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*THE BIOSYNTHESIS OF THE BENZOQUINONE RING OF UBIQUINONE FROM p-HYDROXYBENZALDEHYDE AND p-HYDROXYBENZOIC ACID IN RAT KIDNEY, AZOTOBACTER VINELANDII, AND BAKER'S YEAST**

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In the photosynthetic microorganism *Rhodospirillum rubrum*, p-hydroxybenzaldehyde-U-C^{14} has been discovered to undergo a direct conversion into the benzoquinone ring of ubiquinone-10.[1] (U indicates the uniformly or randomly labeled compound.) The carbonyl carbon is lost in the course of this conversion, since carbonyl-labeled p-hydroxybenzaldehyde does not yield radioactive ubiquinone-10. It has been found that the methyl group attached to the ubiquinone ring is supplied by the one-carbon pool.[1, 2] It was of interest to determine whether the anaerobic pathway discovered in *R. rubrum* also occurs in species using oxygen as a terminal electron acceptor. This paper reports that a conversion of p-hydroxybenzaldehyde-U-C^{14} into the ring of ubiquinone occurs in aerated yeast and *Azotobacter vinelandii*, and in a mince of rat kidney cortex. In addition, p-hydroxybenzoic acid-U-C^{14} has been found to give rise to ubiquinone with about the same efficiency as does the aldehyde.

Materials and Methods.—C^{14}-labeled p-hydroxybenzaldehyde, prepared as described elsewhere,[1]

was purified by paper chromatography in two solvent systems [benzene-2% formic acid (2:1), and chloroform-methanol-water-formic acid (1000:100:96:4)[3]]. As a test of purity, samples were rechromatographed in these systems, and in a third, chloroform-acetic acid-water (2:1:1), (organic phase[4]), and the chromatograms were scanned in a Nuclear-Chicago strip counter. A single radioactive peak was observed. The resolution of the method was such that a discrete impurity which contained approximately 0.5% of the total radioactivity would have been detected.

L-Tyrosine-U-C^{14}, obtained from Nuclear-Chicago Corp., was purified by chromatography on Whatman no. 1 paper with butanol-acetic acid-water (4:1:5).[5]

p-Hydroxybenzoic acid-U-C^{14} was prepared by alkali fusion of tyrosine-U-C^{14} in a small-scale adaptation of the procedure developed by Reio and Ehrensvard.[6] L-Tyrosine-U-C^{14} (6 μmoles) and approximately 150 mg each of KOH and NaOH were mixed in a platinum crucible in a Wood's metal bath, at 270°C. After 6 min, the mixture was cooled, dissolved in a slight excess of 10 N H_2SO_4, and mixed with 10 gm of Celite. p-Hydroxybenzoic acid-U-C^{14} was eluted from the Celite with ether, and purified by paper chromatography in two solvent systems (butanol-acetic acid-water (4:1:5)[5] and 2-propanol-water-concentrated ammonium hydroxide (8:1:1).[8] The yield of purified product was 20–30%. At least 99.5% of the C^{14} appeared in a single peak when a sample was rechromatographed with butanol-acetic acid-water (4:1:2).[7]

Slices prepared from two rat kidneys were shaken with the radioactive substrates for 3 hr at 38°C in 10 ml of Krebs-Ringer bicarbonate buffer.[9, 10] The rats used weighed 300–400 gm. Following the incubation, the nonsaponifiable fraction[10] was extracted with petroleum ether, and the extract was concentrated to 0.2 ml. A small amount of precipitate was removed by centrifugation, and the yellow supernatant solution was applied to a thin layer plate of silica gel (Silica Gel G, Brinkmann Instruments, Inc.), and developed with benzene-chloroform (1:1).[11] The yellow portion containing ubiquinone-9 was scraped off the plate and eluted with ether.

