THE INCORPORATION OF p-HYDROXYBENZOIC ACID AND ISOPENTENYL PYROPHOSPHATE INTO UBIQUINONE PRECURSORS BY CELL-FREE PREPARATIONS OF RAT TISSUES

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SUMMARY. Cell-free preparations of rat kidney, liver and brain supplemented with an extract of Micrococcus lysodeikticus and IPP incorporate p-hydroxybenzoic acid-U-^{14}C into a single lipid component which is shown to be identical with 3-polyprenyl-4-hydroxybenzoic acid. MLE and IPP are essential. These preparations lack the decarboxylase which converts PPHB to PPP. The PHB-polyprenyltransferase activity appears to be located in the membranous components of the mitochondria and is susceptible to inhibition by sulfhydryl reagents, diphenylamine and other agents.

It has been established that in the rat PHB can serve as a precursor of ubiquinone, both in vivo and in tissue slices or minces (1). No reports have appeared however of PHB labeling of UQ, or any of its immediate precursors in mammalian cell-free systems.

A procedure has been described (2) for the preparation of bacterial cell-free systems utilizing the polyprenyl pyrophosphates formed by M. lysodeikticus as the side-chain source.

We report here the application of the same technique to tissue homogenates and crude fractions thereof obtained from rats. We have found that a single metabolite, identified as 3-polyprenyl-4-hydroxybenzoate was formed but no 2-polyprenylphenol could be detected. The effect of several factors on the alkylation of PHB are also discussed.

Materials and Methods - Twenty-four Sprague Dawley rats (250-300 g) were sacrificed and the kidneys, brain and liver quickly removed and chilled in ice.

Abbreviations: PHB, p-hydroxybenzoate; MLE, Micrococcus lysodeikticus extract, PPP, 2-polyprenylphenol; PPHB, 3-polyprenyl-4-hydroxybenzoate; IPP, isopentenyl pyrophosphate; UQ, ubiquinone.

Tissue homogenates were prepared by first mincing the tissue as previously described (2). The mince, suspended in an equal volume of buffer, was homogenized by 12 passes of a Teflon pestle (Potter Elvehjem grinder). The homogenate was diluted with a half volume of buffer and this preparation, after centrifugation at 100 x g for 10 minutes to remove nuclei and cell debris, was used in the initial experiments. A crude centrifugal fractionation obtained by consecutive spins at 7500 x g for 10 minutes and 105,000 x g for 1 hour provided a "mitochondrial" pellet and "microsomal" and soluble fractions.

The MLE, IPP and PHB-U^{14}C were prepared as described (2). IPP-1-^{14}C, tri-lithium salt, was the very generous gift of Dr. C. Donninger, Milstead Laboratory of Chemical Enzymology, Sittingbourne, England.

After incubation the reactions were terminated by addition of non-radioactive PHB (10 mg) followed by heating at 90° C for 10 minutes. The precipitate which formed was washed twice with cold water and then extracted with either acetone (5 x 20 ml) or with hot ethanol (2 x 20 ml) followed by ether:ethanol 3:1 (3 x 20 ml). The extracts were evaporated on a steam bath under a flow of nitrogen and the residue was dissolved in petroleum ether and subjected to t.l.c. on "Chromar 1000" silica gel sheets manufactured by Mallinckrodt Chemical Works, St. Louis. Detection and counting of radioactive compounds was as previously described (2).

Results and Discussion - In contrast to bacterial systems no instances are available of the conversion of PHB to UQ in cell-free mammalian tissues. Recently Nowicki et al. (4) have presented evidence for the presence of 6-methoxy-2-nonaprenylphenol in rat liver slices. Green et al. (5) published a preliminary report using a rat liver homogenate but the full experimental details have not yet appeared.

Bacterial cell-free preparations of R. rubrum (2) and E. coli (3) supplemented with MLE and IPP readily provided 3-polyprenyl-4-hydroxybenzoic acid and 2-polyprenylphenol. Application of the same conditions to minces and homogenates from rat organs resulted in the incorporation of PHB into

Table I. Effect of MLE and IPP on Incorporation of PHB into Compound "R" by Extracts of Rat Tissues

Organ	dpm Incorporated into "R"	dpm/mg Tissue
Kidney		
Mince	0	0
Mince + MLE + IPP	40,000	50
Homogenate	0	0
Homogenate + MLE + IPP	40,000	50
Liver		
Mince	0	0
Mince + MLE + IPP	18,000	30
Homogenate	0	0
Homogenate + MLE + IPP	11,000	18
Brain		
Mince	0	0
Mince + MLE + IPP	18,000	23
Homogenate	0	0
Homogenate + MLE + IPP	20,000	25

