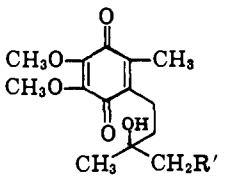
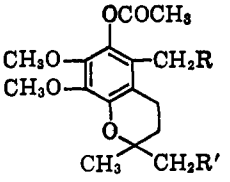
10.1 μ, 14.35 μ; *Anal.* Found: C, 69.62; H, 8.47; P, 3.32; 85% purity based upon elemental analysis and hydrogenation data. Preferential cleavage of the benzyl moieties of the phosphate triester XII yielded the corresponding 5-phosphomethyl-6-chromanyl acetate I: $\lambda_{max}^{isooctane}$ 289 mμ ($E_{1\,cm.}^{1\%}$ 43); λ_{max}^{neat} 4.1–4.5 μ, 5.65 μ, 6.30 μ, 8.30 μ, 8.99 μ, 9.80 μ; *Anal.* Found: C, 60.68; H, 9.00; P, 4.80. The n.m.r. spectra of compounds I and X–XII were in agreement with the assigned structures.



X R′ = $(CH_2CH_2\underset{CH_3}{\underset{|}{C}}HCH_2)_3H$
XIII R′ = H



XI R = Cl
R′ = $(CH_2CH_2\underset{CH_3}{\underset{|}{C}}HCH_2)_3H$
XII R = $OPO(OCH_2C_6H_5)_2$
R′ = $(CH_2CH_2\underset{CH_3}{\underset{|}{C}}HCH_2)_3H$
XIV R = Cl; R′ = H
XV R = $OPO(OCH_2C_6H_5)_2$
R′ = H

The 6-chromanol of coenzyme Q_1 was converted to the corresponding γ-hydroxyquinone XIII by ferric chloride oxidation; $\lambda_{max}^{isooctane}$ 276 mμ ($E_{1\,cm.}^{1\%}$ 588); λ_{max}^{neat} 2.8 μ, 6.1 μ, 6.2 μ, 7.8–7.9 μ; *Anal.* Found: C, 62.40; H, 7.46. When the γ-hydroxyquinone was dissolved in acetyl chloride at room temperature, the 5-chloromethyl-6-chromanyl acetate XIV was formed; m.p. 82–84°; $\lambda_{max}^{isooctane}$ 293 mμ ($E_{1\,cm.}^{1\%}$ 78); λ_{max}^{Nujol} 5.68 μ, 6.35 μ, 8.35 μ, 8.45 μ, 9.0 μ; *Anal.* Found: C, 58.68; H, 6.61; Cl, 10.23, 10.37. The chloromethyl compound on treatment with silver dibenzylphosphate yielded the phosphate triester XV: $\lambda_{max}^{isooctane}$ 291 mμ ($E_{1\,cm.}^{1\%}$ 54); λ_{max}^{neat} 5.6 μ, 6.3 μ, 6.85 μ, 7.8 μ, 8.3 μ, broad 9.8–10.1 μ, 13.4 μ, 14.35 μ. *Anal.* Found: C, 62.48; H, 6.31; P, 5.24. Selective cleavage of the benzyl moieties of the phosphate triester XV yielded the 5-phosphomethyl-6-chromanyl acetate III; potassium salt, $\lambda_{max}^{H_2O}$ 286 mμ ($E_{1\,cm}^{1\%}$ 38.5); λ_{max}^{Nujol} 5.7 μ, 6.3 μ, 8.2 μ, 8.9 μ, 9.8 μ, 10.25 μ, 12.0 μ.

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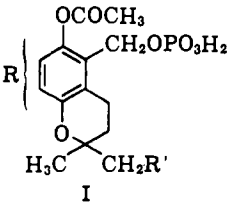
RECEIVED MARCH 30, 1963

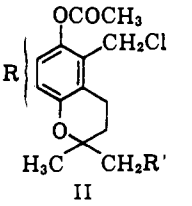
COENZYME Q. XLVIII. DATA ON QUINONE METHINES AS REACTION INTERMEDIATES AND THEIR POSSIBLE ROLE IN OXIDATIVE PHOSPHORYLATION

Sir:

Three new phosphomethyl derivatives I in the coenzyme Q and vitamin K groups have been reported.[1] These phosphates were synthesized from the 5-chloromethyl derivatives II which were derived from the parent quinones. A study of the mechanism of formation of II led to a consideration of quinone methines as intermediates in these reactions. A plausible mechanism involving these reactive intermediates in the biochemical transformations involved in oxidative phosphorylation has evolved.



