Oxidative Phosphorylation in Fractionated Bacterial Systems. XXIII. Enzymatically Reduced Derivatives of Vitamin $K_{1(20)}$ Obtained by Acetylation*

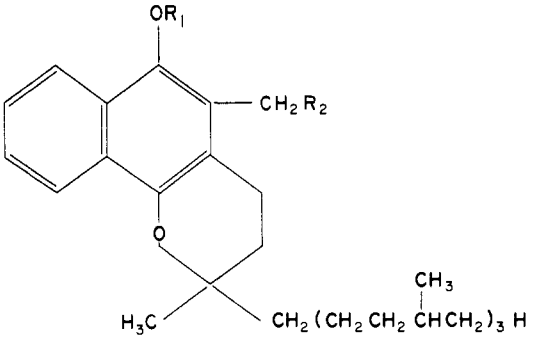
Philip Klubes† and Arnold F. Brodie

ABSTRACT: Anaerobic incubation of a cell-free extract of *Mycobacterium phlei* with vitamin $K_{1(20)}$, DL-malate, and inorganic phosphate followed by acetylation of the concentrated incubation mixture with acetic anhydride–pyridine gave only one acetylated derivative of vitamin $K_{1(20)}$, diacetyldihydrovitamin $K_{1(20)}$. When the 6-hydroxychromanol derivative of vitamin $K_{1(20)}$, a possible intermediate, was added to the incubation mixture it was recovered as the 6-chromanyl acetate of vitamin $K_{1(20)}$ following acetylation with acetic anhydride–pyridine.

Acetylation with acetyl chloride of the ether-extractable material obtained from the incubation mixture yielded only the 5-chloromethyl-6-chromanyl acetate derivative of vitamin $K_{1(20)}$; this compound was of nonenzymatic origin. Thin layer chromatography of the ether-extractable material failed to give rise to any reduced derivative of vitamin $K_{1(20)}$ as detected by the Emmerie–Engel spray reagent. The enzymatic formation of a chromanol derivative of vitamin $K_{1(20)}$ could not be confirmed by trapping techniques.

A role for vitamin $K_{1(20)}$ in electron transport and oxidative phosphorylation in a light-irradiated cell-free system from *Mycobacterium phlei* has been demonstrated by Brodie (1961). Incubation of vitamin $K_{1(20)}$ under anaerobic conditions with a phosphorylating system from *M. phlei* resulted in accumulation of reduced intermediates of vitamin $K_{1(20)}$. Direct extraction of the enzymatic reaction mixture with ether gave a spectrum (λ_{max} 245 mμ) characteristic of a chromanol derivative of vitamin $K_{1(20)}$. Treatment of the concentrated incubation mixture with acetyl chloride, in an attempt to stabilize the enzymatically reduced products, resulted in isolation of diacetyldihydrovitamin $K_{1(20)}$ and a crude product (λ_{max} 245 mμ) identified as the acetylated chromanyl derivative of vitamin $K_{1(20)}$ (Russell and Brodie, 1961, 1962).

Wagner *et al.* (1962) confirmed the presence of the 6-chromanyl acetate of vitamin $K_{1(20)}$ (I) from the enzymatic and acetylated mixture. They noted, however, that the 6-chromanyl acetate might be derived, at least in part, from a nonenzymatic cyclization of dihydrovitamin $K_{1(20)}$. Further studies revealed a second and unexpected chromanyl derivative, 5-chloromethyl-6-chromanyl acetate, of vitamin $K_{1(20)}$ (II) from the enzymatic and acetylated reaction mixture (Wagner *et al.*, 1963a). At the same time the nonenzymatic origin of this derivative by the reaction of the acetyl chloride with vitamin $K_{1(20)}$, in the presence of traces of moisture or acid, was confirmed (Wagner *et al.*, 1963a). Thus, acetylation of the incubation mixture with acetyl chloride resulted in the isolation of diacetyldihydrovitamin $K_{1(20)}$, the 6-chromanyl acetate of vitamin $K_{1(20)}$ (I), and the 5-chloromethyl-6-chromanyl acetate of vitamin $K_{1(20)}$ (II). Nevertheless, it was still not possible to exclude the enzymatic cyclization of vitamin $K_{1(20)}$ during anaerobic incubation with a cell-free system from *M. phlei*. Chromanyl compounds have been postulated as intermediates in oxidative phosphorylation (Chmielewska, 1960; Asano *et al.*, 1962; Erick-