In other experiments, a finely chopped mince of the cortices of 4 rat kidneys was incubated with radioactive substrate in 20 ml of bicarbonate buffer. The tissue was collected by centrifugation, resuspended in distilled H_2O, recentrifuged, homogenized, and lyophilized. The dried preparation was extracted three times with 70-ml portions of ether-ethanol (3:1). The extract was evaporated to dryness, taken up in petroleum ether, and chromatographed on thin layer plates, as described above. Ubiquinone of constant specific activity was obtained by repeated thin layer chromatography on silica gel plates, using benzene-chloroform (1:1) and benzene-ethyl acetate (4:1) as solvents, and by repeated crystallization from ethanol after the addition of carrier ubiquinone. In experiments with p-hydroxybenzaldehyde-U-C^{14} and p-hydroxybenzoic acid-U-C^{14} the specific activity of the ubiquinone was usually constant after a single thin layer chromatographic separation, since there was negligible C^{14} in lipids other than ubiquinone. However, when L-tyrosine-U-C^{14} was used as substrate, constant specific activity was achieved only after much more extensive purification of the ubiquinone.

A. vinelandii was grown in Burk's medium. One liter of medium containing radioactive p-hydroxybenzaldehyde or p-hydroxybenzoic acid was inoculated with growing cells from a smaller flask, and was shaken rapidly on a Gump shaker for 3 days at 30°. The cells were centrifuged, washed, and lyophilized, and then extracted 3 times with 70-ml portions of ether-ethanol (3:1). Ubiquinone-8 was isolated from the extract as described for the kidney mince preparation.

Red Star baker's yeast was transferred several times on agar plates to obtain a pure culture. It was then grown anaerobically as described by Sugimura and Rudney.[12] The harvested yeast was resuspended in phosphate buffer containing 0.5% glucose, and p-hydroxybenzaldehyde-U-C^{14}. A drop of Dow Antifoam A was added, and air was bubbled rapidly through the suspension during a 7-hr incubation at 38°C. Under these conditions, there is an increase in the synthesis of ubiquinone-6.[12] The cells were collected and washed, and ubiquinone-6 was obtained by saponification,[12] extraction with petroleum ether, and chromatography on thin layer plates.

Ubiquinone was assayed by the change in extinction at 275 mμ upon addition of KBH_4 to a solution in 99% ethanol.

The C^{14} of the ubiquinone was determined using a Nuclear-Chicago gas flow counter and that of the tyrosine, p-hydroxybenzaldehyde, and p-hydroxybenzoic acid using a Packard Tri-Carb liquid scintillation spectrophotometer. All radioactivity data were converted to disintegrations per minute (dpm) by calculation based on the activity of C^{14} standards counted at the same time. The total amount of C^{14} incorporated into ubiquinone was calculated from the amount of ubi-

quinone determined in the petroleum ether extracts, and the specific activity of the chromatographed ubiquinone. In the experiments with p-hydroxybenzaldehyde-U-C^{14} and p-hydroxybenzoic acid-U-C^{14}, estimates made in this way agreed fairly closely with the total amount of radioactivity found in the petroleum ether extracts.

Following the addition of 100 mg of carrier, the ubiquinone was degraded to separate the benzoquinone ring from the side chain. Carrier ubiquinone-6, used in the yeast experiments, and ubiquinone-9, used in the kidney and *A. vinelandii* experiments, were gifts of Dr. Isler of Hoffmann-La Roche Co., Basle. Since ubiquinone-8, the homologue occurring in *A. vinelandii*, was not available in sufficient quantity, ubiquinone-9 was substituted. It was assumed that a small difference in the length of the side chain would not influence the course of the degradation. In the degradation, the ubiquinone was reduced with $Na_2S_2O_4$, and acetylated with acetic anhydride,[13] and the dihydrodiacetyl derivative was ozonized by the method of Bentley *et al.*[14] From the side chain, the degradation yields levulinaldehyde, which was converted to the *bis*-dinitrophenylhydrazone. This derivative was recrystallized from chloroform, plated on a weighed filter paper, and the C^{14} was determined in a gas flow counter. The 2,5-diacetoxy-3,4-dimethoxy-6-methylphenylacetic acid obtained from the benzoquinone ring[13] was sublimed, crystallized from ether, determined by titration, and its radioactivity was measured in the scintillation counter.