I



II

Ia, R = 2,3-dimethoxy-5-methyl-1,4-benzoquinonyl, R′ = $(CH_2CH_2\underset{CH_3}{\underset{|}{C}}HCH_2)_3H$

Ib, R = 2-methyl-1,4-benzoquinonyl, R′ = $(CH_2CH_2\underset{CH_3}{\underset{|}{C}}HCH_2)_3H$

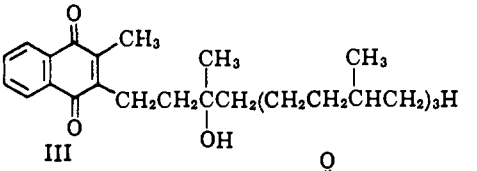
Ic, R = 2,3-dimethoxy-1,4-benzoquinonyl, R′ = H

IIa, R = 2,3-dimethoxy-5-methyl-1,4-benzoquinonyl, R′ = $(CH_2CH_2\underset{CH_3}{\underset{|}{C}}HCH_2)_3H$

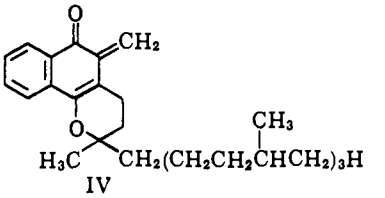
IIb, R = 2-methyl-1,4-benzoquinonyl, R′ = $(CH_2CH_2\underset{CH_3}{\underset{|}{C}}HCH_2)_3H$

IIc, R = 2,3-dimethoxy-1,4-benzoquinonyl, R′ = H

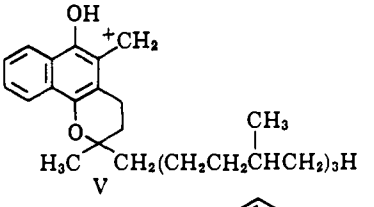
The synthesis of the hydroxyquinone III and its reaction with acetyl chloride to form the 5-chloromethyl derivative IIb have been reported.[1] Compound IIb was obtained directly by the reaction of vitamin $K_{1(20)}$ with acetyl chloride in the presence of water, strong acids or dihydrovitamin $K_{1(20)}$. A reasonable explanation of the formation of IIb involves the 1,4-addition of acetyl chloride to the quinone methine IV, which may be derived from the acid-catalyzed, non-reductive cyclization of vitamin $K_{1(20)}$. Quinone methines are known[2]



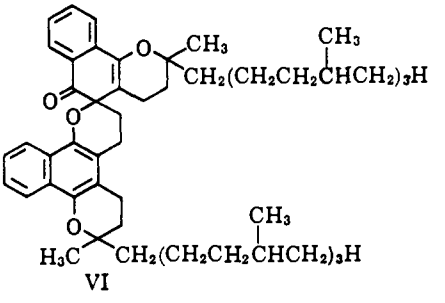
III



IV



V



VI

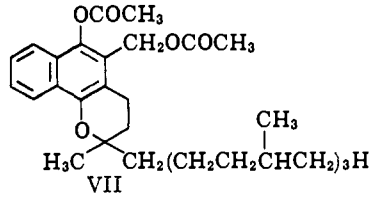
(1) A. F. Wagner, A. Lusi, C. H. Shunk, B. O. Linn, D. E. Wolf, C. H. Hoffman, R. E. Erickson, B. Arison, N. R. Trenner and K. Folkers, *J. Am. Chem. Soc.*, **85**, 1534 (1963).