I. $R_1 = COCH_3$, $R_2 = H$

II. $R_1 = COCH_3$, $R_2 = Cl$

III. $R_1 = H$, $R_2 = H$

* From the Department of Microbiology, University of Southern California School of Medicine, and the Los Angeles County General Hospital, Los Angeles, California. *Received May 18, 1966.* This work was supported by Grant AI-05637 from the National Institutes of Health, U. S. Public Health Service, by a grant from the National Science Foundation (GB-3583), and by the Hastings Foundation of the University of Southern California School of Medicine.

† Bank of America-Giannini Foundation Medical Fellow. Present Address: Department of Pharmacology, The George Washington University School of Medicine, Washington, D. C.

son *et al.*, 1963; Vilkas and Lederer, 1962). Therefore, a study was undertaken to determine whether the enzymatic cyclization of vitamin $K_{1(20)}$ during anaerobic incubation in a cell-free system from *M. phlei* could be confirmed by other acetylation techniques.

Experimental Procedure

Chemicals. Vitamin $K_{1(20)}$ was purchased from Mann Research Laboratories and purified by column chromatography on Permutit before use. All other chemicals and reagents were of the highest purities commercially available.

Thin Layer Chromatography. Thin layer chromatography on alumina was developed in petroleum ether (bp 30–60°)–diethyl ether (85:15) (system A). This procedure is a modification of the method of Wagner *et al.* (1963a). Thin layer chromatography on silica gel G was developed in petroleum ether–isopropyl ether–acetone–glacial acetic acid (85:12:4:1:1) (system B) according to the method of Stowe (1963). The Emmerie–Engel spray reagent was prepared before use according to the method of Dilley (1964).

Ultraviolet Absorption Spectra. The absorption spectra of the quinone derivatives were examined with a Cary Model 11 recording spectrophotometer with spectral grade isooctane as solvent. The concentrations of vitamin $K_{1(20)}$, 6-chromanyl acetate, and diacetyldihydrovitamin $K_{1(20)}$ were calculated from the measured absorbancies according to the values reported in the literature; for vitamin $K_{1(20)}$ $\lambda_{max}^{isooctane}$ 270 mμ, $E_{1cm}^{1\%}$ 424 (Merck Index, 1960); for 6-chromanyl acetate $\lambda_{max}^{isooctane}$ 245 mμ, $E_{1cm}^{1\%}$ 815 (Wagner *et al.*, 1963b); for diacetyldihydrovitamin $K_{1(20)}$ $\lambda_{max}^{isooctane}$ 232 mμ, $E_{1cm}^{1\%}$ 1640 (Brodie, 1963).

Infrared Absorption Spectra. The infrared absorption spectra were determined with a Perkin-Elmer Model 21 infrared spectrophotometer.

Synthesis of 6-Hydroxychromanol of Vitamin $K_{1(20)}$. 6-Hydroxychromanol III was prepared by a modification of the method of Chen and Dallam (1963). The crude product was purified by column chromatography on Permutit. The column was developed with 2% ether in petroleum ether and the product eluted with 25% ether in petroleum ether to give 3,4-dihydro-2,5-dimethyl-2-(4,8,12-trimethyltridecyl)-2H-naphtho[1,2-*b*]pyran-6-ol as a colorless oil in a yield of 70%; $\lambda_{max}^{isooctane}$ 249, 325, and 338 mμ; $E_{1cm}^{1\%}$ 750, 126 and 131; λ_{max}^{neat} 2.95 and 3.45 μ. Thin layer chromatography (system B) of the product gave R_F 0.27 as a blue fluorescent spot under ultraviolet light. An identical R_F was obtained with the Emmerie–Engel spray reagent. The product was stored in the refrigerator under argon.

