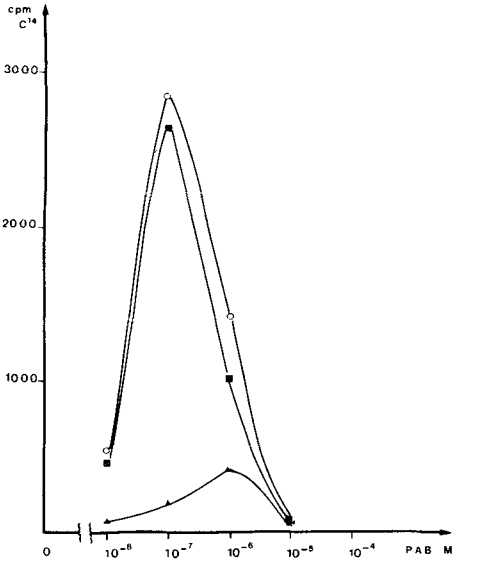

FIG. 2. — *Effect of* p-*aminobenzaote concentration during growth of* E. coli *83-1 on the* in vitro *prenylation of* p-*hydroxybenzoate.*

—O—O— Incorporation into 3-octaprenyl 4-hydroxybenzoate and 2-octaprenyl phenol.

—■—■— Incorporation into 3-octaprenyl 4-hydroxy benzoate.

—▲—▲— Incorporation into 2-octaprenyl phenol.

exhaustion of the endogenous polyprenyl precursor during the growth in the presence of high concentrations of *p*-aminobenzoate. It is to be noted that small changes in the *p*-aminobenzoate concentrations used for aromatic mutant cultures (for example, from 0.1 to 1 μM) may lead to a large difference in the amount of polyprenylated products formed subsequently from [^{14}C]*p*-hydroxybenzoate. For this reason, *p*-aminobenzoate concentration was always maintained to 0.1 μM in subsequent experiments. The loss of prenylating activity for lower *p*-aminobenzoate concentrations can be explained by a general metabolic disturbance arising from *p*-aminobenzoate limitation.

Biochemical test for p-*hydroxybenzoate octaprenyltransferase.*

Table IV shows that an extract of 83-1 strain, grown with *p*-hydroxybenzoate and *o*-succinylbenzoate, *i.e.* unable to accumulate the endogenous side chain precursor, as shown in experiment B, is able to synthesize 2-nonaprenyl 4-hydroxybenzoate when incubated with *p*-hydroxybenzoate plus solanesyl pyrophosphate, a higher homolog of the endogenous polyprenyl precursor (A). Incorporation of ^{14}C (from [^{14}C]*p*-hydroxybenzoate) is 40 p. cent of the incorporation obtained in 2-octaprenyl 4-hydroxybenzoate when an extract of cells grown without *p*-hydroxybenzoate and *o*-succinylbenzoate is incubated, under the same conditions, without the addition of solanesyl-pyrophosphate (C). When an extract of 2-45 strain was incubated under the same conditions (D and E), no incorporation was obtained either with or without solanesyl pyrophosphate, thus confirming the previously assumed enzymic defect localization. A complementation experiment (F) with a mixture of 83-1 and 2-45 extracts, in the absence of solanesyl pyrophosphate, shows a large incorporation in 2-octaprenyl 4-hydroxybenzoate, which indicates that 2-45 strain is able to a accumulate the endogenous chain precursor.

TABLE IV.

Synthesis of polyprenyl p-*hydroxybenzoate with 83-1 and 2-45 strain extracts.*

	Extract from	Additions	Incorporation in 2-polyprenyl 4-hydroxybenzoate	
			^{14}C-dpm	^{3}H-dpm
A	83-1	[^{14}C] *p*-hydroxybenzoate and [^{3} H] solanesyl-PP	1,840	1,870
B	83-1	[^{14}C] *p*-hydroxybenzoate	0	—
C	83-1	[^{14}C] *p*-hydroxybenzoate	4,100	—
D	2-45	[^{14}C] *p*-hydroxybenzoate and [^{3} H] solanesyl-PP	0	0
E	2-45	[^{14}C] *p*-hydroxybenzoate	0	—
F	2-45 + 83-1	[^{14}C] *p*-hydroxybenzoate	5,750 5,650	— —