Each flask contained rat tissue (0.8 g), PHB-U-^{14}C (20 μgrams 284,000 dpm at 9.14 μc/μmole), 100 mg MLE, 1.7 μmoles IPP, and 200 μmoles of $MgCl_2$ in a total volume of 1.0 ml were preincubated for 2 hours at 20° before addition to the tissues. Total volume (5.0 ml) in Krebs Ringer phosphate buffer without calcium chloride. Incubations carried out in air at 37° for 3 hours.

radioactive lipids. These results are shown in Table I. It can be seen that MLE and IPP are essential for activity.

T.l.c. of the radioactive lipids showed that all the activity was in a single peak and the R_f of this material designated as "R" (0.18-0.20 in 10% acetone 90% petroleum ether) was the same regardless of the tissue studied. No radioactive UQ was detected in any of the incubations. The specific activity of the kidney preparations was higher than the liver and brain, and it was decided to concentrate on the kidney homogenate preparations.

The buffer used in Table I was the Krebs-Ringer phosphate buffer, minus calcium chloride. In earlier work with kidney mince preparations (6) the Krebs-Ringer bicarbonate buffer was used and so the experiment was re-run using these conditions to see if any ubiquinone was produced by the homogenate. No UQ was formed and the incorporation of PHB into "R" was reduced. This also was the case when calcium chloride was included in the phosphate buffer.

We repeated the experiment of Green et al. (5) with a liver homogenate. Radioactive peaks labeled by mevalonic acid were obtained, but careful chromatography showed that the activity in UQ was negligible. Use of PHB-U-^{14}C and mevalonic acid did not produce any ^{14}C labeled UQ. In our hands therefore the Green et al. preparation does not produce labeled UQ.

The presence of only one radioactive compound from the incubations was of interest as both of the bacterial systems studied previously had given two compounds (2)(3).

Compound "R" and a sample of ^{14}C labeled 3-polyprenyl-4-hydroxybenzoate isolated from an incubation of an R. rubrum sonicate were co-chromatographed on t.l.c. in 4 solvent systems. In each case a single radioactive peak was obtained suggesting that the two compounds were the same. However, this provided no indication of similarity of side-chain length.

In R. rubrum UQ-50 is the major UQ component, while in the rat UQ-45 predominates. Thus if the spectrum of side-chain lengths is determined in the alkylation step the relative amounts of the "R" prenylogs should parallel those of UQ in the rat. Further information on the nature of "R" came from labeling studies with specifically labeled precursors (Table II). When PHB-7-^{14}C was used as ring precursor "R" was labeled showing the presence of the carboxyl carbon. S-adenosyl methionine-Me-^{14}C did not label "R", suggest-

Table II. The Incorporation of Labeled Precursors into Compound "R"

	Radioactivity in "R" dpm	% Incorporation
1. PHB-U-^{14}C 284,000 dpm, 9.14 μc/ mole	9,800	3.4
2. PHB-7-^{14}C 355,400 dpm, 6.85 μc/ mole	13,700	3.9
3. IPP-1-^{14}C 663,000 dpm, 0.069 μc/ mole	3,100	4.7
4. S-adenosyl-methionine-Me-^{14}C 444,100 dpm, 55 μc/ mole	0	0

Conditions were as described in Table I. Kidney homogenate was used in all experiments; in Experiments 3 and 4, 3 μg of unlabeled PHB was added.

ing that no methyl group is present. When IPP-^{14}C was pre-incubated with MLE and then added to a kidney homogenate along with the PHB compound "R" was labeled showing unequivocally the presence of a terpenyl side-chain.

A radioactive sample of "R" was purified by t.l.c. and incubated with a sonicate preparation of R. rubrum. After incubation part of the initial activity was isolated as a second compound which proved to be chromatographically indistinguishable from a sample of PPP produced in a parallel incubation from PHB-U-^{14}C and MLE and R. rubrum. The percentage conversion of R to PPP was 10% but this rather low value is not unexpected if the decarboxylating enzyme has any specificity with respect to side-chain length which is C-50 for R. rubrum and C-45 for the rat.

All the evidence accumulated points to "R" being a 3-polyprenyl-4-hydroxybenzoic acid. It would seem therefore that in the rat the initial step from PHB is the same as in E. coli and R. rubrum. The similarity with these bacterial systems is increased by the isolation from rat tissue of 2-nonaprenyl-6-methoxyphenol (4).