Synthesis of 6-Chromanyl Acetate of Vitamin $K_{1(20)}$. 6-Chromanyl acetate was prepared according to the method of Wagner *et al.* (1963b) by treatment of 6-hydroxychromanol with acetic anhydride–pyridine. The crude product was purified by column chromatography on Permutit and was eluted with 5% ether in petroleum ether following preliminary development of the column with 2% ether in petroleum ether. The product, 3,4-dihydro-2,5-dimethyl-2-(4,8,12-trimethyltridecyl)-2H-naphtho[1,2-*b*]pyran-6-yl acetate, was obtained as a colorless oil, yield 71%; $\lambda_{max}^{isooctane}$ 245, 308, 315, 322, and 329 mμ; $E_{1cm}^{1\%}$ 852, 117, 107, 91, and 92; λ_{max}^{neat} 3.40 μ and 5.65 μ. Thin layer chromatography (system A) of the product gave R_F 0.42 as a blue fluorescent spot under ultraviolet light.

Anal.[1] Calcd for $C_{33}H_{50}O_3$: C, 80.11; H, 10.19. Found: C, 80.22, H, 9.97.

Synthesis of 5-Chloromethyl-6-chromanyl Acetate of Vitamin $K_{1(20)}$. Vitamin $K_{1(20)}$ (2 g, 4.3 mmoles) was placed in a 100-ml round-bottom flask, placed in an ice bath, and treated with 20 ml of acetyl chloride. After 1 hr the ice bath was removed and the reaction mixture was allowed to stand overnight at room temperature. The mixture was poured onto ice in order to hydrolyze the acetyl chloride and the product was extracted with ether. The extract was washed with 5% (w/v) aqueous sodium bicarbonate and water, dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The residual oil was dissolved in petroleum ether and adsorbed on a column (2.2 × 25 cm) containing Permutit (40 g). After preliminary development with 2% ether in petroleum ether (400 ml) the product was eluted with 5% ether in petroleum ether (400 ml). The column chromatography on Permutit was repeated. The second chromatography was similar to that described above with the exception that 200 ml of 2% ether in petroleum ether was used to develop the column and the product was eluted with 5% ether in petroleum ether (200 ml) to yield 71 mg (3%) of 5-chloromethyl-3,4-dihydro-2-methyl-2-(4,8,12-trimethyltridecyl)-2H-naphtho[1,2-*b*]pyran-6-yl acetate as a colorless oil; $\lambda_{max}^{isooctane}$ 248, 308, 325, and 339 mμ; $E_{1cm}^{1\%}$ 695, 97, 87, and 85; λ_{max}^{neat} 3.40 μ and 5.68 μ. Thin layer chromatography (system A) of the product gave R_F 0.36 as a dark quenching spot under ultraviolet light.

Anal. Calcd for $C_{33}H_{49}ClO_3$: C, 74.89; H, 9.33; Cl, 6.70. Found: C, 74.86; H, 9.36; Cl, 7.00.

Preparation of Extracts. M. phlei, ATCC 354, was grown and cell-free extracts were prepared according to the method of Brodie and Gray (1956). The preparations were routinely tested for coupled phosphorylation by the conventional Warburg method at 30° as previously described (Brodie and Gray, 1956). Only those preparations which showed phosphorylating activity were used in the anaerobic incubations.

Anaerobic Incubation. Anaerobic incubation was carried out in a Thunberg tube which contained a small magnetic stirring bar. The purified vitamin $K_{1(20)}$ or 6-hydroxychromanol derivative of vitamin $K_{1(20)}$ used in the anaerobic incubation was suspended in 1.0 ml of crude extract with sonic oscillation for 2 min. Following addition of *M. phlei* crude extract, $MgCl_2$, inorganic phosphate, vitamin $K_{1(20)}$ or 6-

[1] Microanalyses were done by Elek Microanalytical Laboratories, Torrance, Calif.

