Coleman, J. E., and Vallee, B. L. (1962), *Biochemistry 1*, 1083.
Druyan, R., and Vallee, B. L. (1962), *Fed. Proc. 21*, 247.
Hoch, F. L., Martin, R. G., Wacker, W. E. C., and Vallee, B. L. (1960), *Arch. Biochem. Biophys. 91*, 166.
Hughes, T. R., and Klotz, I. M. (1956), *Methods Biochem. Analy. 3*, 265.
Kaplan, N. O. (1960), *Enzymes 3*, 105.
Kaplan, N. O., and Ciotti, M. M. (1954), *J. Biol. Chem. 211*, 431.
Kaplan, N. O., Ciotti, M. M., and Stolzenbach, F. E. (1957), *Arch. Biochem. Biophys. 69*, 441.
Kaye, M. A. G. (1955), *Biochim. Biophys. Acta 18*, 456.
Li, T. K., Ulmer, D. D., and Vallee, B. L. (1962), *Biochemistry 1*, 114.
Li, T. K., Ulmer, D. D., and Vallee, B. L. (1963), *Biochemistry 2*, 482.
Li, T. K., and Vallee, B. L. (1963), Abstract of papers, 142nd Meeting, American Chemical Society, p. 67c.
Li, T. K., and Vallee, B. L. (1964), *J. Biol. Chem. 239*, 792.
Mahler, H. R., Baker, R. H., and Shiner, V. J. (1962), *Biochemistry 1*, 47.
Plane, R. A., and Theorell, H. D. (1961), *Acta Chem. Scand. 15*, 1866.
Schubert, J. (1956), *Methods Biochem. Analy. 3*, 247.
Snedecor, G. W. (1946), Statistical Methods, Ames, Iowa, Iowa State College Press.
Theorell H., and Chance, B. (1951), *Acta Chem. Scand. 5*, 1127.
Theorell, H. D., and McKinley-McKee, J. S. (1961), *Acta Chem. Scand. 15*, 1811.
Theorell, H. D., Nygaard, A. P., and Bonnichsen, R. (1955), *Acta Chem. Scand. 9*, 1148.
Ulmer, D. D., Li, T. K., and Vallee, B. L. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 1155.
Vallee, B. L. (1955), *Advan. Protein Chem. 10*, 318.
Vallee, B. L., and Coombs, T. L. (1959), *J. Biol. Chem. 236*, 2615.
Vallee, B. L., and Hoch, F. L. (1955), *Proc. Natl. Acad. Sci. U. S. 41*, 237.
Vallee, B. L., Hoch, F. L., Adelstein, S. J., and Wacker, W. E. C. (1956), *J. Am. Chem. Soc. 78*, 5879.
Vallee, B. L., Williams, R. J. P., and Hoch, F. L. (1959), *J. Biol. Chem. 234*, 2621.
Wallenfels, K., and Sund, H. (1957), *Biochem. Z. 329*, 41.
Winer, A. D. (1958), *Acta Chem. Scand. 12*, 1695.
Winer, A. D., and Theorell, H. D. (1960), *Acta Chem. Scand. 14*, 729.
Yonetani, T. (1963), *Acta Chem. Scand. 17*, (*Suppl. 1*), 96.

The 2-Desmethyl Vitamin K_2's. A New Group of Naphthoquinones Isolated from *Hemophilus parainfluenzae**

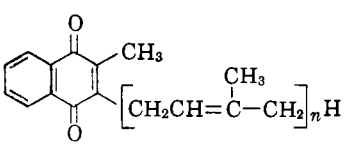
ROBERT L. LESTER, DAVID C. WHITE, AND STANFORD L. SMITH

From the Departments of Biochemistry and Chemistry, University of Kentucky, Lexington

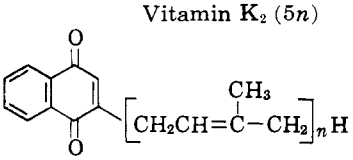
Received March 19, 1964

Three naphthoquinones related to vitamin K_2 have been purified from *Hemophilus parainfluenzae*. Ultraviolet, infrared, and nuclear magnetic resonance spectra are consistent with a structure that differs from vitamin K_2 in that the 2-methyl substituent is replaced with hydrogen; this group of compounds is termed the 2-desmethyl vitamin K_2's. The principal component has a C_{30} polyisoprenoid side chain; lesser amounts of what appear to be the C_{25} and C_{35} isoprenologs have also been detected. A method is described for reversed-phase paper chromatography of these compounds and subsequent detection by ultraviolet absorbance. A reliable technique for reduction of these compounds by KBH_4 is also described.