0.5 ml of extract of 2-45 strain (29 mg proteins/ml) and/or 0.5 ml of extract of 83-1 strain (35 mg proteins/ml) grown with (A, B, F) or without (C) *p*-hydroxybenzoate and *o*-succinylbenzoate, were incubated as described in Materials and Methods with [^{14}C]*p*-hydroxybenzoate (0.30 μmoles ; 720,000 cpm) and [^{3}H]solanesyl pyrophosphate (10 nmoles ; 70,000 cpm) when indicated. Incubation : 30 min. at 37°C. The reaction was stopped with acidified acetone and 2-polyprenyl 4-hydroxybenzoates extracted and purified as described in the experimental part.

Prenylated products formed by 83-1 extracts :

When the prenylated products formed from the endogenous side chain precursor and [^{14}C]*p*-hydroxybenzoate by cell-free extracts of 83-1 strain, harvested in stationary phase, were compared to those previously described from cells harvested in logarithmic phase (*vide supra*), it turned out that the peak ratio of 3-octaprenyl 4-hydroxybenzoate and 2-octaprenyl phenol was generally inverted (fig. 3). However, when the amounts of prenylated products formed in the same conditions by a 3,000 g centrifuged cell-free extract and the centrifugation pellet of sonicated 83-1 strain, harvested in logarithmic phase, were compared (table V), only 50 p. cent of *p*-hydroxybenzoate polyprenyltransferase and 30 p. cent of PPHB decarboxylase activity are recovered in the supernatant, indicating a differential extraction of the two enzymes.

A comparison of activities in the cell-free extract and the homogenate at different stages of growth suggests that the previously found differential ratio of the two enzymic activities is only a reflect of a difference in enzyme extraction at different growth times. Moreover, a loss of PPHB decarboxylase activity occurred when logarithmic phase 83-1 cells were kept frozen a few days before cell-free extract preparation : in some cases, no more [^{14}C] octaprenyl phenol was formed.

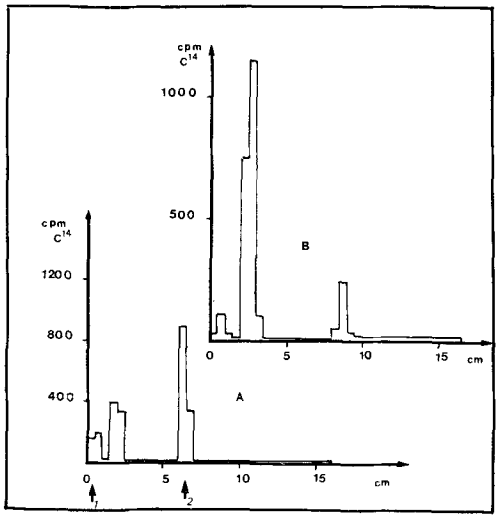

FIG. 3. — *Effect of growth stage on the enzymatic activity of the cell-free extract from* E. coli *83-1. TLC radiochromatogram (in benzene) of the hexane extract of the incubation mixture from cells harvested in :*

A : middle logarithmic phase.

B : stationary phase.

The arrows indicate the position of authentic specimens of : *1*, *p*-hydroxybenzoate ; *2*, 2-nonaprenyl phenol.

[^{14}C]*p*-hydroxybenzoate and various added [^{3}H] polyprenyl pyrophosphates [14], was investigated. Table VI shows that for equally high concentra-