TABLE I: Summary of Reduced Derivatives of Vitamin $K_{1(20)}$ Obtained from Anaerobic Incubation Mixtures by Different Acetylation Procedures.[a]

Method	Compound Added	μmoles	Eluent (ether, %)	Compound Found	μmoles
Acetylation with acetic anhydride–pyridine (3 expt)	Vitamin $K_{1(20)}$	1440	2	Vitamin $K_{1(20)}$	289
			5	—	—
			50	Diacetyldihydrovitamin $K_{1(20)}$	101.0
Recovery of 6-hydroxychromanol by acetylation with acetic anhydride–pyridine (1 expt)	Vitamin $K_{1(20)}$	480	2	Vitamin $K_{1(20)}$	116
			5	6-Chromanyl acetate	1.7
	6-Hydroxychromanol	4.8	50	Diacetyldihydrovitamin $K_{1(20)}$	21
Acetylation of ether-extractable material with acetyl chloride (3 expt)	Vitamin $K_{1(20)}$	2880	2	Vitamin $K_{1(20)}$	868
			5	5-Chloromethyl-6-chromanyl acetate	90
			50	—	—

[a] The procedures used for anaerobic incubation, acetylation, column chromatography, and identification of the products are described in the text.

hydroxychromanol as indicated, and DL-malate, the Thunberg tube was evacuated first at the water pump and then at the vacuum pump. The Thunberg tube was closed and removed from the vacuum pump and the mixture was incubated anaerobically in the dark with stirring at room temperature for 4 hr. The incubation mixture was then treated as described below.

Anaerobic Incubation. ACETYLATION WITH ACETIC ANHYDRIDE–PYRIDINE. The system consisted of crude extract (320 mg of protein), 200 μmoles of $MgCl_2$, 300 μmoles of inorganic phosphate, 240 μmoles of vitamin $K_{1(20)}$, 500 μmoles of DL-malate, and water to a final volume of 10.0 ml. A duplicate system was run simultaneously. Following incubation the mixtures were transferred under argon to a single 100-ml round-bottom flask and concentrated *in vacuo* at 30° on a flash evaporator for 1 hr to yield a solid residue. To the residue was added 20 ml of acetic anhydride and 2 ml of anhydrous pyridine. After 1 hr the mixture was concentrated *in vacuo* at 30° on a flash evaporator for 1 hr. The residue was extracted with two 15-ml portions of ether, the ether extracts were centrifuged, and the supernatant was decanted and concentrated *in vacuo* to a syrup. The syrupy residue was dissolved in a small amount of petroleum ether and adsorbed on a column (1.3 × 30 cm) of Permutit (15 g). The column was eluted with successive 200-ml portions of 2, 5, and 50% ether in petroleum ether. The eluents were concentrated *in vacuo*. The residues were taken up in isooctane and examined spectrophotometrically. The material in the 2% ether fraction had an ultraviolet spectrum which was identical with that of vitamin $K_{1(20)}$ whereas the 5% ether fraction had no absorption. The 50% ether fraction gave a spectrum identical with that of diacetyldihydrovitamin $K_{1(20)}$. The 2% ether fractions obtained from three different experiments (in duplicate) were pooled and concentrated *in vacuo* to a syrup and the amount of vitamin $K_{1(20)}$ obtained was determined spectrophotometrically from its known extinction coefficient (Table I). The 5% ether fractions obtained from three experiments gave no indication of the presence of any type of quinone derivative when examined spectrophotometrically. The syrupy residue of diacetyldihydrovitamin $K_{1(20)}$ obtained from three experiments was pooled and crystallized from 2.0 ml of absolute ethanol to yield 81 mg of a white crytalline product. A second recrystallization from 1.0 ml of absolute ethanol afforded 54 mg (101 μmoles) of diacetyldihydrovitamin $K_{1(20)}$ as white needles, mp 60–61°. A mixture melting point with an authentic specimen prepared according to the method of Fieser (1939), mp 60.5–61.5°, gave 60–61°. The product was further characterized by $\lambda_{max}^{isooctane}$ 232 mμ, $E_{1cm}^{1\%}$ 1580; λ_{max}^{KBr} 5.7 μ. Thin layer chromatography (system B) of the product gave R_F 0.23 as a blue fluorescent spot under ultraviolet light, identical with an authentic specimen.

Anal. Calcd for $C_{35}H_{52}O_4$: C, 78.31; H, 9.77. Found: C, 78.84; H, 9.64.

