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Isolation and Identification of 6-Methoxy-2-nonaprenylphenol as an Intermediate in the Biosynthesis of Ubiquinone-9 in the Rat*

Henry G. Nowicki,† G. Hossein Dialameh, and Robert E. Olson‡

ABSTRACT: 6-Methoxy-2-nonaprenylphenol (6-MNPP) has been identified as an intermediate in the biosynthesis of ubiquinone-9 in rat liver. This metabolite has been purified from the neutral lipids of rat liver and analyzed by spectrometry. Its mass spectrum, ultraviolet absorption spectrum, and chromatographic properties correspond to those of an authentic synthetic specimen of 6-MNPP. It is enriched with radioactivity from benzoate-*U*-^{14}C, *p*-HBA-*G*-^{3}t, methionine-*methyl*-^{14}C, and mevalonate-*2*-^{14}C in liver slices engaged in the biosynthesis of ubiquinone-9 from these precursors. Synthetic 6-MNPP labeled in its methoxyl with tritium and administered intravenously was efficiently converted to hepatic ubiquinone-9 by intact rats.

In the rat, the aromatic ring of ubiquinone is derived from phenylalanine *via* tyrosine by a pathway that involves the transamination and dehydration of tyrosine to yield *p*-hydroxycinnamic acid, which then undergoes β-oxidation to form *p*-hydroxybenzoic acid[1] (Olson, 1966). In plants and microorganisms which synthesize the phenylamino acids, a branch point exists in the shikimate pathway which yields *p*-hydroxybenzoic acid (Rudney and Raman, 1966; Cox and Gibson, 1964; Whistance and Threlfall, 1967). *p*-HBA is thus a focal metabolite in ubiquinone biosynthesis in all organisms in which it has been tested. Argument now exists regarding the universality of the pathway from *p*-HBA to ubiquinone. In 1966, Friis *et al.* (1966) proposed a pathway for *Rhodospirillum rubrum* based upon the isolation of a number of prenylated phenols and quinones from lipid extracts of this organism although only two of the steps have been validated by biosynthetic studies for that organism (Raman *et al.*, 1969). Nonetheless, this scheme (Scheme I) (Nilsson *et al.*, 1968) has served as a useful working hypothesis for the investigation of the pathway from *p*-HBA to ubiquinone in a variety of organisms. Whistance and his collaborators (Whistance *et al.*, 1969–1971) have surveyed a large number of organisms for evidence of the presence of these postulated intermediates by chromatographic and radioisotopic techniques and have drawn tentative conclusions about the validity of the Folkers scheme for given organisms on the basis of the presence or absence of intermediates in the pathway. In some organisms capable of overall ubiquinone biosynthesis, most of the postulated intermediates could be detected although in others none was found. In *Pseudomonas ovalis*, they identified 2-polyprenyl-1,4-benzoquinone as an intermediate which appears "out of order" in the Folkers scheme. It is obvious that much further work must be done in order to exhaustively test this postulated sequence of reactions, including the ultimate identification of the enzymes involved.

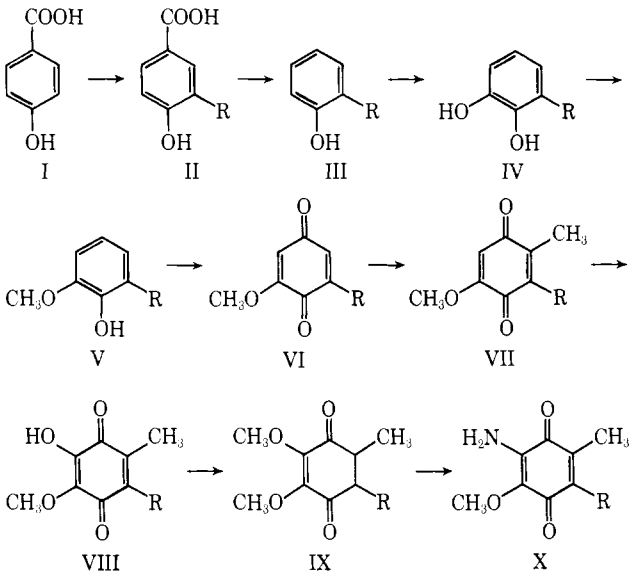
* From the Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri 63104. *Received September 27, 1971.* This investigation was supported in part by Research Grant-in-Aid AM 10004 and Training Grant GM-446 (H. G. N.) from the National Institutes of Health. U. S. Public Health Service, Bethesda, Md.

† Most of this report was taken from a dissertation submitted by H. G. N. to St. Louis University in partial fulfillment of the degree of Doctor of Philosophy (1971). Present address: Department of Biochemistry, University of California, Riverside, Calif.

‡ To whom to address correspondence.

[1] Abbreviations used are: tlc, thin-layer chromatography; Q-9, ubiquinone-9; 5-DMQ-9, 5-demethoxyubiquinone-9; MVA, mevalonate; *p*-HBA, *p*-hydroxybenzoate; 2-NPP, 2-nonaprenylphenol; 6-MNPP, 6-methoxy-2-nonaprenylphenol; the *Chemical Abstracts* form of indicating isotopic labeling (^{14}C, *t*) is used.

SCHEME I



None of these intermediates has previously been unequivocally identified as functional in higher animals.

In 1966, we (Olson and Aiyar, 1966) reported that an unknown nonsaponifiable lipid derived from benzoate-*1*-^{14}C or *p*-hydroxybenzoate-*U*-^{14}C accumulated in rat liver slices during the early stages of the biosynthesis of ubiquinone-9 and seemed, on a temporal basis, to bear a precursor–product relationship to ubiquinone-9. It was shown not to be 2-nonaprenylphenol analogous to the 2-decaprenylphenol which accumulates in *R. rubrum* during the biosynthesis of ubiquinone-10 (Olsen *et al.*, 1965). This metabolite has recently been identified as 5-demethoxyubiquinone-9 (Nowicki *et al.*, 1969; Trumpower *et al.*, 1972) (metabolite VII in Scheme I) and has been shown to arise from *p*-HBA in liver slices and to be converted to ubiquinone in the isolated perfused rat liver. In this paper, we wish to report the identification of another intermediate, 6-methoxy-2-nonaprenylphenol (metabolite V in Scheme I), in rat liver slices undergoing biosynthesis of ubiquinone-9 from various distal precursors, and to present evidence that it is a *bona fide* metabolic intermediate in this process. Preliminary reports of this work have been presented (Nowicki *et al.*, 1969).

Materials and Methods

Animals. Male albino rats of the Sprague-Dawley strain (130–180 g in weight), obtained from Charles River Breeding Laboratories, Brookline, Mass., and female rats obtained from the St. Louis University Colony (Wistar-derived strain of the same size), were used in these studies. They were fed a stock commercial ration (Purina Laboratory Chow, Ralston Purina Co., St. Louis, Mo.) *ad libitum*.