Table III. Incorporation of PHB into PPHB by Kidney Homogenate Fractions

Homogenate Fraction	dpm into PPHB
Whole homogenate	30,400
100 g ——> 7,500 g pellet (mit)	42,900
7,500 g ——> 105,000 g pellet (mic)	9,700
Soluble fraction (sol)	0
Mit + sol	38,700
Mic + sol	9,700
Mic + mit	41,500

Each incubation contains rat tissue from 0.67 g kidney PHB-U-^{14}C 284,000 dpm at 9.14 μc/ mole and MLE and IPP as described in Table I. Centrifugation was 100 x g 10 mins, 7,500 x g 10 mins and 105,000 x g for 1 hour. Incubations were in a total volume of 5 ml of Krebs Ringer phosphate buffer without calcium chloride at 37° C for 3 hours in air.

The kidney homogenate was fractionated by centrifugation. The data obtained (Table III) show that the major alkylating activity resides in the crude "mitochondrial" fraction pelleting between 100 and 7,500 x g. In subsequent experiments this fraction, rather than the complete homogenate, was used. Sonication of the 100 to 7,500 x g fraction followed by centrifugation at 48,000 x g resulted in over 75% of the activity being recovered in the pellet, indicating that the activity is bound to membrane fragments. Attempts to solubilize the enzyme have so far been unsuccessful.

Diphenylamine (1 mM) completely inhibits the alkylation of PHB in a cell-free system of R. rubrum (3). The effect of this and other inhibitors has been tested on the sonicated rat "mitochondrial" fraction (Table IV). Diphenylamine is not as effective as in the bacterial system, but PCMB completely inhibited the reaction. Phosphate also acted as an inhibitor, when added to the tricine buffer used.

The isolation of 3-polyprenyl-4-hydroxybenzoate described here and the

Table IV. Effect of Inhibitors on the Alkylation of PHB

Compound (1 x 10^{-3} M)	dpm in PPHB	% Inhibition
0	14,300	0
Diphenylamine	6,000	58
Iodoacetamide	8,100	43
p-Chloromercuribenzoate	0	100
Mercaptoethanol	6,100	58
Potassium cyanide	7,800	45
Sodium fluoride	6,900	51
Potassium phosphate	5,200	64

Rat kidney mitochondrial fraction suspended in 23 ml. of 0.1 M Tricine buffer, pH 7.4, sonicated for 2 mins (Branson Model W185C, #8 power output) in a rosette cell cooled in ice and 2.0 ml containing 45 mg protein was used. Each incubation contained PHB-U-^{14}C specific activity 8.68 μc/ mole, 283,000 dpm and MLE and IPP as outlined in Table I. Final volume was brought to 5.0 ml with the same buffer.

presence of 2-nonaprenyl-6-methoxyphenol in liver (4) suggests that the pathway for UQ synthesis in the rat is similar to that occurring in R. rubrum, E. coli and other bacterial systems (2,3,7). The complete inhibition of the decarboxylase system remains to be explained. Our kidney preparations are in this sense similar to the recently discovered mutant of E. coli (8) and to E. coli cell-free preparations treated with O_2 (9).

In supplemented mammalian cell-free preparations one of the limiting factors is the supply of polyprenylpyrophosphate. This work provides the first example of a polyprenyl transferase with an aromatic compound as an acceptor. We propose to call this enzyme, PHB:polyprenylpyrophosphate-polyprenyl transferase or PHB:polyprenyl transferase as a trivial name. If the transferase is truly located in mitochondria then there must be a system for transporting a polyprenylpyrophosphate presumably synthesized in the microsomal fraction of the mitochondria. Work designed to define more precisely the location of the transferase is in progress. The localization of subsequent steps of decarboxylation, hydroxylation, and methylation remains to be determined.

The question concerning the specificity of the transferase is an interesting one. It has been suggested by Stoffel and Martius (10) on the basis of cell tissue culture studies that the quinone nucleus is initially formed and addition of the side-chain occurs at a later stage in the pathway. The question now arises as to the specificity of the transferase with regard to the aromatic nucleus. It may be that the enzyme has a broad specificity and can alkylate both p-hydroxybenzoate and UQ-0, and possibly other ring systems. Another point of extreme interest is that the PHB-polyprenyl transferase appears in brain. These questions are being investigated further.

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