Anaerobic Incubation. RECOVERY OF 6-HYDROXYCHROMANOL AS 6-CHROMANYL ACETATE. The system consisted of crude extract (320 mg of protein), 200 μmoles of $MgCl_2$, 300 μmoles of inorganic phosphate, 240 μmoles of vitamin $K_{1(20)}$, 2.4 μmoles of 6-hydroxychromanol, 500 μmoles of DL-malate, and water to a final volume of 10.0 ml. A duplicate system was run simultaneously. The anaerobic incubation, acetylation with acetic anhydride–pyridine, and column chromatography of this single experiment are identical with that described above. The eluents obtained from the column chromatography were concentrated *in vacuo* and the residue was taken up in isooctane and examined

spectrophotometrically. Vitamin $K_{1(20)}$, 6-chromanyl acetate, and diacetyldihydrovitamin $K_{1(20)}$ were identified in the 2, 5, and 50% ether fractions, respectively, on the basis of their ultraviolet adsorption spectra. The concentrations of each compound were determined spectrophotometrically from their known extinction coefficients (Table I).

The 6-chromanyl acetate obtained from the acetylated incubation mixture was further characterized by thin layer chromatography (system A). The material had R_F 0.47 and was identified by its blue fluorescence under ultraviolet light. This material was identical with an authentic specimen. The fluorescent material of R_F 0.47 from the acetylated incubation mixture was removed from the plate with ether and concentrated *in vacuo*. The residue was taken up in isooctane and examined spectrophotometrically. The ultraviolet absorption spectrum was identical with that of an authentic specimen of 6-chromanyl acetate.

Anaerobic Incubation. ACETYLATION OF THE ETHER-EXTRACTABLE MATERIAL WITH ACETYL CHLORIDE. In an attempt to avoid traces of moisture in the concentrated reaction mixture, the dry material was extracted with ether and the acetylation carried out with the dry ether-extractable material. The system consisted of crude extract (300 mg of protein), 400 μmoles of $MgCl_2$, 600 μmoles of inorganic phosphate, 480 μmoles of vitamin $K_{1(20)}$, 1000 μmoles of DL-malate, and water to a final volume of 13.0 ml. A duplicate system was run simultaneously. Following incubation the mixtures were transferred under argon to a single 100-ml round-bottom flask and concentrated *in vacuo* at 30° on a flash evaporator for 1 hr to yield a solid residue. The residue was extracted with three 15-ml portions of ether. The ether extracts were pooled and centrifuged. The supernatant solution was decanted and concentrated to dryness *in vacuo*, and the residue was treated with 15 ml of acetyl chloride overnight. Examination of the spectrum, in isooctane, of an aliquot of the ether extract showed the characteristic absorption maximum at 245 mμ reported by Russell and Brodie (1961). On standing the spectrum reverted to that identical with vitamin $K_{1(20)}$.

The acetylated reaction mixture was poured onto ice and extracted with ether. The extract was washed with 5% (w/v) aqueous sodium bicarbonate and water, dried over anhydrous sodium sulfate, filtered, and concentrated *in vacuo*. The anaerobic incubation (in duplicate) and acetylation was repeated two more times and the crude acetylated material obtained from the three experiments was pooled. The syrupy residue was dissolved in a small amount of petroleum ether and adsorbed on a column (1.3 × 30 cm) of Permutit (15 g). The column was eluted with successive 200-ml portions of 2, 5, and 50% ether in petroleum ether. The eluents were concentrated *in vacuo*. The residues from the 2 and 50% ether fractions were taken up in isooctane and examined spectrophotometrically. The material in the 2% ether fraction had an ultraviolet spectrum identical with that of vitamin $K_{1(20)}$ and its concentration was determined from the known extinction coefficient of vitamin $K_{1(20)}$ (Table I). Spectral examination of the residue obtained from the 50% ether fraction gave no indication of the presence of diacetyldihydrovitamin $K_{1(20)}$ (Table I).