Chemicals. Inorganic reagents were of analytical grade (A. R.) and were obtained from Mallinckrodt Chemical Co. Zinc dust and phosphorous tribromide (A. R.) were obtained from Fisher Scientific Co. Sodium hydride oil dispersion (56.7% NaH by weight) and potassium borohydride (A. R.) were purchased from Metal Hydrides, Inc. Guaiacol (2-methoxyphenol), veratrole (1,2-dimethoxybenzene), *N*,2,6-trichloro-*p*-benzoquinoneimine, *N*,2,6-tribromo-*p*-benzoquinoneimine, α,α-dipyridyl, sulfanilic acid, boron trifluoride etherate, *p*-hydroxybenzoic acid, A. R., and Spectral Grade cyclohexane were obtained from Eastman Organic Chemicals, Inc. Cysteine hydrochloride, cystine, glutathione (oxidized and reduced forms), catechol, methyl iodide, pyrogallol, 3,5-dinitrobenzoyl chloride, trichloroacetic acid, paraffin oil, and sucrose certified ACS were obtained from Fisher Scientific Co. Sodium acetate and triple-distilled phytol were obtained from Matheson Coleman & Bell. Acetic anhydride, sodium acetate, and triethylamine reagent grade were obtained from Mallinckrodt Chemical Co. Solanesol, ubiquinone-9 and ubiquinone-10 were the generous gifts of Dr. O. Wiss of Hoffmann-La Roche Ltd. (Basle, Switzerland).

Radiochemicals. L-Tyrosine-*U*-^{14}C (5.5 mCi/mmole), L-phenylalanine-*U*-^{14}C (10 mCi/mmole), benzoic acid-*U*-^{14}C (50 mCi/mmole), benzoic acid-*7*-^{14}C (carboxyl labeled) (9 mCi/mmole), DL-mevalonate-*2*-^{14}C as the sodium salt (10 mCi/mmole), L-methionine-*methyl*-^{14}C (50 mCi/mmole), benzoic acid-*G-t* (100 mCi/mmole), where G denotes a generally labeled compound, and methyl-*t* iodide (100 mCi/mmole) were obtained from Amersham-Searle Corp., and were used without further purification. *p*-Hydroxybenzoic acid-*G-t* (800 mCi/mmole) was prepared by catalytic exchange by Amersham-Searle Corp., under the direction of Dr. Bryan W. Baker. The product was purified by multiple tlc on silica gel G using a solvent mixture of benzene–dioxane–acetic acid (90:25:4, v/v). The final product was radiochemically pure and had a specific radioactivity of 800 mCi/mmole.

Solvents. Ethyl ether, anhydrous (analytical reagent), was purchased from Mallinckrodt Chemical Co. Spectral-analyzed pyridine, hexane, methanol, carbon tetrachloride, and certified dioxane were obtained from Fisher Scientific Co.

Chromatographic Materials. Silica gel G, silica gel H, alumina H, and silica gel (0.05–0.20 mm) were purchased from Brinkman Instrument Co. SilicAr TLC-7G (equivalent to silica gel G), silicAr TLC-7, silicAr TLC-4, silicAr CC-4 (100–200 mesh), and ChromAr sheets (1000 μ) were purchased from Mallinckrodt Chemical Co.

Preparation and Incubation of Liver Slices. Liver slices were prepared from decapitated and exsanguinated rats as previously described (Gold and Olson, 1966). They were suspended in Krebs–Ringer phosphate (pH 7.4) at 4° and gassed with 100% oxygen. In a usual experiment, ten 30-ml beakers were put into a shallow pan which contained about 1 in. of chipped ice. Each beaker received 9 ml of suspending medium and 2.0–2.5 g of liver slices. A labeled precursor of ubiquinone was usually added to the buffer in advance. The beakers containing tissue and buffer were placed in a Dubnoff metabolic shaking incubator (Precision Scientific Co., Chicago) which was previously set at 37° and shaken at 60–80 strokes/min in an atmosphere of 100% O_2. The time of incubation varied from 30 to 180 min.

Extraction of Lipids. Ubiquinone and related quinones are best extracted from liver by a direct saponification of the tissue, as described by Gold and Olson (1966). Polyprenylphenols, on the other hand, are labile to alkali and are best extracted by methods avoiding heat and alkali. The Folch method of total lipid extraction involving lyophilization of the liver and extraction of the dry powder with heptane according to Rudney and Sugimura (1961) was found to be long and tedious. A rapid and reproducible method which extracts total lipid including all detectable intermediates in ubiquinone biosynthesis involves grinding liver with granular sodium sulfate for dehydration, extraction with peroxide-free anhydrous ethyl ether, and evaporation of the extract to a yellow viscous oil *in vacuo*. Lipid (25–30 mg) per gram of fresh liver was obtained by this method.

TABLE I: Retention Volumes of Synthetic Polyprenylphenols and Ubiquinone on Silica Gel Column Eluted with Mixtures of Hexane and Diethyl Ether.[a]

Column Fraction % E/P	Vol Added (l.)	Compound Eluted	Ret Vol (l.)
Hexane	0.25	Hydrocarbons	0.2
1% Ethyl ether	2.00	6-Methoxy-2-nonaprenylphenol	0.9–1.1
		2-Nonaprenylphenol	1.9–2.3
5% Ethyl ether	2.00	Ubiquinone-9	3.0–3.4
25% Ethyl ether	2.00	5-Demethoxy-ubiquinone-9	4.5–4.8

[a] Silica gel (60 g) would accommodate 0.5–1.5 g of total lipid and effect a good separation of the compounds listed. Carrier compounds added to this column in the amount of 324 mg.

TABLE II: R_F Values of Various Compounds Related to Ubiquinone Biosynthesis in Six Solvent Systems.

Compounds	System I	II	III	IV	V	VI
Ubiquinone-9	0.31	0.24	0.39	0.51	0.39	0.55
Ubiquinone-10	0.30	0.14	0.42	0.53	0.42	0.57
Diacetyldihydro Q-9	0.31	0.48				
Diacetyldihydro Q-10	0.30	0.30				
2-Nonaprenylphenol	0.50	0.29	0.62	0.76	0.51	0.44
6-Methoxy-2-nonaprenylphenol	0.69	0.24	0.81	0.93	0.76	0.36
6-Methoxy-2-phytylprenylphenol	0.85	0.89				
6-Methoxy-2-allylphenol	0.49	1.00				
5-Demethoxy-ubiquinone-9	0.15	0.30	0.24	0.35	0.19	0.53
p-Hydroxybenzoic acid	0.00	1.00		0.00	0.00	0.00

Chromatography. The preliminary fractionation of rat liver lipids for polyprenylphenols was achieved by silica gel and silicic acid chromatography. For amounts of total lipid up to 1.5 g, such as that derived from a metabolic experiment involving 25 g of liver slices, the silica gel column developed by Nilsson *et al.* (1968) was very satisfactory.