The isoprenologs of coenzyme Q and vitamin K_2 occur widely in microorganisms. A growing body of evidence suggests a respiratory function for these quinones. Studies on the development and characterization of the electron-transport system in *Hemophilus parainfluenzae* (White and Smith, 1962) and the demonstration of a menadione-requiring auxotroph of a closely related strain (Lev and Reiter, 1962) led us to attempt to characterize the quinones in *H. parainfluenzae*. Examination of the lipid extracts of this organism revealed the presence of naphthoquinones which differed from vitamin K_2 in that the methyl substituent on the quinone ring was replaced with a hydrogen. Three such compounds which differ in the length of their polyisoprenoid side chains have been recognized in *H. parainfluenzae*. A concise nomenclature for these compounds can be based on the system used for the vitamin K_2 homologs. The group of compounds is designated as the 2-desmethyl vitamin K_2's or DMK_2.[1] As in the vitamin K_2 series the number of carbon atoms in the side chains is given for the particular homolog. Thus in *H. parainfluenzae* we have found large amounts of DMK_2 (30) with lesser amounts of DMK_2 (25) and DMK_2 (35).



Vitamin K_2 (5*n*)



DMK_2 (5*n*)

This communication will describe the isolation and characterization of this group of compounds.

* This investigation was supported by research grants (GM-07627 and GM-10285) from the National Institutes of Health, U. S. Public Health Service.

[1] Abbreviations used in this work: DMK_2, the 2-desmethyl derivatives of vitamin K_2; SFQ, substance purified from lipid extracts of as train of *Streptococcus faecalis* (Baum and Dolin, 1963).

EXPERIMENTAL

Growth of Bacteria.—The strain of *H. parainfluenzae* was that utilized previously (White and Smith, 1962). The growth medium contained 2% proteose peptone, 0.5% yeast extract (Difco), 102 mM NaCl, 9 mM KNO_3,

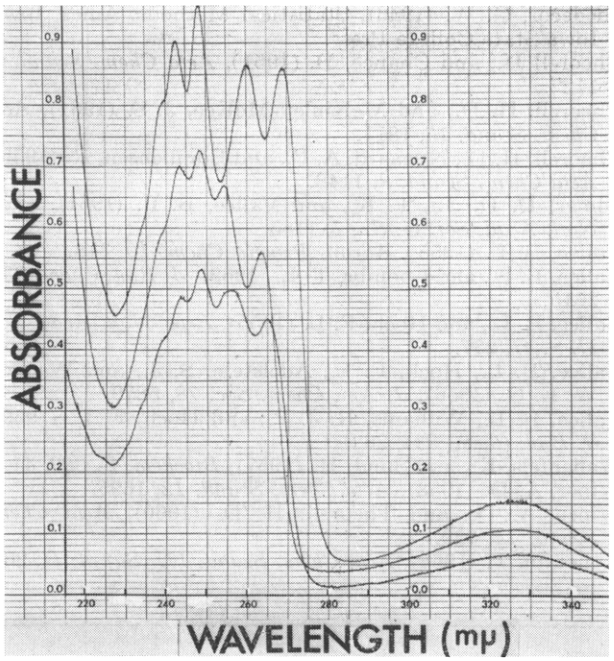


FIG. 1.—Ultraviolet spectra of DMK_2 and related naphthoquinones. Upper, vitamin K_2 (30); lower, 2-undecyl-1,4-naphthoquinone; middle, DMK_2 (fraction I), 0.021 mg/ml. All compounds were dissolved in isooctane.

0.115 mM $Na_2S_2O_4$, 50 mM Na-gluconate, and 20 mM Tris at *p*H 7.6. This medium was boiled, filtered, autoclaved at 118° for 25 minutes, and incubated in 2.5-liter Parrot flasks containing 1 liter of medium. After cooling, filter-sterilized DPN was added to a final concentration of 1.5 μM. Flasks were inoculated with 10^5 late-stationary-phase cells and incubated on a rotary shaker at 37° for 15 hours. Cells were harvested by centrifugation in early-stationary phase at a yield of about 600 mg dry wt per liter of medium. The bacteria were washed in 50 mM potassium phosphate buffer, *p*H 7.6, and stored as pellets at −20°. Lack of contamination was established as described by White (1962).

