TABLE I

REACTIONS OF PYRONES WITH NITRILES

No.	Pyrone	Nitrile	M.P., °	Yield, %	Empirical Formula	Analyses, Calcd. (Found) Carbon	Hydrogen	Nitrogen
I_A	Kojic acid	*p*-Nitrophenylacetonitrile	167.5–168	70	$C_{14}H_{12}N_2O_6$	55.26 55.10	3.97 4.24	9.20 9.04
I_B	α-Chloro-α-deoxy kojic acid	*p*-Nitrophenylacetonitrile	118–120	86	$C_{14}H_{11}N_2ClO_5$	52.10 52.39	3.43 3.58	8.68 8.44
I_C	2-Hydroxymethyl-5-methoxy-4-pyrone	*p*-Nitrophenylacetonitrile	140	94	$C_{15}H_{14}N_2O_6$	56.60 56.41 60.23	4.43 4.29 3.49	8.80 8.86 5.40
I_D	Comenic acid	Benzonitrile	Sublimes above 200, discolors above 270	66	$C_{12}H_9NO_5$	60.51	4.70	5.12
I_E	2,6-Dimethyl-4-pyrone	*p*-Nitrophenylacetonitrile	115.5–116.5	76	$C_{15}H_{14}N_2O_4$	62.93 62.42	4.92 4.55	9.78 10.12
I_F	α-Chloro-α-deoxy kojic acid	Cyanoacetic acid	162–163	54	$C_9H_7ClO_6$[a]	43.83 44.10	2.86 3.11	—

[a] As hydrolysis of the compound failed to change the melting point and composition, the original product was the ketone and not the imide.

TABLE II

REACTIONS OF PYRONES WITH SUBSTITUTED ACRYLIC ACIDS

No.	Pyrone	Unsaturated Acid	Yield, %	M.P.	Formula	Analyses, Calcd. (Found) Carbon	Hydrogen	Chlorine
II_A	2,6-Dimethyl-4-pyrone	Cinnamic acid	98	128–129	$C_{16}H_{16}O_4$	70.59 70.91	5.92 5.70	
II_B	Coumarin	Cinnamic acid	61	142.5–143.5	$C_{18}H_{14}O_4$	73.45 73.19	4.79 4.59	
II_C	α-Chloro-α-deoxy-kojic acid	Crotonic acid	29	145–146	$C_{10}H_{11}O_5Cl$	48.69 48.52	4.49 4.29	14.37 14.19

TABLE III

CARBETHOXY DERIVATIVES OF PYRONES

No.	Pyrone	Moles of Ethyl Chlorocarbonate per Mole Pyrone	M.P.	Formula	Yield, %	Analyses, Calcd. (Found) Carbon	Hydrogen
III_A	Kojic acid	1	162–163	$C_9H_{10}O_6$	41	50.23 50.41	4.68 4.41
III_B	α-Chloro-α-deoxykojic acid	2	140–141	$C_{12}H_{13}ClO_7$	32	47.30 47.54	4.30 4.14
III_D	6-Nitrocoumarin	2	160–161.5	$C_{15}H_{13}NO_8$	51	53.73 53.48	3.90 3.68

refluxed for 90 min. and then poured into 400 ml. of water. Ten milliliters of concd. hydrochloric acid and 1 g. of aluminum chloride was then added; the resulting precipitate was filtered and dried in air, yield 2.5 g. Recrystallization of the precipitate twice from ethanol produced crystals melting at 155°.

Anal. Calcd. for $C_{24}H_{21}BrO_5$: C, 61.41; H, 4.51; Br, 17.02. Found: C, 61.02; H, 4.23; Br, 17.45.

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TEXAS SOUTHERN UNIVERSITY
HOUSTON 4, TEX.

Coenzyme Q. XIV. Reactions of Ethyl Cyanoacetate with Dimethoxybenzoquinones

CLIFFORD H. SHUNK, JAMES F. MCPHERSON, AND KARL FOLKERS

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A sensitive color reaction[1] for certain quinones, has been modified so that it is applicable to co-

(1) R. Craven, *J. Chem. Soc.*, 1605 (1931).

enzyme Q_{10} (I) and other dimethoxybenzoquinones. We find that tetrasubstituted dimethoxybenzoquinones give a positive test contrary to the early concept.[1]

Craven[1] reported an intense bluish-violet coloration, changing to blue, green, and finally reddish brown when certain quinones are treated with ethyl cyanoacetate and excess alcoholic ammonia. He stated that the reaction requires the presence of a labile hydrogen or halogen atom adjacent to a carbonyl group of the quinone.