Silica gel (60 g; 0.05–0.02 mm) was suspended in freshly distilled hexane, and poured into a column (ACE Glass Inc., Vineland, N. J.), 52.5 cm × 21 mmole i.d.) fitted with a glass wool plug at the bottom. A 500-ml reservoir (Kontes Glass Inc., Vineland, N. J.) was fitted at the top with a $^{24}/_{40}$ ground-glass connection. The total lipid (0.5–1.5 g) was charged to the column in a few milliliters of hexane and 250 ml of hexane percolated through the column. The elution was continued with increasing quantities of ethyl ether in hexane, *e.g.*, 2000 ml of 1% ether in hexane followed by 2000 ml of 5%, and 2000 ml of 25% ether in hexane, 1000 ml of pure ether, and 500 ml of methanol. Fractions of 100 ml were collected at a flow rate of 400 ml/hr. The elution pattern for 6-methoxy-2-nonaprenylphenol, 2-nonaprenylphenol, ubiquinone-9, and 5-demethoxyubiquinone is shown in Table I.

For larger amounts of total lipid, such as that employed for the isolation of 6-MNPP (10–15 g), a preliminary resolution of the neutral fraction was carried out on a silicic acid column. After heating in an oven overnight at 110°, silicic acid (SilicAr CC-4, 100–200 mesh) was deactivated by adding water at a 6% level. The watered silicic acid was then slurried with hexane and put into an appropriate sized column. Silicic acid (20 g) was used per gram of lipid. The lipid was charged to the column in a small amount of hexane. The elution of neutral lipids was accomplished stepwise by increasing the polarity of the mobile phase by adding benzene to the hexane (15, 22.5, 30.0, 37.5, 45, 52.5, 60, 80, and 100% benzene). Ubiquinone-9 usually appeared in the 30.0% benzene in hexane fraction.

The definitive separation of ubiquinone metabolites and relative compounds was achieved by tlc. Tlc was carried out on plates with an adsorbent thickness of 0.50–1.0 mm. The plates were prepared with a Quickfit Reeve Angel apparatus (N. J.) and allowed to stand at ambient temperature 1–2 hr. The plates were activated by heating at 120° for 2–3 hr and then stored in a dry atmosphere. Table II shows the six systems which were employed. They were: I = silica gel G, benzene–hexane (50:50, v/v); II = ChromAr sheet impregnated with 10% light paraffin, acetone–water (90:10, v/v); III = silica gel G, cyclohexane–ethyl ether (80:20, v/v); IV = silicAr TLC-7G, hexane–ethyl ether–acetic acid (80:20:1, v/v); V = silica gel G, petroleum ether (bp 30–60°)–ethyl ether (85:15, v/v); VI = alumina H, petroleum ether–ethyl ether (80:20, v/v). All chromatograms were run 15 cm (origin solvent front); the R_F values were calculated by measuring from the origin to the center of the spot using a plastic template (Desaga) calibrated in 0.50-cm increments.

Some lipids were visible as pigments (carotenoids and ubiquinones). Others were revealed with iodine vapor. The Emmerie–Engel reagent was used to search for chromanols by spraying the chromatogram with 0.5% $FeCl_3$ and oversprayed with 0.2% α,α-dipyridyl in methanol. Originally, phenols were detected with the commercially available fast blue salt B and diazotized sulfanilic acid (Pauly's reagent). The best diagnostic spray, however, has been Gibb's reagent, 100 mg of *N*,2,6-trichloro-*p*-benzoquinoneimine in 10–15 ml of methanol. This was sprayed onto a developed chromatogram, oversprayed with saturated aqueous sodium bicarbonate, and warmed. A positive test is a characteristic blue color with absorption between 500 and 700 nm. The spray is somewhat specific for phenols with an unsubstituted position para to the hydroxyl function (Gibbs, 1927), albeit exceptions have been found (King *et al.*, 1957), *e.g.*, phenols with a carboxyl or halogen in the position para to the hydroxyl group will give a positive test.

For determination of the distribution of radioactivity on the chromatoplate, the majority of the plate was protected with a clean glass cover 20 × 20 cm and authentic compounds run on the side of the plate were sprayed lightly. Bands of 1 cm were scrapped off of the plate. The lipid from the scraped silica gel was extracted with ethyl ether and the adsorbent removed by centrifugation or filtration. An aliquot from each 1-cm band was taken for counting.

Crystallizations. Ubiquinone-9 and -10, their dihydrodiacetate derivatives, and 6-methoxy-2-nonaprenylphenol were crystallized from ethanol. About 1 ml of alcohol was used per 3–6 mg of lipid to be crystallized. The solution was allowed to stand overnight at 0–2° and then put in the freezer at −20°.

The crystals were recovered by filtration, and washed with a small volume of cold ethanol. Melting points were determined on a Fisher-Johns melting point block and recorded as read.

Dihydrodiacetyl Derivative of Ubiquinone. The diacetyl derivative of dihydroubiquinone was prepared by reductive acetylation according to Lester *et al.* (1958).

Analytical Methods. Ubiquinone-9 was determined in chromatographic fractions by measuring uv absorption at 275 nm before and after reduction with borohydride. A value of $\Delta E^{1\%}_{ox\rightarrow red}$ of 158 was used. Ultraviolet absorbancies were measured in a Zeiss PMQ II spectrophotometer. For uv absorption spectra, a Cary Model 14 double-beam spectrophotometer was used.

Nuclear magnetic resonance (nmr) spectra were obtained on a Varian HA 60 MC nmr spectrometer. Carbon tetrachloride was used as the solvent with tetramethylsilane as the internal standard. Infrared absorption spectra were obtained with a Perkin-Elmer Model 21 double-beam spectrophotometer equipped with ordinate scale expansion. The solvent was carbon tetrachloride. Mass spectra were obtained on an LKB Model 9000 single-focusing mass spectrometer. A direct probe was used at a recorded vacuum of 10^{-8} Torr, an ionizing energy of 70 eV, ionizing current of 240 μA, an accelerating energy of 3.5 kV, and slit settings of 0.20 and 0.25 mmole. The temperature of the direct probe was varied from ambient to 145°.

Radioactivity was routinely measured using a Packard Model 4322 liquid scintillation spectrometer, equipped with an IBM electric print-out. Appropriate aliquots were pipetted into 20-ml counting vials (Wheaton Glass, Millville, N. J.). The solvents were evaporated with hot air from a hair dryer. About 10 ml of scintillator, 2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene–toluene (supplied as a concentrated solution, Liquifluor was diluted with toluene), was then added to the vial. Quenching was determined by the channel ratio technique. All values were corrected for background and instrument efficiency. ^{14}C was determined with an 85% efficiency and tritium was determined with 41% efficiency.