Isolation of DMK_2.—Pooled pellets representing 119 g dry wt were blended in 610 ml of 50 mM phosphate buffer, *p*H 7.6, and 9 volumes of acetone was slowly added with stirring. All subsequent operations were carried out in as dark a laboratory as possible. The suspension was stirred at room temperature for 17 hours and then filtered through paper. To the filtrate was added $^1/_5$ volume of water and $^1/_5$ volume of isooctane. After shaking in a separatory funnel the isooctane layer was collected. The acetone-water phase was reextracted after additional $^1/_5$ volumes of isooctane and 4 M KCl had been added. The cell residue was washed with $^1/_4$ volume of 9:1 acetone-water which was partitioned as above. All isooctane fractions were pooled and evaporated to dryness under reduced pressure at a temperature of less than 30°. The lipid was then dissolved in 32 ml absolute ethanol at 45° and cooled to 0° overnight. The mixture was centrifuged and the supernatant was removed. Further impurities were precipitated from the supernatant at −20°. The final supernatant (27.5 ml) was again evaporated to dryness with reduced pressure and dissolved in isooctane.

Column Chromatography.—Further purification was achieved by chromatography on silicic acid (100 mesh, Mallinckrodt) treated to remove fines by decantation in water, and further washed and dried according to Rouser *et al.* (1961).

TABLE I
PURIFICATION OF DMK_2

Purification Stage[a]	Total Absorbance Units[b]	$\Delta E^{1\%}_{1\,cm}$ (263–280 mμ)
1. Cells (119 g)	2780[c]	0.2[c]
2. Initial isooctane extract	2780	9.4
3. Ethanol supernatant (−20°)	2100	23
4. Final silicic acid column		
Fraction I	1310	253
Fraction II	300	

[a] See Experimental for details of purification procedure. [b] Δ absorbance (263–280 mμ) × volume (ml); 1-cm light path. [c] Calculated on the assumption that the DMK_2 is quantitatively extracted.

A 1.5 × 15-cm column of silicic acid was equilibrated with isooctane and the sample obtained after low temperature alcohol fractionation was applied in isooctane. The column was eluted with 80 ml of isooctane, followed by 10% $CHCl_3$–90% isooctane. A yellow band containing DMK_2 was immediately eluted with 60 ml of the latter solvent. Since it seemed that this column was overloaded, probably due to the presence of contaminating phospholipids, the DMK_2 fraction was taken to dryness, redissolved in isooctane, and applied to a 1.5 × 21-cm silicic acid column. The sample was washed on with 120 ml isooctane and the DMK_2 fraction was eluted with 45% $CHCl_3$–55% isooctane. The bulk of the DMK_2 was eluted between 300 and 400 ml as a yellow band (fraction I). The rest of the DMK_2 emerged between 400 and 560 ml and had a brownish-orange color (fraction II). Each fraction was taken to dryness and redissolved in isooctane. The ultraviolet spectrum of fraction I is shown in Figure 1. The ultraviolet spectrum of fraction II differed from that of fraction I in that it had somewhat more end absorption in addition to the typical DMK_2 absorption bands.

Paper Chromatography.—A reversed-phase paper-chromatographic system was developed that separated the DMK_2 homologs. With this system it was possible to elute the DMK_2 from the paper and directly take ultraviolet-absorption spectra with negligible blank absorption from the paper and stationary phase. Whatman 3MM paper was dipped into a $CHCl_3$ solution containing 10% (w/v) No. 200 Dow Corning silicone oil (100 centistokes) and allowed to dry. Samples containing 10–200 μg were applied to 36-cm (14-inch) chromatograms and developed ascending with 95% methanol–5% H_2O to which was added 0.1% glacial acetic acid. The spots of DMK_2 could be observed as quenching areas with an ultraviolet lamp. After extended exposure the spots acquired a red fluorescence. If ultraviolet spectra were required as soon as the chromatogram was dry the spots were quickly circled with a pencil, cut out, and eluted with isooctane.

Absorption Spectra.—Ultraviolet-absorption spectra were measured with a Cary Model 15 spectrophotometer. Infrared spectra were obtained in CCl_4 with a Perkin Elmer Model 21 spectrometer.

Nuclear Magnetic Resonance Spectra.—Nuclear magnetic resonance spectra were run on CCl_4 solutions containing 1% tetramethylsilane as internal standard using a Varian Associates HR-60 spectrometer equipped with a flux stabilizer and electronic integrator. Spectra were calibrated by the usual side-band technique (Packard and Arnold, 1951). Integrated signal areas are the average of three or more alternate up- and downfield sweeps and are accurate to ±2% or better.