Results and Discussion.—Tables 1, 2, and 3 show that rat kidney, *A. vinelandii*, and yeast convert p-hydroxybenzaldehyde-U-C^{14} to ubiquinone. Degradations were performed on three samples marked with an asterisk in Tables 1–3, and the results are presented in Table 4. In each case the specific activity of the derivative from the ring agreed fairly well with the initial specific activity of the ubiquinone, and no radioactivity was detected in the levulinaldehyde-*bis*-dinitrophenylhydrazone derived from the side chain of the ubiquinone. Lipids in the ether-ethanol extracts, other than ubiquinone, also contained very little C^{14}. These observations do not exclude the occurrence of pathways for complete oxidation of p-hydroxybenzaldehyde, since radioactive fragments entering the acetate pool, for example, might be greatly diluted by endogenous materials. Evidence for such a degradative

TABLE 1

INCORPORATION OF p-HYDROXYBENZALDEHYDE-U-C^{14}, p-HYDROXYBENZOIC ACID-U-C^{14}, AND L-TYROSINE-U-C^{14} INTO UBIQUINONE-9 BY RAT KIDNEY

Expt.	C^{14}-Aromatic Compound Added			Ubiquinone	Incorporation	
		μmoles	dpm/mole	(dpm/μmole)	mμmoles	Per cent
1	p-Hydroxybenzaldehyde-U-C^{14}	0.011	3.94×10^7	1.83×10^4*	0.137	1.07
2	"	0.011	3.94×10^7	7.1×10^3	0.038	0.30
	"	5.0	8.7×10^4	87	0.176	0.003
3	"	0.011	3.94×10^7	8.1×10^3*	0.107	0.83
4	"	0.0055	3.94×10^7	4.40×10^3	0.085	1.30
	p-Hydroxybenzoic acid-U-C^{14}	0.011	2.84×10^7	3.64×10^3	0.096	0.74
5	p-Hydroxybenzaldehyde-U-C^{14}	0.011	3.94×10^7	5.43×10^3	0.118	0.91
	p-Hydroxybenzoic acid-U-C^{14}	0.022	2.84×10^7	3.40×10^3	0.103	0.40
	"	0.0055	2.84×10^7	2.53×10^3	0.076	1.24
6	p-Hydroxybenzoic acid-U-C^{14}	0.50	3.6×10^5	83	0.173	0.03
7	L-Tyrosine-U-C^{14}	0.536	3.69×10^7	3.91×10^2	...	0.00083
8a	p-Hydroxybenzaldehyde-U-C^{14}	0.011	3.94×10^7	3.89×10^3	0.68	0.53
8b	L-Tyrosine-U-C^{14}	1.11	3.69×10^7	8.3×10^2	...	0.0028

* Pooled for degradation; see Table 4.

Experiments 1 and 2: tissue slices from 2 kidneys in each flask. Experiments 3–7: mince from 4 kidney cortices in each flask. Experiment 8: mince from 4 kidneys in the flask receiving p-hydroxybenzaldehyde-U-C^{14}, mince from 8 kidneys in the flask receiving L-tyrosine-U-C^{14}. Other conditions as described in the text. "Per cent incorporation" means 100 × total dpm found in ubiquinone divided by the total dpm added to the flask. "mμmoles incorporation" means $^7/_6$ × total dpm found in ubiquinone divided by the specific activity of the p-hydroxybenzaldehyde-U-C^{14} or p-hydroxybenzoic acid-U-C^{14} in dpm per mμmole. The factor $^7/_6$ allows for loss of one carbon from the substrate.