Chemical Synthesis of 2-Nonaprenylphenol. The basic procedure of Daves *et al.* (1967) was used. Solanesol was purified by silica gel column chromatography and 0.01 mole of the pure alcohol was dissolved in 10 ml of petroleum ether, and placed into a 25-ml round-bottom flask which was immersed in an ice bath and equipped with a magnetic stirrer. An ice-cold solution of pyridine (0.22 ml) and 0.41 ml of phosphorus tribromide in 3 ml of petroleum ether was added in small increments to the alcoholic solution over a 1-hr period. The reaction mixture was then stirred for 2 hr, poured into crushed ice, and stirred for 10 min. The product was extracted with ether and washed with 5% $NaHCO_3$ and finally with water. Solanesol bromide, which is slightly yellow-brown, was used the same day as prepared.

The sodium salt of phenol was prepared by adding 0.1 mole of NaOH in 85% aqueous methanol to 0.102 mole of phenol dissolved in 20 ml of methanol. The flask was flushed with nitrogen and evaporated to dryness. Excess phenol was removed by heating the white powder to 60° at 1 Torr for 24 hr.

To a 5-ml (ground glass jointed) round-bottom flask, 0.0027 mole of solid sodium phenoxide was added. Anhydrous benzene, 1 ml containing 0.0027 mole of solanesyl bromide, was then layered over the salt. The flask was fitted with a calcium chloride drying tube and the heterogeneous medium was stirred magnetically for 24 hr. The next day the solvent was reduced by about 50% with a gentle stream of nitrogen. The reaction was continued for another 4 hr and was terminated by adding crushed ice. The products were extracted with ethyl ether, dried over sodium sulfate, and then evaporated *in vacuo* to an oil. Chromatography on a silica gel column yielded 1.68 g (85% yield) of pure 2-nonaprenylphenol showing the expected nmr and uv spectra.

Chemical Synthesis of 6-Methoxy-2-Nonaprenylphenol. The same basic method was employed for the synthesis of this polyprenylphenol as was used for 2-nonaprenylphenol. Solanesyl bromide was prepared from its alcohol, and sodium *o*-methoxyphenoxide was prepared from guaiacol and NaOH as indicated above. The desired 6-methoxy-2-nonaprenylphenol was then synthesized from solanesyl bromide and the sodium phenoxide in 35% yield by the same method described for the synthesis of 2-nonaprenylphenol. Its nmr and uv spectra corresponded to that described by Daves *et al.* (1967).

Chemical Synthesis of 6-Methoxy-t-2-nonaprenylphenol. In order to synthesize a radioisomer of 6-MNPP, tritiated guaiacol was synthesized from methyl-*t* iodide and catechol. 8.0 ml of a solution of 0.2 N NaOH containing 1.6 mmoles of catechol was added to an ampoule containing 0.25 mmole of radioactive methyl-*t* iodide (100 mCi/mmole). The tube was stoppered and heated to 80° for 8 hr. The reaction mixture was then poured on cracked ice and the product was extracted with ethyl ether after acidification to pH 3. The ether solution was dried over sodium sulfate and evaporated *in vacuo* to yield a pale oil. Guaiacol (100 mCi/mmole) was easily separated from unreacted catechol and *o*-quinone formed during the reaction by tlc on silica gel G with mobile phase of benzene–methanol–acetic acid (90:16:8, v/v). Guaiacol had an R_F of 0.79 and catechol 0.52. The yield was 91% based on the recovery of tritium and the compound was radiochemically pure as judged from the distribution of its radioactivity on a chromatoplate. The sodium salt was formed by reaction of the radioactive guaiacol with equimolar NaH. 6-Methoxy-*t*-2-nonaprenylphenol was then synthesized from 0.23 mmole of sodium salt of guaiacol-*methyl-t* and 0.23 mmole of solanesyl bromide in 1 ml of benzene. The flask was fitted with a calcium chloride drying tube and stirred magnetically for 24 hr. The reaction mixture was poured into crushed ice and extracted with ethyl ether. The ether was dried with $NaSO_4$ and evaporated *in vacuo* to an oil. Chromatography on silica gel column resulted in a partial separation of desired product from unreacted guaiacol, which moved with a mobility similar to ubiquinone. The desired product (30% yield) was then purified to radiochemical purity by tlc using system 1 (Table II). Radioassay and uv analysis of the desired product demonstrated the theoretical specific activity of 100 mCi/mmole and a $E^{1\%}_{1\,cm}$ in hexane at 279 nm of 42.7. The product was solubilized in isotonic saline (0.90%) containing 1% Tween 80 for injection (2 mg/0.1 ml).

Results

Detection of a Lipophilic Metabolite of Benzoate Less Polar Than Ubiquinone in Lipid Extracts of Rat Liver. In 1967, we (Dialameh *et al.*, 1967) reported the presence of an inconspicuous metabolite of benzoate-*U*-^{14}C in nonsaponifiable extracts of rat liver slices which was less polar than ubiquinone on columns of alumina (Brockmann Grade III). It seemed possible that it represented a polyprenylphenolic intermediate in ubiquinone biosynthesis in the rat which was poorly recovered because of the use of saponification and alumina chromatography. We, therefore, decided to reinvestigate this compound with the aid of better methods, more highly radioactive precursors, and authentic synthetic markers. Liver slices were incubated with a variety of potential radioactive pre-

TABLE III: Incorporation of Isotopically Labeled Compounds into Ubiquinone-9 and Suspected 6-Methoxy-2-nonaprenylphenol in Rat Liver Slices.[a]

Labeled Precursor	Suspected 6-MNPP (dpm)	Q-9 (dpm)
Benzoate-*U*-^{14}C	4,450	3510
Benzoate-*G*-*t*	2,895	0
Tyrosine-*U*-^{14}C	490	675
Phenylalanine-*U*-^{14}C	365	595
Methionine-*methyl*-^{14}C	895	4900
Benzoate-*carboxyl*-^{14}C	0	0
p-Hydroxybenzoate-*G*-*t*	36,790	0
Mevalonic Acid-*2*-^{14}C	3,990	8500[b]

[a] Each experiment represents ten beakers with the isotopically labeled precursor present at a level of 10 μCi/beaker. Liver slices were incubated for 1 hr and the lipid was analyzed by silica gel column and tlc. [b] Not purified to radiochemical purity. Corrected dpm on basis of other experiments should be *ca.* 5000.

cursors for 1 hr. The total lipids were extracted without saponification and chromatographed on silica gel. The 6-MNPP and Q-9 fractions were evaporated and subjected to tlc in system 1. The results are shown in Table III. The pattern of labeling observed is consistent with that of a ubiquinone precursor. The incorporation of benzoate-*U*-^{14}C, tyrosine-*U*-^{14}C, and phenylalanine-*U*-^{14}C into the suspected polyprenylphenol (X) in the same proportions as in Q-9 support the presence of an aromatic ring.