TABLE II
ULTRAVIOLET-ABSORPTION SPECTRA OF DMK$_2$ AND RELATED COMPOUNDS[a]

Compound	$\lambda\lambda_{max}$ (A)					
Vitamin K$_2$ (30)	3259	2691	2603	2484	2429	2390 sh
ϵ mM[b]				18.6		
2-Undecyl-1,4-naphthoquinone	3272	2652	2562	2487	2435	2390 sh
Fraction I	3265	2637	2544	2485	2436	2390 sh
$E^{1\%}_{1\,cm}$	52.7	272	321	348	336	
ϵ mM[c]	3.1	15.5	18.3	19.9	19.2	
Fraction I (ethanol):						
Oxidized	3300	2650 sh	2510	2460		
Reduced	~3240			2460		
	~3370					
Absorbance reduced ÷ oxidized		0.211		2.08		

[a] Spectra obtained with isooctane as solvent unless otherwise indicated. Reduced spectrum obtained by reduction with KBH_4 as indicated under Experimental. [b] Isler *et al.* (1960). [c] ϵ mM calculated for fraction I assuming composition given in Table III.

Chemical shifts are expressed as parts per million downfield from tetramethylsilane and are accurate to ±0.05 ppm or better for sharp peaks and signals showing first-order splittings. For broad unresolved signals or complex multiplets the location of the estimated weighted center is given and uncertainty is indicated by the prefix *ca.*

Reduction of DMK$_2$.—DMK$_2$ was converted to the hydroquinone form by reduction with KBH_4. In this reaction basic conditions must be avoided. The reaction was carried out by dissolving the quinone in ethanol (0.02 mM) containing 1/100 volume of 1 M ammonium acetate buffer (aqueous), *p*H 5.0. Addition of 4 μmoles KBH_4 from a freshly prepared aqueous solution to 1 ml of the quinone solution results in immediate and usually complete reduction. If addition of more KBH_4 causes no further absorbance changes at 246 mμ and 265 mμ, complete reduction can be assumed.

RESULTS

The purification procedure employing solvent fractionation and silicic acid chromatography led to a 1250-fold concentration of product with a 60% recovery (Table I). After the initial lipid extraction the principal impurities were phospholipids. The final product (fraction I) was a dark-orange oil. The purification procedure was monitored throughout by the ultraviolet spectrum. The principal absorbing impurities had high end absorption in the far ultraviolet. Hence it was felt that, instead of measuring the absolute absorbance at 249 mμ, a more reliable assay procedure would be to measure the difference in absorbance between the longest-wave major ultraviolet band (263 mμ) and a nearby minimum (280 mμ).

Ultraviolet Spectra.—A comparison of the ultraviolet spectrum of DMK$_2$ with other known naphthoquinones was made (Table II, Figure 1). This comparison, along with literature data (Morton and Elam, 1941), shows that the spectrum of DMK$_2$ most closely matched that of a monosubstituted 1,4-naphthoquinone. As can be seen from Figure 1, vitamin K$_2$, DMK$_2$, and 2-undecyl-1,4-naphthoquinone all have absorption maxima at 239, 243, and 326 mμ. These maxima have been interpreted as due to the benzenoid chromophore, —C_6H_4—CO—, in naphthoquinones whereas the other two principal maxima are attributed to the quinoid moiety (Morton and Elam, 1941). The exact positions of these latter maxima depend on the nature of the substituents on this portion of the molecule. It can be seen that in the monosubstituted naphthoquinones these latter maxima are shifted to a shorter wavelength. It was felt that the slight differences between the spectra of DMK$_2$ and 2-undecyl-1,4-naphthoquinone could be explained by the presence of an allyl substituent in DMK$_2$ which is typical of other naturally occurring quinones with polyisoprenoid side chains.

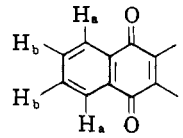
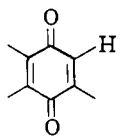
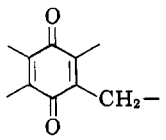
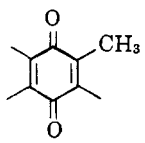
Detection of Homologs.—Reversed-phase paper chromatography of fractions I and II revealed the presence of one main spot with lesser amounts of two other spots. After elution from the paper each of the lesser spots gave an ultraviolet spectrum that was qualitatively identical to that of the major spot. By analogy with the findings on coenzyme Q (Lester *et al.*, 1959), it was felt that we were dealing with homologs that had the same ultraviolet chromophore but differed in the side-chain length. As can be seen from Table III, the R_F values of the DMK$_2$'s matched closely with those for vitamins K$_2$ (25–35). The R_F of the major DMK$_2$ component was closest to that of vitamin K$_2$ (30). The extinction coefficient at 249 mμ (Table II) indicated high purity with respect to the naphthoquinone moiety. No other substances could be detected with general lipid sprays on chromatograms. Therefore, in view of the small amount of pure compound available, separation of each of the homologs in pure form was not attempted. It was also thought that more concrete evidence was necessary to establish the proposed structure and that this could be obtained with nuclear magnetic resonance spectroscopy.