When structure studies on coenzyme Q[2,3] first indicated that it is a dimethoxybenzoquinone containing one or two alkyl substituents, it was thought that Craven's test might indicate a tri- or a tetrasubstituted benzoquinone. It appeared that coenzyme Q_{10} is not a tetrasubstituted benzoquinone, since no color was produced in the test; however, coenzyme Q_{10} is not soluble in alcoholic aqueous ammonia. When the test was modified by dissolving coenzyme Q_{10} and ethyl cyanoacetate in absolute ethanol and then adding gaseous ammonia, a blue color developed which reached maximum intensity in about five minutes. After about thirty minutes, the color had changed to green; after twenty hours, a tan color had developed.

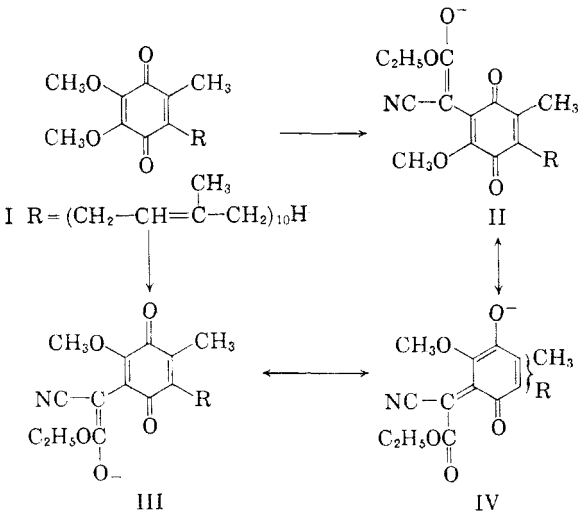
All three of the possible dimethoxydimethylbenzoquinones (2,3-dimethoxy-5,6-dimethyl-, 2,5-dimethoxy-3,6-dimethyl-, and 2,6-dimethoxy-3,5-dimethylbenzoquinone) gave positive tests. The blue color given by 2,5-dimethoxy-3,6-dimethylbenzoquinone was very persistent, and lasted for twenty-four hours. The blue colors formed by the other two compounds had changed to green after about one-half hour. Therefore, the test could not exclude a tetrasubstituted benzoquinone for the structure of coenzyme Q_{10} and did indicate that it is not a 2,5-dimethoxy-3,6-dialkylbenzoquinone.

Jeffreys[4] recently reported that the methoxy group of methoxybenzoquinone could be displaced by the anion of ethyl cyanoacetate. Previous investigators[5] had shown that methoxy groups in benzoquinones were readily displaced. Therefore, the blue color produced from coenzyme Q_{10} with ethyl cyanoacetate in the presence of ammonia is probably due to the reaction of one or both of the methoxy groups with the formation of ions represented by II, III, and IV. Although it is likely that only one of the methoxy groups is reactive under these conditions, one cannot tell without further studies whether just one or both methoxy groups participate; thus, two orientations are possible.

TABLE I

COLOR CHANGES FROM REACTION OF ETHYL CYANOACETATE WITH VARIOUS BENZOQUINONES

Benzoquinone	Color after 5 Min.	30 Min.	24 Hr.
Coenzyme Q_{10}	Blue	Green	Tan
2,3-Dimethoxy-5,6-dimethylbenzoquinone	Blue	Green	Tan
2,5-Dimethoxy-3,6-dimethylbenzoquinone	Blue	Blue	Blue
2,6-Dimethoxy-3,5-dimethylbenzoquinone	Blue	Green	Tan
2,5-Dimethoxybenzoquinone	Blue	Blue	Blue
2,6-Dimethoxybenzoquinone	Blue	Green	Tan
2,3-Dimethoxybenzoquinone	Blue	Green	Tan
2,5-Dihydroxybenzoquinone	Pink color with separation of pink crystals		
Diethoxy homolog of coenzyme Q_{10}	Light blue	Light green	Light tan
Duroquinone	Colorless	Colorless	Colorless



An ethoxy group is displaced with greater difficulty since the diethoxy homolog of coenzyme Q_{10}[6] gives a much weaker color.