TABLE 2

INCORPORATION OF p-HYDROXYBENZALDEHYDE-U-C^{14}, p-HYDROXYBENZOIC ACID-U-C^{14}, AND p-HYDROXYBENZALDEHYDE-CARBONYL-C^{14} INTO UBIQUINONE-8 BY GROWING *Azotobacter vinelandii*

Expt.	C^{14}-Aromatic Compound Added	μmoles	dpm/μmole	Ubiquinone (dpm/μmole)	Incorporation μmoles†	Incorporation Per cent†
1	p-Hydroxybenzaldehyde-U-C^{14}	5.0	4.34×10^4	4.58×10^{3}*	0.38	6.5
2	"	8.0	2.51×10^4	9.45×10^3	0.14	1.5
3	p-Hydroxybenzaldehyde-carbonyl-C^{14}	6.3	6.25×10^4	0	—	—
4	"	6.3	6.25×10^4	0	—	—
5	p-Hydroxybenzoic acid-U-C^{14}	1.0	3.6×10^5	2.74×10^4	0.156	13.4

* Degraded; see Table 4. † See legend to Table 1. Conditions as described in the text.

TABLE 3

INCORPORATION OF p-HYDROXYBENZALDEHYDE-U-C^{14} INTO UBIQUINONE-6 BY AERATED YEAST

Expt.	C^{14}-Aromatic Compound Added	μmoles	dpm/μmole	Ubiquinone (dpm/μmole)	Incorporation μmoles†	Incorporation Per cent†
1	p-Hydroxybenzaldehyde-U-C^{14}	8.0	2.51×10^4	2.6×10^{4}*	5.7	6.1
2	"	8.0	2.51×10^4	8.95×10^{3}*	0.08	0.87

* Pooled for degradation; see Table 4. † See legend to Table 1. Conditions as described in the text.

TABLE 4

DEGRADATION OF RADIOACTIVE UBIQUINONE SAMPLES

Expt.	Ubiquinone Origin	Ubiquinone dpm/μmole	Ring fragment (dpm/μmole)
1	Rat kidney	36	38
2	Yeast	21	17
3	*A. vinelandii*	93	119

The figures give the specific activities of the ubiquinone and the substituted phenylacetic acid obtained from the benzoquinone ring. Details given in the text. The samples used for degradation are marked with an asterisk in Tables 1–3.

pathway has been obtained in *A. vinelandii*. Cell suspensions in 0.006 *M* K phosphate buffer, pH 7.2, were shaken at 38°C for 3 hr with 5 μmoles of p-hydroxybenzaldehyde-I-C^{14}. The CO_2 was trapped in KOH in a center well and was counted as $BaCO_3$ in the gas flow counter. It contained 67% of the added C^{14}. From the results of the degradations, it is concluded that in the rat, baker's yeast, and *A. vinelandii* (and in *R. rubrum*[1]) there exists a direct pathway from p-hydroxybenzaldehyde to the benzoquinone ring which does not involve degradation of the p-hydroxybenzaldehyde to two-carbon units.

R. rubrum does not utilize the carbonyl carbon atom of p-hydroxybenzaldehyde in the formation of ubiquinone,[1] and experiments 3 and 4 in Table 2 indicate that the same is true of *A. vinelandii*. In the rat[2] and in *R. rubrum*,[1] evidence has been obtained that the methyl group on the ubiquinone ring is provided by the one-carbon pool.