TABLE III
PAPER CHROMATOGRAPHY OF DMK$_2$ FRACTIONS AND VITAMIN K$_2$ HOMOLOGS[a]

Sample	R_F	Per Cent of Total Absorbance (263–280 mμ)
Fraction I		
DMK$_2$ (35)	0.21	10
DMK$_2$ (30)	0.29	85.5
DMK$_2$ (25)	0.40	4.5
Fraction II		
DMK$_2$ (35)	0.21	3.2
DMK$_2$ (30)	0.29	91.4
DMK$_2$ (25)	0.40	5.4
Vitamin K$_2$ (35)	0.18	
Vitamin K$_2$ (30)	0.27	
Vitamin K$_2$ (25)	0.36	

[a] Chromatography and elution of spots carried out as indicated under Experimental. Recovery of total DMK$_2$ absorbance was 80%.

TABLE IV
COMPARISON OF NUCLEAR MAGNETIC RESONANCE SPECTRA[a]

	Compound					
	2-DMK_2 (30) (Fraction I)	2-Undecyl-1,4-naphthoquinone	Vitamin K_2 (30)	Vitamin K_9 (H)	Chlorobium-quinone	CoQ_{10}
	(a) 7.99 m (2) (b) 7.62 m (2)	(a) 8.00 m (2) (b) 7.63 m (2)	7.9 m		7.9 m	
	6.59 t (1)	6.63 t (1)				
	3.16 d (2)	2.47 m (2)	3.4 d	3.3 d		3.07 d (2)
			2.2 s	2.17 s	2.1 s	2.0 sh
—CH=	5.13 t 4.99 b[b]		5.1 b	5.02 (8)	5.1 b (6) 6.2 d (1)	5.03 b (10)
=C—CH_2—CH_2—C(=CH_3)=	1.91 (20.5)		2.0 b	1.98	2.0	1.97 (37)
=C(—CH_3)—	1.51, 1.63 (21.1)		1.6 b, 1.8 d	1.59, 1.68, 1.80	1.7 d, 1.6 b, (21)	1.58, 1.67, 1.73 (36)
sat —CH_2—	1.21 (trace)	1.23 b		1.38 (6)		
sat —CH_3	0.86 (trace)	0.86 m		0.88 (3)		
Reference			Frydman and Rapoport (1963)	Gale *et al.* (1963)	Frydman and Rapoport (1963)	Gale *et al.* (1963)

[a] Chemical shifts are in parts per million downfield from tetramethylsilane; s = singlet, d = doublet, t = triplet, m = multiplet, b = broad, sh = shoulder; numbers in parentheses are relative numbers of protons.

Nuclear Magnetic Resonance and Infrared Spectra.—The structure of DMK_2 is unambiguously established by its nuclear magnetic resonance spectrum (Table IV), the most significant feature of which is a one-proton triplet ($J \cong 1.4$ cps) at 6.59 ppm. The small splitting is characteristic of four-bond allylic couplings (Jackman, 1959) and must be due to a hydrogen on C_2 of the naphthoquinone ring coupled to the $—CH_2—$ on C_3. This assignment is verified by comparison with the nuclear magnetic resonance spectrum of 3-undecyl-1,4-naphthoquinone, which shows an identical one-proton triplet ($J \cong 1.4$ cps) at 6.63 ppm.

The remaining signals in the nuclear magnetic resonance spectrum of DMK_2 are characteristic of a 1,4-naphthoquinone (two proton multiplets at *ca.* 7.62 and 7.99 ppm corresponding to protons on C_6,C_7 and C_5,C_8, respectively) with an isoprenoid side chain. The side chain $—CH_2—$ next to the ring appears as a broadened two-proton doublet ($J \cong 7.2$ cps) at 3.16 ppm. An identical spacing is observed in a broad triplet at *ca.* 5.13 ppm (olefinic proton on the second carbon from the ring) which is partially obscured by the broad signal at *ca.* 4.99 ppm owing to the remaining olefinic protons in the side chain. The relative signal areas of olefin to allylic $—CH_2—$ (1.91 ppm) to allylic $—CH_3$ (1.51 and 1.63 ppm) in the side chain are 6.00:20.5:21.1 (theory for a C_{30} unit; 6.00:20.0:21.0). The ratio of aromatic protons to total side-chain protons is 4.00:48.5 (theoretical 4.00:49.0).