The incorporation of methionine-*methyl*-^{14}C into X shows the presence of a methyl group, although fewer than in Q-9 which had four times the total radioactivity of X. Benzoate-*G*-*t* and *p*-HBA-*G*-*t* strongly labeled X, but not Q-9, signifying unsubstituted positions in the aromatic ring of X. Carboxyl-labeled benzoate was not incorporated into X, indicating the absence of a carboxyl group.

Finally, the incorporation of mevalonate-*2*-^{14}C suggests the presence of an isoprenyl side chain. The very high incorporations noted with *p*-HBA-*t* (800 mCi/mmole) led to the expectation that *p*-HBA-*t* would be the ideal probe to guide the isolation of the compound.

Isolation of Radioactive 6-Methoxy-2-nonaprenylphenol with Carrier from Liver Slices Metabolizing p-Hydroxybenzoate-G-t. Rat liver slices totaling 25 g were distributed in ten beakers each containing 100 μCi of *p*-HBA-*G*-*t* and incubated for 1 hr at 37°. Three milligrams each of synthetic 6-MNPP and 2-NPP were added to the total lipid extract, which was then chromatographed on silica gel. Of the 1 mCi of substrate used, 169 μCi was recovered from the column, including 166 μCi of unaltered substrate. Of the 3 μCi of radioactivity incorporated into lipid-soluble metabolites, 2.5 was found in a very polar metabolite, possibly identical with 3-nonaprenyl-4-hydroxybenzoate, detected by Winrow and Rudney (1969) in rat liver homogenates. Virtually all of the remaining 0.5 μCi was distributed among three compounds as shown in Table IV. The two most prominent ones were 6-MNPP and 5-DMQ-9. Much less radioactivity was associated with 2-NPP. About 1.0 mg of solids was recovered from the 6-MNPP fraction; 0.33 mg representing 2.0×10^5 DPM of biosynthetically labeled 6-MNPP was combined with 80 mg of synthetic 6-MNPP to give a mixture with a calculated specific activity of 2.5×10^3 dpm/mg and crystallized to constant specific activity. The data are shown in Table V. No significant change from the calculated specific activity was noted in four recrystallizations.

TABLE IV: Distribution of Tritium from *p*-HBA-*G*-*t* in Neutral Lipid Fractions from Rat Liver Slices.

Fraction	Radioactivity	
	Dpm $\times 10^{-5}$	% Total
Hydrocarbons	0	0
6-MNPP	5.97	52.8
2-NPP	0.03	0.3
Ubiquinone	0.00	0.0
5-DMQ-9	5.33	46.9

TABLE V: Cocrystallization of Synthetic 6-Methoxy-2-nonaprenylphenol with Biosynthetically Labeled 6-Methoxy-2-nonaprenylphenol.[a]

Crystallizn	6-MNPP (mg)	Radioactivity	
		Total Dpm	Sp Act. (dpm/mg)
1	77.50	193,750	2492
2	73.49	183,200	2497
3	70.00	175,700	2510
4	68.12	173,045	2540

[a] The original specimen of biosynthetically labeled 6-MNPP had a specific activity of 6×10^5 dpm/mg. It was diluted 240 times to give a calculated specific activity of 2500 dpm/mg. The weight of each recrystallized sample was determined by uv spectrometry. The melting point of 6-MNPP was 30–31° and constant.

Time Course of Incorporation of Benzoate-U-^{14}C into 6-MNPP and Q-9 in Rat Liver Slices. Rat liver slices were incubated with benzoate-*U*-^{14}C for various times from 15 to 180 min. 6-MNPP and Q-9 were extracted from a 4-g sample of liver slices. The time course of incorporation into the two compounds is shown in Figure 1. The incorporation into 6-MNPP was greatest at 1 hr whereas that into Q-9 was greatest at 3 hr. The time lag in biosynthesis of Q-9 in liver slices first noted by Gold and Olson (1966) and by Trumpower *et al.* (1972) is seen here again. The temporal relation between incorporation of benzoate-*U*-^{14}C into 6-MNPP and Q-9 suggests a precursor–product relationship between the two compounds.

Isolation and Identification of 6-MNPP from Rat Liver. The lipid isolated from 500 g of rat livers (12 g) was separated into pre-Q-9 (2.54 g) and post-Q-9 fractions by silicic acid column chromatography. To the pre-Q-9 (which contains 6-MNPP) a biosynthetically labeled suspected 6-MNPP was added as a marker. The labeled metabolite was generated *in vitro* by incubation of liver slices with *p*-hydroxybenzoic acid-*G*-*t*. The combined lipid weighing 4.60 g (2.54 g of unlabeled pre-Q-9

TABLE VI: Summary of the Isolation of Suspected 6-Methoxy-2-nonaprenylphenol from Rat Liver.

Chromatographic System	Charge Wt	6-MNPP Fraction Wt	Sp Act. of 6-MNPP (dpm/mg)
Silicic acid column 1	12 g	2.54 g	
Silicic acid column 2	4.60 g[a]	450 mg	106
Silica gel column	450 mg	96 mg	458
Two tlc systems			
Silica gel G	96 mg	15 mg	1,960
Reverse-phase tlc	15 mg	5 mg[b]	4,320
Silica gel column	5 mg[b]	3 mg	6,900
Tlc silica gel G	3 mg	<1 mg	>19,000

[a] ^{3}H-Labeled lipids (2.1×10^5 dpm) from rat liver slices incubated with *p*-HBA-*G*-*t* were added at this step. [b] Excludes paraffin oil.

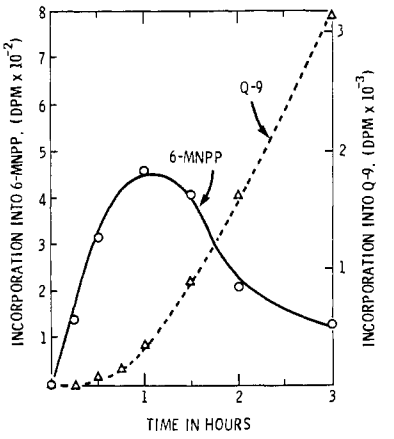


FIGURE 1: Time course of incorporation of benzoate-*U*-^{14}C into 6-methoxy-2-nonaprenylphenol and ubiquinone-9 in rat liver slices. Each value represents 4 g of liver slices incubated with 20 μCi of benzoate-*U*-^{14}C for the time indicated.

fraction) isolated from 500 g of rat liver and 2.06 g of total lipid isolated from *in vitro* incubation of ten rat livers with 100 μCi each of *p*-hydroxybenzoate-*G*-*t* was chromatographed on a silicic acid column. 6-MNPP fractions were counted for ^{3}H. The data for this and subsequent steps are presented in Table VI. This fraction was then rechromatographed on a silica gel column. It was known that the yellow carotenoids migrate just in front of 6-MNPP. These were partially separated from the radioactive material which followed. The labeled material (96 mg) was combined and chromatographed repeatedly on tlc (Table II, system 1, 20 mg per 20 × 20 cm plate) until a single spot was obtained which remained positive to Gibb's reagent.