The residue obtained from the 5% ether fraction was subjected to column chromatography on Permutit two more times, the product being eluted with 5% ether in petroleum ether following development of the column with 2% ether in petroleum ether. This procedure afforded 48 mg (90 μmoles) of 5-chloromethyl-3,4-dihydro-2-methyl-(4,8,12-trimethyltridecyl)-2H-naphtho[1,2-*b*]pyran-6-yl acetate as a colorless oil: $\lambda_{max}^{isooctane}$ 248, 308, 325, and 339 mμ; $E_{1cm}^{1\%}$ 682, 88, 78, and 70; λ_{max}^{neat} 3.40 and 5.68 μ. The product had R_F 0.35 following thin layer chromatography (system A) and was identical with an authentic sample. The product and authentic sample exhibited a dark quenching spot under ultraviolet light. An authentic specimen of 6-chromanyl acetate gave R_F 0.43 as a blue fluorescent spot under ultraviolet light.

Anal. Calcd for $C_{33}H_{49}ClO_3$: C, 74.89; H, 9.33; Cl, 6.70. Found: C, 74.78; H, 9.25; Cl, 6.98.

Anaerobic Incubation. THIN LAYER CHROMATOGRAPHY OF THE ETHER-EXTRACTABLE MATERIAL. An attempt was made to examine the enzymatically formed derivative in the absence of a trapping agent. The system consisted of crude extract (320 mg of protein), 100 μmoles of $MgCl_2$, 150 μmoles of inorganic phosphate, 120 μmoles of vitamin $K_{1(20)}$, 250 μmoles of DL-malate, and water to a final volume of 9.0 ml. A second system, which contained in addition 1.2 μmoles of 6-hydroxychromanol, was run simultaneously. Following incubation the mixtures were transferred under argon to separate 100-ml round-bottom flasks and concentrated *in vacuo* at 30° in a flash evaporator for 1 hr to

TABLE II: Thin Layer Chromatography of the Ether-Extractable Material Obtained from the Anaerobic Reaction.[a]

Conditions	R_F
Reaction mixture + $K_{1(20)}$	0.64
Reaction mixture + $K_{1(20)}$ + 6-hydroxychromanol	0.27, 0.64
Authentic 6-hydroxychromanol (25 μg)	0.27
Authentic $K_{1(20)}$ (500 μg)	0.64

[a] The reaction mixture is described in the text. As indicated 1.2 μmoles of 6-hydroxychromanol was added. The thin layer chromatography was carried out on silica gel G with the solvent system B described previously. The 6-hydroxychromanol was detected as a pink spot after treatment with the Emmerie–Engel spray reagent, whereas vitamin $K_{1(20)}$ was detected by visual observation.

yield a solid residue. Each residue was extracted with two 15-ml portions of ether. The ether extract was centrifuged and the supernatant solution was decanted and concentrated to dryness *in vacuo*. The residue obtained from each extract was subjected to thin layer chromatography on silica gel G (system B) and the plate sprayed with the Emmerie–Engel reagent. The results are shown in Table II. When an aliquot of the ether extract obtained from the first system, which contained only vitamin $K_{1(20)}$, was examined spectrally in isooctane it showed the characteristic absorption maximum at 245 mμ reported by Russell and Brodie (1961). On standing the spectrum reverted to that identical with vitamin $K_{1(20)}$.

Results and Discussion

Acetylation with Acetic Anhydride–Pyridine. In order to exclude the possibility of the nonenzymatic cyclization of either vitamin $K_{1(20)}$ or enzymatically formed dihydrovitamin $K_{1(20)}$ the concentrated incubation mixture was acetylated with acetic anhydride–pyridine. Fractionation of the acetylated mixture by column chromatography on Permutit resulted in isolation of vitamin $K_{1(20)}$ and diacetyldihydrovitamin $K_{1(20)}$ in the 2 and 50% ether fractions, respectively, based on spectrophotometric identification. Recrystallization from absolute ethanol of the syrupy residue obtained from the 50% ether fraction gave a single crystalline derivative which was characterized as diacetyldihydrovitamin $K_{1(20)}$.

The 5% ether fraction which would be expected to contain an acetylated chromanyl derivative of vitamin $K_{1(20)}$, such as 6-chromanyl acetate, did not contain any spectrophotometrically detectable quinol derivative. Model experiments showed that the sensitivity of the column chromatographic procedure was such that 50 μg of 6-chromanyl acetate could be recovered quantitatively, in the 5% ether fraction; however, quantitative recovery with amounts less than 25 μg was not achieved.