The presence of small quantities of both higher and lower homologs indicated by chromatographic analysis does not significantly alter the calculated side-chain signal ratios. However, an increase or decrease of one isoprenoid unit in the side chain of the major constituent would drastically alter the ratio of aromatic protons to total side-chain protons (4:57 and 4:41, respectively).

The relative signal intensities observed, the coupling patterns of those protons adjacent to the naphthoquinone ring, and the absence of any signals due to paraffinic protons[2] clearly indicate that the side chain is a C_{39} isoprenoid chain.

The infrared spectrum obtained with fraction I is consistent with the proposed structure. Maxima were observed at the following wavelengths (microns): 3.43 s; 3.47 sh; 5.98 s; 6.31 m; 6.49 m; 6.90 m; 7.23 m; 7.53 m; 7.69 s; 7.90 w; 8.03 m; 11.6 m; 14.42 bw.

Reduction of DMK_2 with KBH_4.—The ultraviolet-absorption spectrum of fraction I in ethanol before and after treatment with KBH_4 is shown in Figure 2. It can be seen that the absorption bands of the quinone

[2] Weak signals due to trace impurities appear at 0.86 and 1.21 ppm corresponding to paraffinic $-CH_3$ and $-CH_2-$, respectively. These are probably due to traces of isooctane remaining from the chromatographic purification. However, the presence of small amounts of 2-DMK_2's with partially saturated side chains can not be excluded.

are not as well defined as those obtained with isooctane as solvent. The spectrum of the hydroquinone could be reproducibly obtained only if care were taken to prevent accumulation of an excess of base produced by hydrolysis of the borohydride.

Apparently both the quinone and hydroquinone forms are unstable in dilute ethanolic KOH solutions as indicated by the following spectral observations. To an ethanol solution of DMK_2 was added KOH to a final concentration of 0.03 N. This resulted in the appearance of a broad band at 302 mμ (λ_{min} = 260 mμ) and disappearance of the maxima in the 240–270-mμ region. The absorbance at 302 mμ increased *ca.* 13-fold, reached a maximum after 2 minutes, and declined linearly for at least 7 minutes at 2–3%/minute. The solution had a violet color after addition of alkali. When an aqueous solution of KBH_4 was added to a solution of DMK_2 in ethanol to a final concentration of 10^{-3} M KBH_4, the absorbance at 246 mμ fell instantaneously *ca.* 10%, then rose and reached a maximum in 30 seconds, and then dropped rapidly. The initial drop in absorbance is probably due to the alkali in the borohydride solution. The rapid increase is due to formation of the hydroquinone, and the subsequent decrease is probably due to decomposition of the hydroquinone since addition of HCl at this point stops the decrease in absorbance. It was found that smooth reduction could be obtained if the system were buffered with slightly acidic ammonium acetate as described under Experimental. In this case reduction was prompt and the resulting product was stable. This procedure should prove to be valuable for the spectrophotometric assay of DMK_2 and other alkali-unstable quinones in lipid extracts. In crude extracts end absorption is usually high owing to other compounds, so that absolute absorbance measurements would give erroneously high results. Measurement of the change in absorbance at 246 mμ after borohydride treatment should give a more reliable estimate of DMK_2.

Discussion

Recently, Baum and Dolin (1963) reported briefly on a substance purified from lipid extracts of a strain of *Streptococcus faecalis*. This substance, termed SFQ, was considered to be a 1,4-naphthoquinone monosubstituted with a C_{40} or C_{45} polyisoprenoid side chain. This conclusion was based on its ultraviolet-absorption spectra and its reaction in ethanolic alkali. The evidence for the length of the side chain was based on the extinction coefficient [$E^{1\%}_{1cm}$ (248 mμ) = 264] and on its R_F in reversed-phase paper chromatography.