The lipid was then subjected to reverse-phase tlc (Table II, system 2). The natural product developed a Gibb's color faster than authentic 6-MNPP before reverse-phase tlc. During reverse-phase tlc, a yellow fast moving contaminant was separated from the metabolite 6-MNPP. The lipid was extracted with ethyl ether; the paraffin was removed by chromatography on silica gel column with petroleum ether, and the suspected 6-MNPP was eluted with 10% ethyl ether in petroleum ether (estimated to be 2–3 mg).

The lipid was transferred to a 1-ml ground-glass container and 0.02 ml of spectra-analyzed *n*-hexane was added. Tlc of 5 μl of this material (Table II, systems 1 and 2) showed it to be homogeneous with the same R_F as authentic 6-MNPP. This material also developed the characteristic beautiful blue color with Gibb's reagent at the same rate as authentic 6-MNPP. From this reaction, it was estimated that 200 μg of 6-MNPP had been isolated. Both the actual and synthetic 6-MNPP had the following properties: uv spectrum at pH 6 in ethanol 273 nm ($E^{1\%}_{1\,cm}$ 41.6) and 279 nm (42.7); at pH 13 in ethanol 241 nm (92.5) and 291 nm (83.2); nmr spectrum (in τ values) showed protons at 3.44 (aromatic), 4.63 (hydroxyl), 4.98 (vinylic), 6.19 (methoxyl), 6.75 (benzylic), and 7.8–8.5 (alkyl). The mass spectrum showed the molecular ion at *m/e* 736 with fragmentation formula, *m/e* 736 − (69 + *n*68); *n* = 0, 1, 2, 3, 4, 5, 6, 7, and 8. A stable benzylic ring fragment was seen at *m/e* 137 and side chain hydrocarbon fragments for $C_5H_9^+$ and $C_6H_9^+$ at *m/e* 68 and 81, respectively. The comparative mass spectra for the natural product and the synthetic 6-MNPP are shown in Figure 2.

The Conversion of 6-Methoxy-t-2-nonaprenylphenol to Ubiquinone-9-t by Intact Rats. Two sets of three adult male rats received 84 μCi of tritiated 6-MNPP (specific activity 100 mCi/mmole) per set intravenously by tail vein (28 μCi/rat) and were sacrificed in three hours. The livers were excised and their total lipids extracted and chromatographed on a column of silica gel. Most of the radioactivity was found in the ubiquinone fraction, which was purified by direct (system 1) and reverse phase (system 2) tlc. Approximately 1.0 mg of ubiquinone-9 was isolated from each set of three livers. After measurement of specific radioactivities, each was diluted sixfold withcold ubiquinone-9 and the diacetyldihydro derivatives were prepared and counted again. The results are presented in Table VII. The specific radioactivities remained essentially constant through reverse-phase chromatography and crystallization of the diacetyldihydro derivative.

Discussion

Since 1965, the bulk of the work on the biosynthesis of ubiquinone in plant and animal tissues and in microorganisms

TABLE VII: Incorporation of Methyl-Labeled 6-Methoxy-2-nonaprenylphenol (100 mCi/mM) into Ubiquinone-9, *in Vivo* in Rat.[a]

Expt	Dose (μCi/Rat)	Sp Act. of Ubiquinone and Its Diacetate Derivative (dpm/μmole)			
		Silica Gel Column	Straight-Phase Q-9	Reverse-Phase Q-9	Diacetate of Q-9
1	27.7	17,050	14,600	14,750	15,600
2	27.7	18,020	14,710	14,200	14,800

[a] Labeled 6-MNPP was suspended into 1% Tween 80; each rat was given the indicated amount in 0.15 ml *via* the tail vein. The animals were allowed to live for three hours before sacrifice. Ubiquinone was isolated as described in the text; the diacetate was prepared by reductive acetylation. Specific activity was determined by a combination of uv spectra and liquid scintillation counting.