Olson *et al.*[2] have reported that addition of p-hydroxybenzoic acid to a rat liver slice preparation almost completely prevents the incorporation of C^{14} from tyrosine-U-C^{14} into ubiquinone. Since oxidation of p-hydroxybenzaldehyde to p-hydroxybenzoic acid is known to occur in mammals,[15, 16] and in some bacterial species,[17] it is possible that p-hydroxybenzoic acid is an intermediate in the conversion of the aldehyde to ubiquinone. Table 5 shows the results of several dilution experiments with the rat kidney mince. Each flask contained 0.011 μmoles of p-hydroxybenzaldehyde-U-C^{14}, and, as stated in the table, some contained 5.0 μmoles of an addi-

TABLE 5

THE EFFECT OF OTHER AROMATIC COMPOUNDS ON THE INCORPORATION OF p-HYDROXYBENZALDEHYDE-U-C^{14} INTO UBIQUINONE BY RAT KIDNEY MINCE

Expt.	Flask	Unlabeled substance added	Ubiquinone (dpm per μmole)
1	1	None	5,810
	2	DL-Tyrosine	4,130
	3	p-Hydroxybenzoic acid	44
	4	3,4-Dihydroxybenzoic acid	1,660
	5	3,4-Dihydroxybenzaldehyde	1,670
2	1	None	3,890
	2	p-Aminobenzoic acid	3,410

Each flask contained mince from 4 kidneys, 0.01 μmole of p-hydroxybenzaldehyde-U-C^{14} (3.94×10^7 dpm per μmole), and 5.0 μmoles of an additional aromatic material, as shown in the table. Other conditions as given in the text.

tional aromatic substance. Of the aromatic compounds tested, only p-hydroxybenzoic acid markedly reduced the incorporation of C^{14} from p-hydroxybenzaldehyde-U-C^{14} into ubiquinone, although it must be recognized that the rates of entry into the cells may differ considerably among the substances tested.

Since p-hydroxybenzoic acid may inhibit the incorporation of p-hydroxybenzaldehyde into the benzoquinone ring, the data in Table 5 do not allow the conclusion that the acid is actually a precursor of ubiquinone. Similarly, the results of Olson *et al.*[2] do not lend themselves to unequivocal interpretation, since the addition of p-hydroxybenzoic acid to their rat liver slice preparation appears to have prevented the expected incorporation of C^{14} from tyrosine-U-C^{14} into the ubiquinone side chain, as well as into the benzoquinone ring. To circumvent this difficulty, it was necessary to prepare and test p-hydroxybenzoic acid-U-C^{14}. Experiments in which varying amounts of this material were incubated with the rat kidney mince are shown in Table 1, and a single experiment with growing *A. vinelandii* is reported in Table 2. In some of the experiments in Table 1, p-hydroxybenzaldehyde-U-C^{14} was included for comparison. It can be seen that p-hydroxybenzoic acid-U-C^{14} and p-hydroxybenzaldehyde-U-C^{14} were converted to ubiquinone in approximately the same amounts. Experiments 5 and 6 in Table 1 show that when small amounts of p-hydroxybenzoic acid-U-C^{14} are added, the amount of radioactivity incorporated into ubiquinone is substantially above the level of detectable impurities in the p-hydroxybenzoic acid-U-C^{14} preparation.

It seems clear from these experiments that the rat and *A. vinelandii* possess a biosynthetic pathway from p-hydroxybenzoic acid to ubiquinone. Since very little radioactivity was found in lipids other than ubiquinone, it is likely that p-hydroxybenzoic acid enters the benzoquinone moiety and not the isoprenoid side chain, although this has not been demonstrated. The present experiments do not distinguish between the following three possibilities: (*a*) p-hydroxybenzoic acid is an intermediate in the conversion of the aldehyde to ubiquinone; (*b*) p-hydroxybenzaldehyde is an intermediate in the conversion of the acid to ubiquinone; or (*c*) the pathway from the aldehyde to ubiquinone and that from the acid to ubiquinone converge with the formation of a third, unidentified compound. Although the oxidation of the aldehyde to the acid has been demonstrated,[15-17] it is conceivable that this oxidation is not relevant to ubiquinone biosynthesis, especially since *A. vinelandii* is able to carry out a total oxidation of p-hydroxybenzaldehyde to CO_2. In *Pseudomonas* species, the acid is an intermediate in the total oxidation of the aldehyde.[17]