Morton and co-workers[7] have reported that ubiquinone (coenzyme Q_{10}) and aurantiogliocladin (2,3-dimethoxy-5,6-dimethylbenzoquinone) give a blue color only slowly with ethyl cyanoacetate in ammonia-ethanol.

The blue color produced by the action of potassium hydroxide on an ethanolic solution of coenzyme Q_{10} and ethyl cyanoacetate has been de-

(2) R. L. Lester, F. L. Crane, and Y. Hatefi, *J. Am. Chem. Soc.*, **80**, 4751 (1958).

(3) D. E. Wolf, C. H. Hoffman, N. R. Trenner, B. H. Arison, C. H. Shunk, B. O. Linn, J. F. McPherson, and K. Folkers, *J. Am. Chem. Soc.*, **80**, 4752 (1958).

(4) J. A. D. Jeffreys, *J. Chem. Soc.*, 2153 (1959).

(5) D. Buckley, H. B. Henbest, and P. Slade, *J. Chem. Soc.*, 4891 (1957).

(6) B. O. Linn, N. R. Trenner, C. H. Shunk, and K. Folkers, *J. Am. Chem. Soc.*, **81**, 1263 (1959).

(7) R. A. Morton, U. Gloor, O. Schindler, G. M. Wilson, L. H. Chopard-dit-Jean, F. W. Hemming, O. Isler, W. M. F. Leat, J. F. Pennock, R. Rüegg, U. Schwieter, and O. Wiss, *Helv. Chim. Acta.*, **41**, 2343 (1958).

veloped into a quantitative determination of coenzyme Q_{10} in urine.[8]

EXPERIMENTAL

About 1 mg. of a substituted benzoquinone was dissolved in 2 ml. of absolute ethanol, if necessary, with warming. The solution was cooled to room temperature, 2 drops of ethyl cyanoacetate was added, and then anhydrous ammonia was absorbed in the solution for 1–2 min. The colors formed by various substituted benzoquinones are listed in Table I.

MERCK SHARP & DOHME RESEARCH LABORATORIES
RAHWAY, N. J.

(8) F. R. Koniuszy, P. H. Gale, A. C. Page, Jr., and K. Folkers, *Arch. Biochem. and Biophys., in press.*

The Preparation of C^{14}-Labeled Spermine and C^{14}-Labeled Spermidine

E. L. JACKSON AND S. M. ROSENTHAL

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The reaction of acrylonitrile with putrescine to produce *N,N'*-bis(2-cyanoethyl)putrescine (I) and catalytic reduction of this nitrile to yield spermine (II) has been reported by Schultz.[1] Spermidine (III) was not obtained in this process by Schultz. The method has been adapted by us to the small-scale preparation, in one operation, of both C^{14}-labeled spermine and C^{14}-labeled spermidine. The cyanoethylation of putrescine-1-C^{14} with the use of nonisotopic acrylonitrile was carried out in ethanol solution. Reduction of the products, without isolation, by Raney nickel and hydrogen at 136–142° and 4700 p.s.i. yielded spermine and spermidine labeled with C^{14} in the putrescine moiety. The spermine and spermidine were separated chromatographically. Application of the procedure to the products of the cyanoethylation of nonisotopic putrescine by acrylonitrile-1-C^{14} afforded spermine and spermidine labeled with C^{14} in the propylamine moiety. The production of spermidine in this way may be due to the presence of its parent nitrile[2] (IV) among the products of the cyanoethylation of putrescine. As the reaction of acrylonitrile with amines is reversible,[3] the spermidine also could owe its origin to the partial dissociation of *N,N'*-bis-(2-cyanoethyl)putrescine to form IV; such a dissociation would be promoted by the elevated reduction temperature.