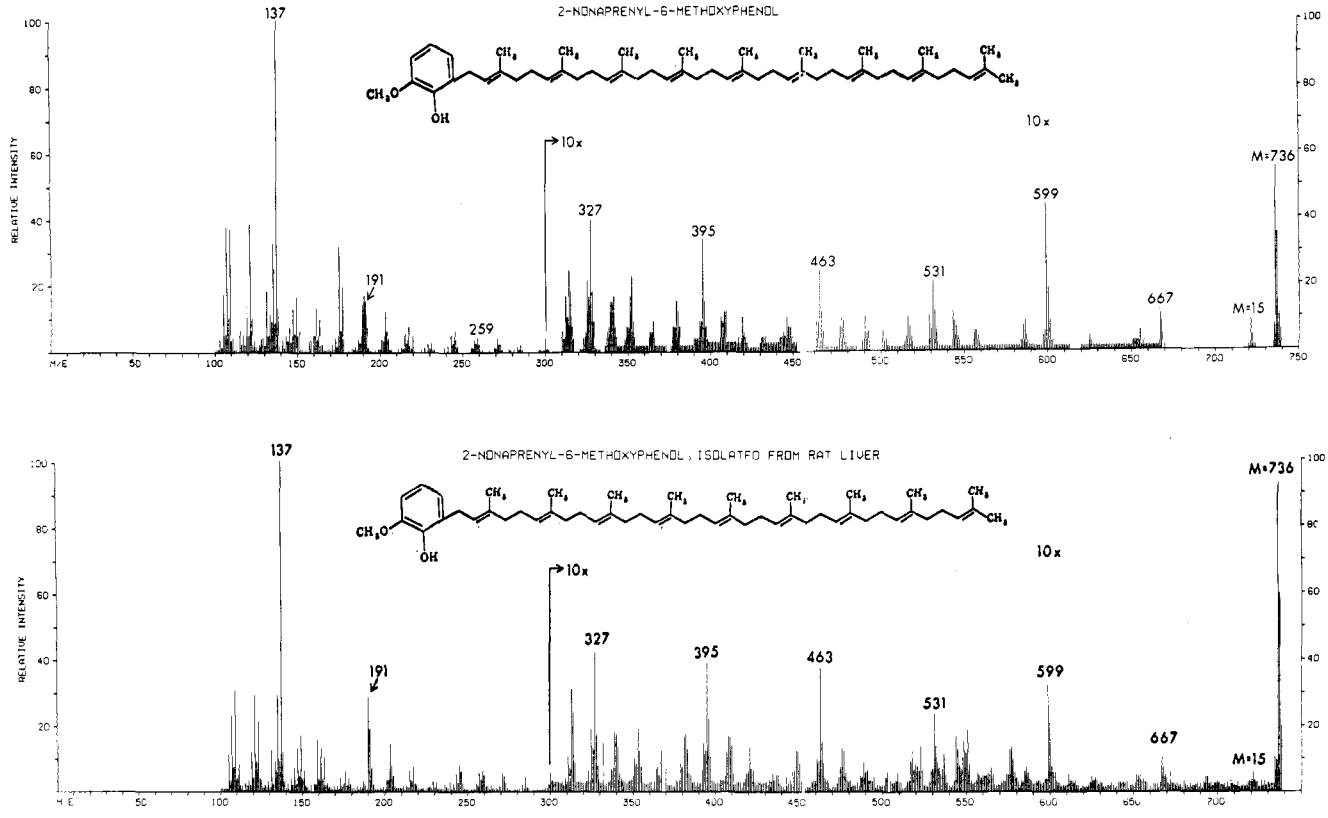


FIGURE 2: Mass spectra of natural and synthetic 6-methoxy-2-nonaprenylphenol.

has been devoted to determination of the sequence of biochemical events in the metabolic pathway between *p*-hydroxybenzoate and ubiquinone. Stoffel and Martius (1960) had claimed that alkylation of a fully substituted ring precursor of ubiquinone was the *last* step in the biosynthetic sequence, a claim which could not be verified by Gloor and Wiss (1959) or by us (Olson, 1965). The first significant development in the solution of this problem was the report by Parson and Rudney (1965) that a lipophilic compound derived from *p*-hydroxybenzoate-*U*-^{14}C accumulated in cultures of *R. rubrum* incubated anaerobically in the dark. This compound could then be converted into ubiquinone-10 by continued incubation in the light. It was less polar than ubiquinone on tlc, and could be labeled by acetate, but not by methionine. At this point, Rudney joined forces with Karl Folkers, then at the Stanford Research Institute, and a large-scale isolation of the new compound was achieved. It was identified as 2-decaprenylphenol (Olsen *et al.*, 1965). It seemed at this time from our own work, as well as that of the Rudney–Folkers team, that alkylation of *p*-hydroxybenzoate with a multiprenyl pyrophosphate was an early, rather than late, event in Q biosynthesis, and further that the carboxyl bearing carbon of Q was the 4-carbon meta to the isoprenyl side chain.

In 1967, we (Dialameh *et al.*, 1967) noted the appearance of a radioactive compound less polar than ubiquinone-9 which appeared in the nonsaponifiable fraction of rat liver slices synthesizing ubiquinone-9 from benzoate-*U*-^{14}C. It was distinct from 2-nonaprenylphenol but we were unable to isolate it. With the adoption of methods better suited to the fractionation of polyprenylphenols described in this paper and *p*-HBA of high specific activity, we have now been able to unequivocally identify this compound as 6-methoxy-2-nonaprenylphenol. It has been isolated from rat liver slices biosynthesizing ubiquinone-9 from labeled precursors and crystallized to constant specific activity. In addition, a tritiated synthetic sample of 6-MNPP was incorporated into hepatic ubiquinone-9 in intact rats. It appears to exist in rat liver at a concentration of about 1 μg/g fresh weight.

We have not been able to identify 2-nonaprenylphenol with certainty in the experiments reported in this investigation. Very small amounts of radioactivity derived from *p*-HBA-*t* have been associated with carrier 2-NPP in chromatographic studies, but reisolation of the 2-NPP and crystallization of a derivative to constant specific activity have not been achieved. It may be possible that 2-NPP is a very trace constituent of rat liver, but we now regard it as on a side path rather than the main path from *p*-hydroxybenzoyl-CoA to 6-MNPP (Dialameh *et al.*, 1970). Because a metabolite similar or identical with 3-nonaprenyl 4-hydroxybenzoate is formed in high yield from *p*-HBA-*G*-*t*, it would appear that decarboxylase activity yielding the former compound is negligible in animal tissues. We have also recently reported the unequivocal identification of 5-DMQ-9 as an intermediate in ubiquinone-9 biosynthesis in rat liver (Trumpower *et al.*, 1972). This intermediate is present in much lower amounts, of the order of 0.1 μg/g fresh weight.

On the basis of the present evidence, we would favor the following pathway (Scheme II) for the biosynthesis of ubiquinone-9 in the rat, which differs somewhat from the general pathway proposed by the Folkers group (Friis *et al.*, 1966).

This sequence, though tentative, would fit our data for the rat better than the original proposal of Folkers. We visualize that the CoA linkage to the benzoate carboxyl is essential for directing alkylation and the first hydroxylation step. Then in concert with O-methylation, or in series with it, the carboxyl group is removed, possibly by reduction. This would put 2-

NPP on a side pathway when compared to 6-MNPP or 5-DMQ-9. Further work is required to determine the mechanism of decarboxylation in animal tissues.

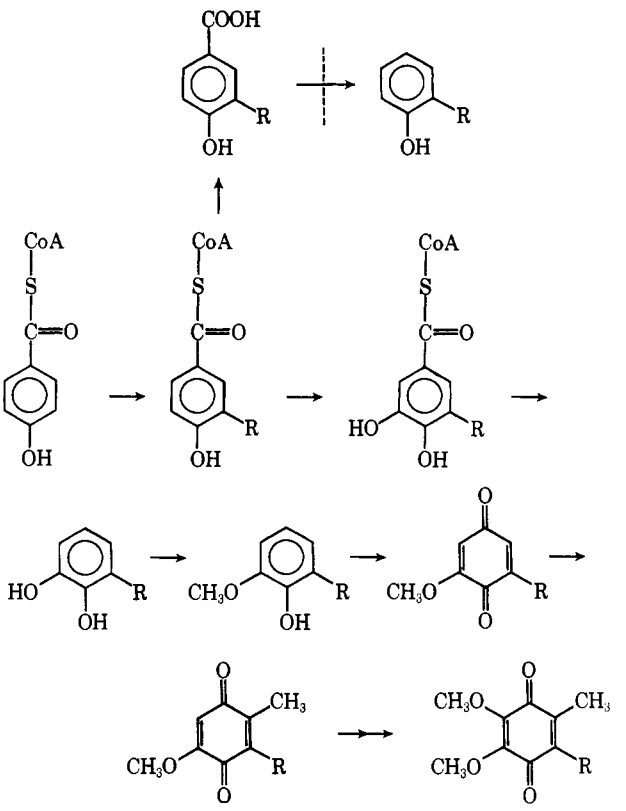
Jones (1967) discovered an auxotroph for *Escherichia coli* which requires *p*-hydroxybenzo c acid for growth. In the absence of hydroxybenzoate, ubiquinone deficiency resulted in this organism with a gross reduction in aerobic respiration. Cox *et al.* (1968) found similar mutants in *E. coli* K_{12} and subsequently (Cox *et al.*, 1969) described the accumulation of 3-octaprenyl-4-hydroxybenzoic acid and 2-octaprenylphenol by two mutants. They found, furthermore, that the two genes, ubiD and ubiB, concerned with these two successive reactions in ubiquinone biosynthesis were closely linked. They also showed that cell extracts in the mutant strain carrying the ubiD$^-$ allele lacked 3-octaprenyl-4-hydroxybenzoic decarboxylase activity. It is of interest that the mutant having the gene ubiD$^-$, which accumulated 3-octaprenyl-4-hydroxybenzoate, had reduced but significant levels of ubiquinone-8 (25% normal) and grew slowly on succinate as a source of carbon. This suggests that an alternate pathway exists in *E. coli* for decarboxylation, which may be identical with the one postulated by us for animal cells involving reductive decarboxylation of the benzoyl-CoA derivative.