DMK_2 isolated from *H. parainfluenzae* has an ultraviolet spectrum that is qualitatively identical to that of SFQ. It also has a similar alkali lability. These facts suggest that the basic features of the structure are the same. The only possible difference would be in the length of the polyisoprenoid side chain. The lower extinction coefficient obtained for SFQ could be explained by the presence of a larger side chain (C_{40}–C_{45}) or possibly by the presence of impurities which do not contribute to the absorbance. NMR and/or molecular weight data on SFQ should settle this point and fully characterize SFQ. The only other discrepancy is the reported 40% increase in absorbance of SFQ at 245 mμ after reduction with KBH_4. The conditions used for reduction of DMK_2 result in a 100% increase in absorbance upon reduction with KBH_4 (Table II). This apparent discrepancy is probably explained by partial decomposition of the quinone under alkaline conditions as outlined under Results. Assuming a C_{40} or C_{45} side chain for SFQ, evidence now exists for at least four homologs of this new family of compounds. Thus this group is already almost as large as the coenzyme Q group in which five homologs have already been described.

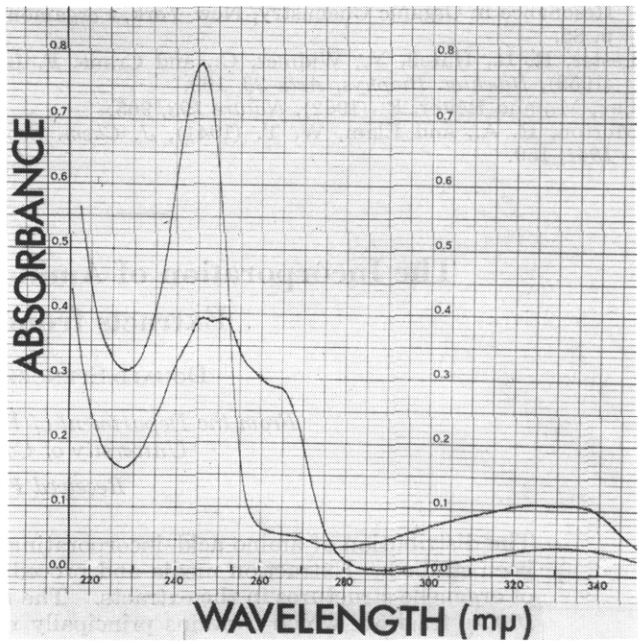
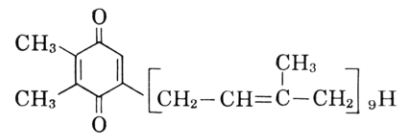


Fig. 2.—Ultraviolet spectra of oxidized and reduced forms of DMK_2 in ethanol. Reduction of DMK_2 (0.011 mg/ml) with KBH_4 carried out as described under Experimental. The curve with the highest absorbance is the reduced form.

The basic structure of this compound is in a sense analogous to plastoquinone:



in that it is a trisubstituted quinone with a long polyisoprenoid side chain.

One might speculate that DMK_2 in *H. parainfluenzae* serves as an electron carrier in the electron-transport system and/or as a component of the oxidative phosphorylation apparatus. The only data bearing on this point are that in preliminary experiments the level of DMK_2 in *H. parainfluenzae* under various growth conditions is correlated with the level of cytochromes and the respiration rate.

Acknowledgments

The authors gratefully acknowledge the technical assistance of W. A. Duke and E. M. Farley. The generous gift of synthetic 2-undecyl-1,4-naphthoquinone from Dr. L. Fieser of Harvard University and homologs of vitamin K_2 from O. Isler of Hoffman La Roche are gratefully acknowledged.

References

Baum, R. H., and Dolin, M. I. (1963), *J. Biol. Chem. 238*, PC 4109.

Frydman, B., and Rapoport, H. (1963), *J. Am. Chem. Soc. 85*, 823.

Gale, P. H., Arison, B. H., Trenner, N. R., Page, A. C., and Folkers, K. (1963), *Biochemistry 2*, 196, 200.

Isler, O., Rüegg, R., and Langemann, A. (1960), *Chem. Weekblad 56*, 613.

Jackman, L. M. (1959), Applications of Nuclear Magnetic

Resonance in Organic Chemistry, New York, Pergamon, p. 85.
Lester, R. L., Hatefi, Y., Widmer, C., and Crane, F. L. (1959), *Biochim. Biophys. Acta 33*, 169.
Lev, M., and Reiter, B. (1962), *Nature 193*, 905.
Morton, R. A., and Elam, W. T. (1941), *J. Chem. Soc. 1941*, 159.
Packard, J. T., and Arnold, M. T. (1951), *J. Chem. Phys. 19*, 1608.
Rouser, G., O'Brien, J., and Heller, D. (1961), *J. Am. Oil Chemists' Soc. 38*, 14.
White, D. C. (1962), *J. Bacteriol. 83*, 851.
White, D. C., and Smith, L. (1962), *J. Biol. Chem. 237*, 1332.