(2) For a discussion of quinone methines see: K. Hultzsch, *Angew. Chem.*, **60**, 179 (1948); R. W. Martin, "The Chemistry of Phenolic Resins," John Wiley and Sons, Inc., New York, N. Y., 1956, pp. 129, 139–146.

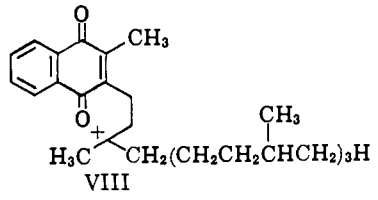
to be very reactive compounds and have been characterized either as dimers and trimers or as adducts with carbonyl compounds, strong acids and dienophiles.

Evidence was found for at least the transitory involvement of IV or its ion V in the acid-catalyzed reactions of vitamin $K_{1(20)}$. Sulfuric acid converted vitamin $K_{1(20)}$ to a yellow oil which exhibits ultraviolet, infrared and nuclear magnetic resonance spectra in agreement with structure VI. This compound is identical with that obtained by the alkaline ferricyanide oxidation of the 6-chromanol of vitamin $K_{1(20)}$ and is structurally analogous to the oxidation products of α-tocopherol[3] and 2,2,5,7,8-pentamethyl-6-chromanol.[4]

The reaction of vitamin $K_{1(20)}$ with strong acids is accompanied by the formation of an intense red-brown color and the dilution of a sulfuric acid solution with acetic anhydride gave the diacetate VII which is identical with that obtained by the reaction of the 5-chloromethyl derivative IIb with silver acetate.[1]



VII



VIII

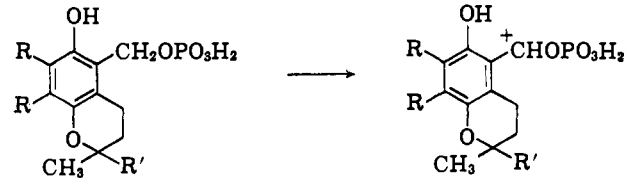
These data support a mechanism involving the reversible protonation of vitamin $K_{1(20)}$ to give the cations VIII and V; the latter is presumably responsible for the red-brown color. Reaction of VIII with water gives the hydroxyquinone III. Reaction of V or its unprotonated form IV with acetyl chloride, acetic anhydride or itself gives IIb, VII and VI, respectively.

Pertinent to this argument are the sulfuric acid-catalyzed conversion[5] of lapachol to β-lapachone; the greater basicity of *o*-quinones compared to the corresponding *p*-quinones[6]; the facile and reversible 1,4-addition to the quinone methine system[2]; and the electronic structure and reactivity of quinodimethanes.[7] Consideration of this chemistry suggests that the stability of the cation V provides considerable driving force for its formation from vitamin $K_{1(20)}$ in strong acid solution.

The formation of VI both by the action of sulfuric acid on vitamin $K_{1(20)}$ and the oxidation of the 6-chromanol derivative is presumptive evidence for a common intermediate. If oxidative attack on the 6-chromanol is presumed to occur at the phenolic hydroxyl, resulting in the corresponding oxonium ion, then rearrangement to the benzylic carbonium ion V is understandable and apparently occurs more readily than does opening of the chroman ring to a quinone. Alternatively, a one electron oxidation may be involved, but the net result, in either case, appears to be an electron-deficient 5-methyl group.