Whistance *et al.* (1971) were unable to detect any intermediates between *p*-hydroxybenzoate and ubiquinone-9 in rat tissues except ubiquinol-9 and suggested that our preliminary reports of the isolation of 5-demethoxyubiquinone-9 and 6-methoxy-2-nonaprenylphenol were in error. The evidence presented here for the identity and metabolic role of 6-MNPP and elsewhere for 5-DMQ-9 (Trumpower *et al.*, 1972) should erase all doubts about the validity of this work. The question arises, however, why were Whistance *et al.* unable to find these intermediates in their preparation of rat liver? Spiller *et al.* (1968) furthermore did not detect 5-demethoxyubiquinone-9 in yeast cells metabolizing *p*-hydroxybenzoate to ubiquinone-6, although Law *et al.* (1971) have more recently found 5-demethoxyubiquinone-6 to be a precursor of ubiquinone-6 in yeast. The answer to this experimental inconsistency may lie in one or more of the following variables: (a) the use of insufficient tissue for extraction and characterization of the intermediates, (b) the use of a strain of organism in which the rate-limiting reaction in the multienzyme pathway shifts so that a given intermediate does not accumulate, (c) the use of inappropriate extraction procedures, (d) the use of insufficient radioactivity in the tracer experiments, and (e) the use of insensitive techniques for the identification of intermediates. It is, of course, true that negative evidence does not have the same force as positive evidence. Indubitably, the controversy can only be settled by additional investigation of the critical variables sited.

Winrow and Rudney (1969) have reported the incorporation of radioactivity from *p*-hydroxybenzoate into 3-polyprenyl-4-hydroxybenzoate by a rat liver homogenate fortified with a *Micrococcus lysodeikticus* extract and isopentenyl pyrophosphate. No polyprenylphenol was found.

At the present time, therefore, it would appear that several of the key intermediates postulated for the biosynthesis of ubiquinone in microorganisms also exist in the rat. These have been demonstrated to be derived from *p*-hydroxybenzoate and to be, in turn, converted to ubiquinone. The absence of certain postulated intermediates in measurable quantities in tissue preparations metabolizing *p*-hydroxybenzoate does not rule out the presence of the intermediate as transient, infinitesimal in concentration, and perhaps enzyme bound. At the moment, evidence for pathways alternate to those proposed by Folkers is present for both microorganisms and mammals, but

SCHEME II



the general outline appears to be correct. Further intensive work will be necessary to determine the exceptions and describe all the enzymes involved.

Acknowledgments

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A Tetrapyrrylmethane Intermediate in the Enzymatic Synthesis of Uroporphyrinogen†

Richard Radmer‡ and Lawrence Bogorad*

ABSTRACT: An uncyclized tetrapyrrylmethane has been identified and recovered from reaction mixtures of the enzyme uroporphyrinogen I synthetase and the pyrrole porphobilinogen incubated with ammonium ions. The polypyrrole has been judged to be a tetrapyrrylmethane on the basis of its electrophoretic behavior and the observation that it forms uroporphyrinogen I *via* a nonenzymatic first-order reaction. The effect of pH on the rate of formation of uroporphyrinogen can be taken to suggest that the tetrapyrrylmethane bears an aminomethyl group on an α position. The tetrapyrrylmethane is not converted to uroporphyrinogen III in an enzymatic system capable of forming this isomer from porphobilinogen. Some hypotheses for the formation of uroporphyrinogen III are reevaluated in the light of these observations.

The enzymatic formation of uroporphyrinogen III (urogen III)[1] from the monopyrrole porphobilinogen (PBG) requires the action of two enzymes—urogen I synthetase and urogen III cosynthetase (Bogorad, 1958b). Urogen III is a precursor of hemes and chlorophylls.

Urogen I is formed when PBG is incubated with urogen I synthetase (Bogorad, 1958a). Although urogen I is not a substrate for the synthesis of urogen III by urogen III cosynthetase, it seems likely that some earlier product of urogen I synthetase is utilized for the enzymatic formation of urogen III.

It has been observed that polypyrrolic precursors of urogen I accumulate in reaction mixtures of PBG and urogen I synthetase in the presence of hydroxylamine or ammonium ions (Bogorad, 1963; Pluscec and Bogorad, 1970). These products can be separated by paper or thin-layer electrophoresis. One of the intermediates which accumulates in hydroxylamine-inhibited reactions has been identified as a dipyrrylmethane by Pluscec and Bogorad (1970). It was also observed in these experiments that hydroxylamine and ammonium ions inhibited the formation of urogen I in different ways;

† From the Department of Biology, University of Chicago, Chicago, Illinois, and from the Biological Laboratories, Harvard University, Cambridge, Massachusetts. *Received July 19, 1971.* Supported in part by grants from the National Institute of Arthritis and Metabolic Disease and the National Aeronautics and Space Administration.

‡ Submitted in partial fulfillment of the requirements of the Ph.D. degree from the Department of Biology, University of Chicago, Chicago, Ill.

Present address: Research Institute for Advanced Studies (RIAS), Baltimore, Md. 21227.

* Permanent address: The Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge, Mass. 02138; to whom correspondence should be addressed.

[1] Abbreviations used: urogen I, uroporphyrinogen I; urogen III, uroporphyrinogen III; PBG, porphobilinogen; tle, thin-layer electrophoresis; uro, uroporphyrin; Copro, coproporphyrin.