Isolation of 6-Chromanyl Acetate. As another check on the effectiveness of the acetylation and fractionation procedure, 6-hydroxychromanol was added to the enzymatic incubation mixture. 6-Hydroxychromanol was used since it is a reduced derivative of vitamin $K_{1(20)}$ which could be converted to 6-chromanyl acetate by acetylation with acetic anhydride–pyridine.

As shown in Table I, incubation and acetylation with acetic anhydride–pyridine followed by fractionation of the acetylated mixture on Permutit resulted in isolation of material which was spectrophotometrically identified as vitamin $K_{1(20)}$, 6-chromanyl acetate, and diacetyldihydrovitamin $K_{1(20)}$ in the 2, 5, and 50% ether fractions, respectively. 6-Chromanyl acetate was obtained in a yield of 35% based on the amount of 6-hydroxychromanol used in the reaction mixture. The derivative obtained in the 5% ether fraction was further characterized by thin layer chromatography on alumina (system A) and shown to have an R_F identical with that of an authentic specimen of 6-chromanyl acetate. In addition, the spectrum obtained after elution from the chromatogram of the ultraviolet-absorbing spot was identical with that of 6-chromanyl acetate.

The acetylation and fractionation procedure appeared to be adequate to detect the presence of a chromanol derivative of vitamin $K_{1(20)}$ when it was added to the incubation mixture. However, the failure to isolate an acetylated chromanyl derivative of vitamin $K_{1(20)}$ following acetylation of the incubation mixture with acetic anhydride–pyridine does not exclude the possibility that a cyclized derivative of vitamin $K_{1(20)}$ may be formed during anaerobic incubation. It is possible that such an intermediate, in the presence of acetic anhydride and/or pyridine, may be converted either to vitamin $K_{1(20)}$ or diacetyldihydrovitamin $K_{1(20)}$.

Isolation of 5-Chloromethyl-6-chromanyl Acetate. Russell and Brodie (1961) demonstrated that the enzymatically reduced intermediates of vitamin $K_{1(20)}$ could be extracted from the concentrated incubation mixture with ether. Therefore, the presence in the ether extract of an enzymatically formed chromanol derivative might be detected following its conversion to 6-chromanyl acetate upon acetylation with acetyl chloride. In addition, since the possibility of traces of moisture in the concentrated incubation mixture could not be excluded (Wagner *et al.*, 1962, 1963a), acetylation of the dried ether extract with acetyl chloride would minimize any nonenzymatic cyclization of dihydrovitamin $K_{1(20)}$ present in the ether extract to 6-chromanyl acetate.

When the ether-extractable material was concentrated *in vacuo* to a syrup and then treated overnight with acetyl chloride a single chromanyl derivative, 5-chloromethyl-6-chromanyl acetate, was obtained. The nonenzymatic origin of this compound has been previously noted (Wagner *et al.*, 1963a). The failure to obtain 6-chromanyl acetate following acetylation of the ether-extractable material with acetyl chloride supports the suggestion of Wagner *et al.* (1962, 1963a) that the 6-chromanyl acetate obtained from the acetylated incubation mixture was predominantly of nonenzymatic origin.

Thin Layer Chromatography. An attempt was made to detect the enzymatic formation of a chromanol compound by a procedure which did not involve acetylation. As shown in Table II, when the ether-extractable material obtained from the concentrated incubation mixture was subjected to thin layer chromatography on silica gel G and then sprayed with the Emmerie–Engel reagent a chromanol compound could not be detected. In addition, enzymatically formed dihydrovitamin $K_{1(20)}$ was not detected, presumably due to the rapid autoxidation which occurs with this compound. A second incubation mixture which contained, in addition, 6-hydroxychromanol gave a positive Emmerie–Engel reaction with an R_F identical with that of 6-hydroxychromanol. Dihydrovitamin $K_{1(20)}$ was not detected. The sensitivity of the Emmerie–Engel reagent was such that 25 μg of 6-hydroxychromanol could be detected on the thin layer plates by

visual observation after spraying. The failure to obtain chromatographic evidence for the enzymatic formation of a chromanol compound by this technique is, however, subject to the reservation that a reduced intermediate of vitamin $K_{1(20)}$ could go undetected due to its decomposition by either the adsorbant and/or the solvent.