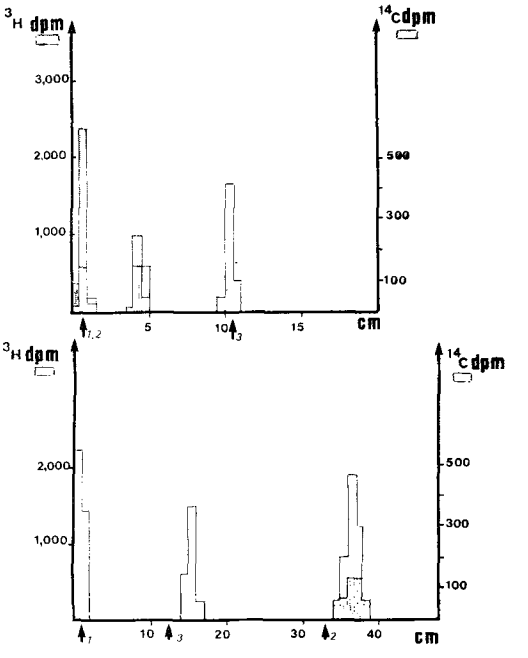

FIG. 4. — *Formation of PPHB and polyprenylphenol from* [^{3}H] *solanesyl pyrophosphate and* [^{14}C]p-*hydroxybenzoate by a 83-1 extract obtained from a logarithmic phase culture (grown without* p-*hydroxybenzoate).*

A : TLC radiochromatogram (in benzene) of the hexanic extract of the incubation mixture.

B : Paraffin-impregnated paper chromatogram.

The arrows indicate the position of authentic specimens of : *1*, solanesyl pyrophosphate ; *2*, *p*-hydroxybenzoate ; *3*, nonaprenyl phenol.

TABLE V.

Incorporation of radioactivity from [^{14}C]p-*hydroxybenzoate into PPHB and octaprenylphenol by a cell-free extract (3,000 g supernatant) and a 3,000 g pellet from 0.5 ml of sonicated 83-1 cells (grown without* p-*hydroxybenzoate).*

	Incorporation in			
	Proteins mg	PPHB cpm	Octaprenylphenol cpm	PPHB + Octaprenylphenol cpm
3.000 g pellet	7.5	560	720	1280
3.000 g supernatant	14.5	912	310	1220

The nature of prenylated products formed by extracts of 83-1 strain grown with *p*-hydroxybenzoate and *o*-succinylbenzoate, *i.e.* lacking the endogenous side chain precursor, incubated with tions of polyprenyl pyrophosphates, the farnesyl homolog is a better side chain donor than the phytyl and the solanesyl homologs. In each case, all the radioactivity was associated only with the

corresponding polyprenyl *p*-hydroxybenzoate ; no incorporation could be detected in any polyprenyl phenolic compound, even when the best conditions for such an incorporation were realized (logarithmic phase cells, fresh extracts, *vide supra*).

When increasing amounts of phytyl pyrophosphate were incubated with [^{14}C]*p*-hydroxybenzoate, no saturation curve could be obtained : concentrations of pyrophosphate ester higher than 12 μM produced a strong inhibition of the prenylation reaction.

Competition between the octaprenyl precursor and synthetic solanesyl pyrophosphate.

In order to obtain more information about the utilization of the endogenous octaprenyl precursor or of an exogenous polyprenyl pyrophosphate, an extract of 83-1 strain, grown without *p*-hydroxybenzoate and harvested in logarithmic phase, was incubated with [^{14}C]*p*-hydroxybenzoate and [1-^{3}H]solanesyl pyrophosphate. Analysis of prenylated products by TLC and reverse phase chromatography showed that the octaprenyl phenol peak was only ^{14}C-labelled, while the 3-polyprenyl 4-hydroxybenzoate peak contained ^{3}H and ^{14}C-labelling (fig. 4). Identical results were obtained in competition experiments with [1-^{3}H] phytyl or [1-^{3}H]farnesyl pyrophosphate as exogenous precursor. These results confirm the previously found incorporation of C_{15}, C_{20}, and C_{45} pyrophosphate esters into the corresponding PPHB *but not* in polyprenyl phenols even under conditions where large amounts of octaprenyl phenol are formed from the endogenous precursor.

In a competitive experiment, increasing concentrations of [^{3}H] solanesyl pyrophosphate were incubated with a cell-free extract of stationary phase cells of 83-1 strain, grown without *p*-hydroxybenzoate, *i.e.* accumulating octaprenyl pyrophosphate but unable to convert 3-octaprenyl 4-hydroxybenzoate to octaprenyl phenol. Different ^{3}H/^{14}C ratios were obtained in the mixture of 3-nonaprenyl and 3-octaprenyl 4-hydroxyben-

TABLE VI.

Incorporation from various [^{3}H]polyprenyl pyrophosphates into PPHB with 83-1 extracts.