Olson *et al.*[2, 14] have reported that intact rats incorporate a small amount of C^{14} from phenylalanine-U-C^{14} and tyrosine-U-C^{14} into the ubiquinone ring. Rat liver slices also incorporate C^{14} from the aromatic amino acids into ubiquinone,[10, 14] although *in vitro* the C^{14} has not been unequivocally shown to enter the benzoquinone ring. Since the formation of p-hydroxybenzoic acid from tyrosine can occur in mammals,[21] a path linking tyrosine and the benzoquinone ring of ubiquinone appears to exist. Our results with tyrosine agree with those of Olson *et al.* Table 1 includes data from two experiments in which chromatographed L-tyrosine-U-C^{14} was added to the rat kidney mince. A small amount of C^{14} was incorporated into ubiquinone. The ubiquinone samples were not degraded in these experiments, but part of the C^{14} presumably entered the side chain.[2, 10, 14] However, more work is required to clarify the relationship of tyrosine to p-hydroxybenzoic acid and p-hydroxybenzaldehyde since Pitt[22] has found that p-hydroxyphenylpyruvate can undergo nonenzymatic oxidation to p-hydroxybenzaldehyde under mildly alkaline conditions. This decomposition should be kept in mind in interpretation of experiments with tyrosine.

In bacteria, p-hydroxybenzoic acid can probably arise from 3-enolpyruvyl-5-phosphoshikimic acid.[18, 19] Here it seems likely that at least one route of ubiquinone biosynthesis does not pass through phenylalanine or tyrosine. Some of the bacterial mutants isolated by Davis and Mingioli[18] required p-hydroxybenzoic acid, and this requirement was not satisfied by tyrosine. Braun *et al.*[20] have recently provided compelling evidence that the aromatic amino acids are not precursors of the ubiquinone ring in *Tetrahymena pyriformis*, a protozoan requiring phenylalanine, tyrosine, and tryptophan for growth.

In conclusion, there may be species differences in the pathways leading to p-hydroxybenzaldehyde and p-hydroxybenzoic acid. It appears that in a variety of species, both aerobic and anaerobic, there occurs a pathway extending from p-hydroxybenzaldehyde and p-hydroxybenzoic acid to ubiquinone. In the species thus far investigated, formation of the benzoquinone ring appears to involve loss of the carboxyl or aldehyde group, accompanied by C-methylation and further introduction of oxygen into the aromatic ring. The details of the mechanisms by which the aromatic ring is hydroxylated may differ considerably among aerobic and anaerobic organisms.[23-25]

Summary.—(1) p-Hydroxybenzaldehyde-U-C^{14} is incorporated into ubiquinone by proliferating *Azotobacter vinelandii*, by an aerated suspension of yeast, and by a mince of rat kidney cortex. (2) Within the limits of measurement, all of the C^{14} incorporated into ubiquinone in these systems was in a fragment consisting of the benzoquinone ring and two carbons of the side chain. (3) Proliferating *A. vinelandii* does not incorporate C^{14} from p-hydroxybenzaldehyde labeled in the carbonyl group into ubiquinone. (4) In the kidney mince and in *A. vinelandii*, p-hydroxybenzoic acid-U-C^{14} is incorporated into ubiquinone with about the same efficiency as is the aldehyde.

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*IN VITRO SYNTHESIS OF VIRAL RNA BY THE POLIOVIRUS RNA POLYMERASE**

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Infection of cultured mammalian cells by either Mengovirus or poliovirus causes the appearance of a new cytoplasmic RNA polymerase.[1, 2] The enzymes induced by the two viruses are quite similar. Both show a requirement for magnesium ions, are inhibited by manganese ions, require all four nucleoside triphosphates for maximal activity, and catalyze the incorporation of each of the nucleoside monophosphates into RNA. The two polymerases are found in particulate cytoplasmic