In this regard it was found that when the ether-extractable material was subjected to thin layer chromatography on silica gel G and then examined under ultraviolet light a fluorescent spot with an R_F identical with that of synthetic 6-chromanyl phosphate of vitamin $K_{1(20)}$ was observed. However, on elution this material failed to give an ultraviolet absorption spectrum characteristic of either 6-chromanyl phosphate or vitamin $K_{1(20)}$. This fluorescent material may be identical with the phosphorylated derivative of vitamin $K_{1(20)}$ recently reported by Watanabe and Brodie (1966). This latter compound, which is formed upon anaerobic incubation of a cell-free system from *M. phlei*, has been tentatively identified as a phosphorylated quinol derivative of vitamin $K_{1(20)}$. This derivative comigrates with 6-chromanyl phosphate upon chromatography on liquid paraffin impregnated paper, gives a negative Emmerie–Engel reaction, and was found to decompose on silica gel thin layer plates.

The report of a phosphate-dependent incorporation of tritium into vitamin $K_{1(20)}$ during oxidative phosphorylation in a cell-free system from *M. phlei* (Gutnick and Brodie, 1965) is of interest since it indicates the formation of a cyclic ring during quinone reduction and oxidation. Under anaerobic conditions, however, incorporation of tritium was not observed.

The incubation and acetylation procedures employed in this work failed to confirm the enzymatic formation of a cyclized derivative of vitamin $K_{1(20)}$ during anaerobic incubation in a cell-free system from *M. phlei*. Moreover, the difficulties associated with attempting to isolate reduced derivatives of quinones, which may be intermediates in oxidative phosphorylation, by the use of acetylation techniques is apparent.

Acknowledgments

We express our appreciation to Mrs. Jane Ballantine Klubes for her technical assistance.

References

Asano, A., Brodie, A. F., Wagner, A. F., Wittreich, P. E., and Folkers, K. (1962), *J. Biol. Chem. 237*, PC-2411.

Brodie, A. F. (1961), *Federation Proc. 20*, 995.

Brodie, A. F. (1963), *Methods Enzymol. 6*, 295.

Brodie, A. F., and Gray, C. T. (1956), *J. Biol. Chem. 219*, 853.

Chen, L. H., and Dallam, R. D. (1963), *Nature 198*, 386.

Chmielewska, I. (1960), *Biochim. Biophys. Acta 39*, 170.

Dilley, R. A. (1964), *Anal. Biochem. 7*, 240.

Erickson, R. E., Wagner, A. F., and Folkers, K. (1963), *J. Am. Chem. Soc. 85*, 1535.

Fieser, L. F. (1939), *J. Am. Chem. Soc. 61*, 3467.

Gutnick, D. L., and Brodie, A. F. (1965), *J. Biol. Chem. 240*, PC3698.

Merck Index (1960), 7th ed, Rahway, N. J., Merck and Co.

Russell, P. J., Jr., and Brodie, A. F. (1961), *Biochim. Biophys. Acta 50*, 76.

Russell, P. J., Jr., and Brodie, A. F. (1962), Quinones in Electron Transport, Boston Mass., Little Brown, p 205.

Stowe, A. D. (1963), *Arch. Biochem. Biophys. 103*, 42.

Vilkas, M., and Lederer, E. (1962), *Experientia 18*, 546.

Wagner, A. F., Wittreich, P. E., Arison, B., Trenner, N. R., and Folkers, K. (1963b), *J. Am. Chem. Soc. 85*, 1178.

Wagner, A. F., Wittreich, P. E., Hoffman, C. H., Folkers, K., and Brodie, A. F. (1962), *Biochem. Biophys. Res. Commun. 8*, 38.

Wagner, A. F., Wittreich, P. E., Lusi, A., Erickson, R. E., Arison, B., Trenner, N. R., Folkers, K., and Brodie, A. F. (1963a), *Arch. Biochem. Biohys. 102*, 11.

Watanabe, T., and Brodie, A. F. (1966), *Proc. Natl. Acad. Sci. U. S.* (in press).