	Polyprenyl pyrophosphate added			PPHB formed	
		dpm	nmoles	dpm	nmoles
1	[^{3}H] farnesyl	132,000	15	11,400	1.3
2	[^{3}H] phytyl	36,000	15	1,670	0.71
3	[^{3}H] solanesyl	350,000	15	1,500	0.064
4	[^{3}H] phytyl	12,000	5	338	0.14
		24,000	10	1,470	0.61
		36,000	15	735	0.31
		48,000	20	645	0.27
		60,000	25	510	0.21
		120,000	50	400	0.17
		240,000	100	265	0.11

1, 2, 3 : 1 ml of enzyme extract from cells grown with *p*-hydroxybenzoate and *o*-succinylbenzoate (30 mg proteins/ml) was incubated at 37°C for 50 min. with $MgCl_2$ (10 μmoles), [^{14}C]*p*-hydroxybenzoate (150 nmoles) and [^{3}H]polyprenyl pyrophosphate in a final volume of 1.2 ml.

4 : 0.5 ml of the same enzyme extract ; $MgCl_2$, 10 μmoles ; final volume : 0.8 ml.

3-polyprenyl 4-hydroxybenzoates were extracted as described in experimental part and recovered after TLC with solvent B [16] : 3-farnesyl 4-hydroxybenzoate, Rf = 0.4 ; 3-phytyl 4-hydroxybenzoate, Rf = 0.4 ; 3-solanesyl 4-hydroxybenzoate, Rf = 0.5 ; 2-polyprenyl phenols, Rf = 0.9.

Amounts of PPHB formed were determined from the specific activities of [^{3}H]precursors. The same calculation from [^{14}C]*p*-hydroxybenzoate gave similar results, but lower amounts of PPHB were found, probably resulting from a dilution of [^{14}C]*p*-hydroxybenzoate by unlabelled *p*-hydroxybenzoate carried along in the cell free extract.

zoates ; using the specific activity of [^{14}C]*p*-hydroxybenzoate as a measurement of the amount of PPHB formed and the specific activity of [^{3}H]solanesyl pyrophosphate for the measurement of the amount of 3-nonaprenyl 4-hydroxybenzoate, it was possible to compare the respective amounts of prenyl derivatives formed competitively from each precursor in the same experiment. Table VII shows that the endogenous octaprenyl precursor is preferentially incorporated even in the presence of high concentrations of exogenous solanesyl pyrophosphate (up to 77 μM). However a significant decrease of 3-octaprenyl 4-hydroxybenzoate and of total PPHB formation occurs in the presence of concentrations of solanesyl pyrophosphate up to 20 μM ; higher concentrations do not result in further inhibition and about 30 p. cent of the activity remain unchanged.

DISCUSSION.

It has been shown that the presence of *p*-hydroxybenzoate octaprenyl-transferase activity is easily detected in *E. coli* by the formation of labelled 3-nonaprenyl 4-hydroxybenzoate in an incubation mixture containing a cell-free extract to which is added labelled/or unlabelled *p*-hydroxybenzoate and unlabelled/ or labelled solanesyl-pyrophosphate as an exogenous side chain donor. This activity has been found in *E. coli* 83-1 supplemented with *p*-hydroxybenzoate, *i.e.* unable to accumulate the endogenous side chain precursor ; no activity was found in *E. coli* 2-45, a ubiquinone-less mutant unable to use *p*-hydroxybenzoate. A very efficient reconstituted biosynthetic system could be obtained by mixing a cell-free extract of 83-1 and 2-45 strains, indicating that accumulated octaprenyl pyrophosphate from 2-45 strain was a good precursor for the octaprenyltransferase system of 83-1. This result is very different from that obtained by Young *et al.* [8] where very low complementation effects were observed, even with menaquinone-less strains which accumulate high amounts of octaprenyl pyrophosphate. It is to be noted that 2-45 strain, though unable to produce ubiquinone, does not overproduce desmethylmenaquinone and can probably accumulate octaprenyl pyrophosphate to a similar extent as menaquinone-less mutants described by Young *et al.* The results described above allow to conclude that the 2-45 strain is a *p*-hydroxybenzoate octaprenyltransferase-less mutant.