$$NC(CH_2)_2NH(CH_2)_4NH(CH_2)_2CN \quad (I)$$

$$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2 \quad (II)$$

$$H_2N(CH_2)_4NH(CH_2)_3NH_2 \quad (III)$$

$$H_2N(CH_2)_4NH(CH_2)_2CN \quad (IV)$$

EXPERIMENTAL

Spermine and spermidine from putrescine-1-C^{14} and acrylonitrile. To a suspension of 223 mg. of putrescine-1-C^{14} dihydrochloride[4] (1.38 mmoles) in 2 ml. of absolute ethanol was added 1.44 ml. of 1.92*N* sodium hydroxide which gave a solution of putrescine-1-C^{14} base. A solution of 164 mg. of nonisotopic acrylonitrile (3.09 mmoles) in 2 ml. of absolute ethanol was mixed with the putrescine-1-C^{14} solution. After being shaken for 5 min., the solution was kept at room temperature for 18 hr. It was diluted with 1 ml. of absolute ethanol, then refluxed for 1 hr. and kept at room temperature for 2 hr. The solution was transferred to a hydrogenation bomb and mixed with *ca.* 0.3 g. of Raney nickel catalyst and 20 ml. of absolute ethanol which had been saturated with ammonia at 20–23°. The mixture was shaken with hydrogen at 136–142° and 4700 p.s.i. for 30 min. The catalyst was filtered and washed thoroughly with absolute ethanol. Nearly all of the solvent was evaporated, with the use of a column, on the steam bath and the residue was neutralized to *p*H 7.0 with hydrochloric acid. Chromatographic separation of the spermidine and spermine was carried out upon Dowex 50 resin (2% cross linked, 100–200 mesh) in the hydrogen form. A column 24 × 1.5 cm. inside diameter was employed with gradient chromatography[4] using 300 ml. of water in the mixing flask and 2.5*N* hydrochloric acid in the reservoir. Spermidine appeared in the eluate between 344 and 468 ml. Spermine was eluted between 484 and 650 ml. These fractions were evaporated to dryness *in vacuo* over potassium hydroxide, and each was subjected to a second chromatography. The yields were 115 mg. (0.45 mmole) of spermidine trihydrochloride (0.034 μc per μ mole) and 114 mg. (0.33 mmole) of spermine tetrahydrochloride (0.034 μc per μ mole). The specific activity of the starting putrescine-1-C^{14} dihydrochloride was 0.034 μc per μ mole.

The above radioactive spermidine trihydrochloride and spermine tetrahydrochloride were found to be contaminated with small amounts of unknown material, and required further purification. An aliquot of the spermidine hydrochloride was recrystallized by dissolving it in a minimum quantity of absolute methanol, acidifying the solution with ethanolic hydrochloric acid, adding an equal volume of ethanol, and then adding ethyl acetate dropwise until precipitation occurred. After standing overnight at 5°, the precipitate was collected by centrifugation. The spermine was recrystallized by dissolving the hydrochloride in 1 ml. of water and adding a slight excess of 1*M* sodium phosphate solution of *p*H 7.2. On standing overnight at 5° spermine phosphate crystallized.

For identification of spermidine and spermine, the compounds were prepared as described with the use of nonisotopic putrescine dihydrochloride and acrylonitrile. An aqueous solution of the spermidine trihydrochloride from the column was treated with a few drops of 37% hydrochloric acid, then concentrated to a small volume and diluted with ethanol to start crystallization; m.p. 257–258° (uncorr.). The crystals showed an infrared spectrum identical with that of authentic spermidine trihydrochloride.

Anal. Calcd. for $C_7H_{22}Cl_3N_3$: C, 33.01; H, 8.71; Cl, 41.77; N, 16.50. Found (dried at 100° *in vacuo*): C, 33.30; H, 8.60; Cl, 41.85; N, 16.78.

The spermine tetrahydrochloride was thrice recrystallized from a mixture of 12% hydrochloric acid and ethanol. The infrared spectrum of these crystals was identical with that of authentic spermine tetrahydrochloride.

(1) H. P. Schultz, *J. Am. Chem. Soc.*, **70**, 2666 (1948).
(2) See O. Bayer, *Angew. Chem.*, **61**, 235 (1949).
(3) F. C. Whitmore, *et al.*, *J. Am. Chem. Soc.*, **66**, 725 (1944).
(4) H. Tabor, S. M. Rosenthal, and C. W. Tabor, *J. Biol. Chem.*, **233**, 907 (1958).