The Incorporation of Amino Acids into Protein by Cell-free Extracts from Tobacco Leaves*

DONALD SPENCER† AND S. G. WILDMAN

From the Department of Botany and Plant Biochemistry, University of California, Los Angeles

Received February 4, 1964

The distribution of amino acid–incorporating activity in cell-free extracts of tobacco leaves has been studied. Extraction media and procedures were designed to give maximum preservation of organelle structures in the extracts. The major portion of the activity is associated with the 1000-*g* fraction, which contains principally nuclei and chloroplasts, together with some mitochondria, spherosomes, and fragments of the nuclei and chloroplasts. Evidence is presented that the nuclei are not contributing to amino acid incorporation by the 1000-*g* fraction. Incorporation of [^{14}C]valine into this fraction is strongly dependent on exogenous adenosine triphosphate, and in addition requires Mg^{2+}, a mixture of amino acids, a mixture of guanosine, uridine, and cytidine triphosphates, and an adenosine triphosphate–generating system for maximum activity. The system is extremely sensitive to ribonuclease (0.02 μg/ml gives 50% inhibition), puromycin, and chloramphenicol, but is relatively insensitive to deoxyribonuclease and actinomycin D. Incorporation rate is approximately linear for 30 minutes and falls to zero in about 60 minutes. Evidence is presented for the intermediate formation of the aminoacyl–soluble ribonucleic acid complex by the 1000-*g* fraction. Added tobacco mosaic virus–ribonucleic acid has no effect on incorporation, whereas polyuridylic acid stimulates the incorporation of [^{14}C]phenylalanine, but not of [^{14}C]valine.

Because most of the visible growth of a plant leaf is the result of cell expansion occurring after cell division has ceased, this organ affords the opportunity to study net protein synthesis in the absence of net nuclear synthesis. During the expansion phase tobacco leaves accumulate protein at an exponential rate (Dorner *et al.*, 1957). When expansion ceases net protein accumulation stops, and thereafter the amount of protein steadily declines as the leaf senesces. Thus, it appeared that young leaves in the process of rapid net protein synthesis might provide a readily accessible source of a system capable of *in vitro* synthesis of protein.

Recent studies in this laboratory (Honda *et al.*, 1962) have led to the development of extraction media which facilitate the preparation from higher plant leaves of cell-free extracts in which the microscopically visible organelles are preserved in a morphologically intact state, closely resembling their condition in the living cell. In these extracts a high proportion of nuclei are intact, many chloroplasts retain the outer mobile jacket seen in the living cell, and the pleomorphic character of mitochondria is also preserved. This finding suggested that such cell-free extracts from young leaves may provide an opportunity for the *in vitro* study of complex processes such as the synthesis of nucleic acids and proteins. This paper describes the distribution of amino acid–incorporating activity in such extracts, and some of the characteristics of an active system associated with the 1000-*g* fraction.

* This work was supported in part by research grants from the Division of Biology and Medicine, U. S. Atomic Energy Commission, Contract AT (11-1)-34, Project 8; the National Institutes of Health (AI-00536); and the National Science Foundation (B-14729).

† Permanent address: Division of Plant Industry, C.S.I.R.O., Canberra, Australia.

MATERIALS AND METHODS

Plant Material.—Both *Nicotiana glutinosa* or *N. tabacum* var. Turkish Samsun plants were grown under standard greenhouse conditions. The former were grown in 10-cm pots and were used when they had reached a height of about 15 cm. Leaves which were about 20–40 mm were used. The *N. tabacum* plants were grown in 7-cm peat pots containing vermiculite and were watered twice daily with a mineral nutrient solution. Leaves 50–70 mm in length were harvested when the plants had reached a height of about 7 cm.

Preparation of Extracts.—The midrib and large veins were cut out of the leaves and, after cooling in ice, 5 g fresh weight of the laminae together with 6 ml of extracting medium were placed in a flat-bottomed, shallow, polythene dish and chopped to a fine mince with a sharp razor blade. The object of the method was to cut the leaf cells so that their protoplasmic contents would be released into the extraction medium with as little crushing of the cells and organelles as possible. Mincing required about 10 minutes, during which time the chopping dish was standing on crushed ice. The extracting medium was essentially that developed by Dr. Shigeru Honda for maximum structural preservation of organelles. Honda medium consisted of ficoll (2.5%), dextran (5%) sucrose (0.25 M), Tris, *p*H 7.8 (0.025 M), magnesium acetate (1 mM), and mercaptoethanol (4 mM). The resulting *brei* was