A careful study of the accumulated intermediates formed from the endogenous side chain precursor and labelled *p*-hydroxybenzoate by the cell-free extract showed that the harvest time was critical for a full recovery of octaprenyl phenol synthesis. Octaprenyl phenol formation was higher in young cells and nearly undetectable during the stationary phase. On the contrary, nearly constant amounts of PPHB were recovered all along the growth period. In fact these results reflect probably a difference in extractibility of the two enzymes from cells at different growth stages : *p*-hydroxybenzoate polyprenyltransferase seems to be more easily solubilized than the decarboxylase activity especially in stationary phase cells. Moreover the latter activity is lost when harvested cells are kept frozen.

When exogenous [^{3}H] polyprenyl pyrophosphates were used as side-chain donors in the absence of endogenous octaprenyl pyrophosphate (strain 83-1 grown with *p*-hydroxybenzoate), ^{3}H-label was found only in PPHB, no detectable labelling being associated with polyprenyl phenol. Short chain pyrophosphates (phytyl-, farnesyl) were far more effective as polyprenyl donors than solanesyl-pyrophosphate. In a competitive experi-

TABLE VII.

Competitive utilization of endogenous octaprenyl pyrophosphate and exogenous [^{3}H]solanesyl pyrophosphate in cell-free extracts of 83-1 strain.

[^{3}H] solanesyl pyrophosphate		Radioactivity in PPHB formed			Total PPHB nmoles	3-nonaprenyl 4-hydroxy-benzoate nmoles	3-octaprenyl 4-hydroxy-benzoate nmoles
dpm	nmoles	^{14}C dpm	^{3}H dpm	^{3}H/^{14}C			
0	0	696	—	—	0.26	—	0.26
116,000	5	432	530	1.22	0.15	0.023	0.12
290,000	12.5	300	750	2.5	0.10	0.032	0.07
580,000	25	500	1320	2.6	0.17	0.056	0.11
1160,000	50	426	1240	2.8	0.14	0.053	0.09

0.5 ml of enzyme extract (36 mg proteins/ml), from cells grown without *p*-hydroxybenzoate, are incubated with 10 μmoles of $MgCl_2$ and 0.15 μmole (0.2 μCi) of [^{14}C]*p*-hydroxybenzoate ; final volume : 0.65 ml.

ment it was possible to compare the relative donor efficiency of exogenous solanesyl pyrophosphate and of endogenous octaprenyl pyrophosphate. The results indicate that the octaprenyl homolog was preferentially used ; some competitive inhibition appears to occur with high amounts of solanesyl pyrophosphate (table VII). These results can be put together with the inhibition similarly occurring when high concentrations of an exogenous polyprenyl pyrophosphate is used in the absence of endogenous precursor (table VI). A similar short chain polyprenyl pyrophosphate utilization has been described previously in a yeast system using an endogenously synthesized polyprenyl pyrophosphate mixture [16]. The polyprenyltransferases of *R. rubrum* or liver mitochondria have been essentially tested with a mixture of long chain polyprenyl pyrophosphates, obtained from the *M. lysodeikticus* or an endogenous polymerizing enzyme [9, 22]. In our system, the use of pure synthetic polyprenyl pyrophosphates demonstrates clearly the non-specificity of the alkylating enzyme with respect to the polyprenyl side chain length ; the higher activity noted here with shorter isoprenologs strikingly recalls that observed for desmethylmenaquinone-methylase, another membrane-bound enzyme from *M. phlei* [17].

Interpretation of similar results have led Rudney and coworkers to attribute the specificity of the chain length of bacterial or mammalian ubiquinones to the availability of one (or two) long chain polyprenyl pyrophosphate(s) endogenously synthesized [22]. However, our results show that, when various exogenous [^{3}H] polyprenyl pyrophosphates, differing from the C_{40} homolog, are introduced into the system, in the absence of the endogenous precursor or in competition with it, all the label coming from the synthetic pyrophosphate is localized in PPHB, while only the endogenous octaprenyl pyrophosphate allows the formation of polyprenyl phenol (fig. 4). These results signify that solubilized PPHB decarboxylase is highly specific for 3-octaprenyl 4-hydroxybenzoate, whereas polyprenyltransferase is not. Thus, the origin of the specificity of side chain length in ubiquinones is not only dependent on the role of the polyprenyl pyrophosphate synthesis system, but probably involves also a contribution of the strict specificity of the decarboxylating enzyme. This fact brings up new questions about the formation of traces of shorter ubiquinone homologs previously described by Daves *et al.* in *E. coli* [3].

Acknowledgments.

This work was supported by grants from the Fondation pour la Recherche Médicale Française, Paris, and from the Commissariat à l'Energie Atomique, Saclay.

RÉSUMÉ.

Des conditions expérimentales permettant la mesure de l'activité polyprénylpyrophosphate *p*-hydroxybenzoate transférase à partir de [^{14}C]*p*-hydroxybenzoate ou de [^{3}H]-polyprénylpyrophosphate ont été mises au point afin de tester la mutation *ubi* A$^-$ dans des couches de *E. coli* incapables de synthétiser l'ubiquinone. La nature des produits de prénylation formés par les extraits de la souche 83-1 en présence de différents polyprényl pyrophosphates, montre que la 3-polyprényl 4-hydroxybenzoate décarboxylase est fortement spécifique pour son substrat octaprénylique tandis que la *p*-hydroxybenzoate polyprényltransférase ne l'est pas.

REFERENCES.

1. Crane, F. L. (1965) in *Biochemistry of quinones* R. A. Morton Ed.), pp. 186-206, Academic Press, New York.
2. Bishop, D. L. H. & King, H. K. (1962) *Biochem. J.*, **85**, 550-554.
3. Daves, G. D. Jun., Muraca, R. F., Whittick, J. S., Friis, P. & Folkers, K. (1967) *Biochemistry*, **6**, 2861-2866.
4. Cox, G. B., Young, I. G., Mc Cann, L. M. & Gibson, F. (1969) *J. Bacteriol.*, **99**, 450-458.
5. Stroobant, P., Young, I. G. & Gibson, F. (1972) *J. Bacteriol.*, **109**, 134-139.
6. Young, I. G., Mc Cann, L. M., Stroobant, P. & Gibson, F. (1971) *J. Bacteriol.*, **105**, 769-778.
7. Young, I. G., Stroobant, P., Mac Donald, C. G. & Gibson, F. (1973) *J. Bacteriol.*, **114**, 42-52.
8. Young, I. G., Leppik, R. A., Hamilton, J. A. & Gibson, F. (1972) *J. Bacteriol.*, **110**, 18-25.
9. Rudney, H. (1970) in *Natural substances formed biologically from mevalonic acid*, (T. W. Goodwin Ed.), pp. 89-103, Academic Press, New York.
10. Cox, G. B., Gibson, F. & Pittard, J. (1968) *J. Bacteriol.*, **95**, 1591-1598.
11. Vogel, H. J. & Bonner, D. M. (1956) *Microb. Genet. Bull.*, **13**, 43.
12. Dansette, P. & Azerad, R. (1968) *Bull. Soc. Chim.*, **6**, 2492-2493.
13. Cramer, F. & Böhm, W. (1959) *Angew. Chem.*, **71**, 775.
14. Samuel, O., El Hachimi, Z. & Azerad, R., *Biochimie*, **56**, 1279-1281.
15. Daves, G. D. Jr., Moore, H. W., Schwab, D. E., Olsen, R. K., Wilczynski, J. J. & Folkers, K. (1967) *J. Org. Chem.*, **32**, 1414-1417.
16. Thomas, G. & Threlfall, D. R. (1973) *Biochem. J.*, **134**, 811-814.
17. Samuel, O. & Azerad, R. (1972) *Biochimie*, **54**, 305-317.
18. Whistance, G. R. & Threlfall, D. R. (1968) *Biochem. J.*, **108**, 505-507.
19. Weiss, U., Davis, B. D. & Mingioli, E. (1953) *J. Am. Chem. Soc.*, **75**, 5572-5576.
20. Dansette, P. & Azerad, R. (1970) *Biochem. Biophys. Res. Comm.*, **40**, 1090-1095.
21. Hamilton, J. A. & Cox, G. B. (1971) *Biochem. J.*, **123**, 435-443.
22. Momose. K. & Rudney, H. (1972) *J. Biol. Chem.*, **247**, 3930-3940.