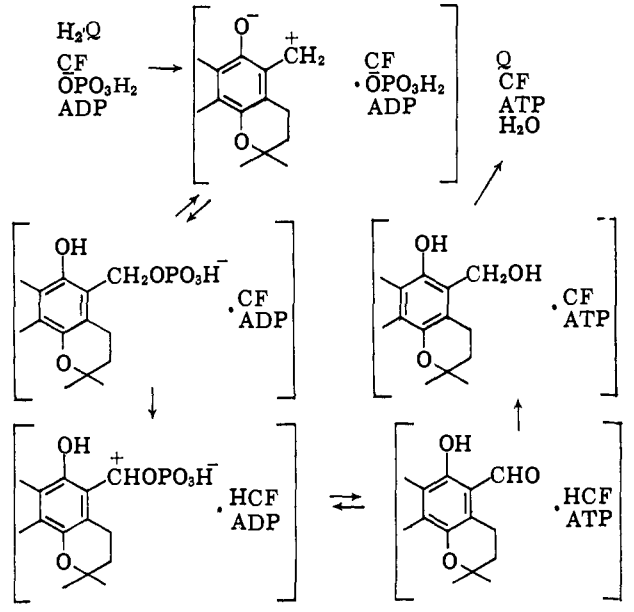
These reactions of vitamin $K_{1(20)}$ leading to electron deficiency and substitution on the 2-methyl group, and the demonstration that such reactions may occur by both acid catalysis and oxidation, suggest a possible role for 2-methyl-3-isoprenyl substituted quinones in biochemical oxidative phosphorylation. A key step of the sequence involves the addition of inorganic phosphate to the quinone methine intermediate[8] followed by the oxidation of the resulting 6-hydroxy-5-phosphomethyl derivative IX to give "active phosphate."

The generation of "active phosphate" by the oxidation of quinol phosphates has been demonstrated by Todd and co-workers.[9] The essential feature of the reaction is the oxidative withdrawal of electrons from the atom bearing the phosphate group and either the concomitant formation of monomeric metaphosphate or its equivalent or the concerted transfer of phosphate to a suitable receptor. The oxidation of IX might also be expected to lead to "active phosphate" by the analogous generation of an electron deficient species X.



IX X

Study of these new reactions of the coenzyme Q and vitamin K group and some aspects of the biochemistry of oxidative phosphorylation[10-12] have led us to consider the following biochemical sequence. The bracketed structures imply the mitochondrial substrate-coupling factor (CF) complexes[13] which are formed in the oxidation and which react with *Pi* and ADP to give ATP. An intracomplex oxidation and reduction step is postu-



(3) D. R. Nelan and C. D. Robeson, *J. Am. Chem. Soc.*, **84**, 2963 (1962).

(4) P. Schudel, H. Mayer, R. Ruegg and O. Isler, *Chimia*, **16**, 368 (1962).

(5) R. H. Thompson, "Naturally Occurring Quinones," Academic Press, Inc., New York, N. Y., 1957, pp. 59-73.

(6) M. G. Ettlinger, *J. Am. Chem. Soc.*, **72**, 3090 (1950).

(7) J. M. Tedder, *Ann. Repts. Chem. Soc.*, **48**, 225 (1961).

(8) In a recent paper [*Experientia*, **18**, 546 (1962)] M. Vilkas and E. Lederer have proposed on theoretical grounds the possible involvement of the addition of inorganic phosphate to a quinone methine as a step in the biochemical synthesis of quinol phosphates.

(9) V. M. Clark, D. W. Hutchinson, G. W. Kirby and A. Todd, *J. Chem. Soc.*, 715 (1961); V. M. Clark, D. W. Hutchinson and A. Todd, *ibid.*, 722 (1961); V. M. Clark and A. Todd, "Quinones in Electron Transport," Ciba Foundation Symposium, J. & A. Churchill, Ltd., London, 1961, pp. 190-200.

(10) E. R. Redfearn, *Ann. Repts. Chem. Soc.*, **57**, 395 (1960).

(11) E. Racker, *Advan. Enzymol.*, **23**, 323 (1961).

(12) A. L. Lehninger and C. L. Wadkins, *Ann. Rev. Biochem.*, **31**, 47 (1962).

(13) R. L. Prairie, T. E. Conover and E. Racker, *Biochem. Biophys. Res. Commun.*, **10**, 422 (1963), and references quoted therein.

lated and may involve a vicinal dithiol grouping[14] on the coupling factor.

(14) A. Fluharty and D. R. Sanadi [*Proc. Natl. Acad. Sci. U. S.*, **46**, 608 (1960)], have implicated a vicinal dithiol–disulfide oxidation-reduction system in oxidative phosphorylation.

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RECEIVED MARCH 30, 1963

EVIDENCE FOR A FUNCTIONAL CARBOXYL GROUP IN TRYPSIN AND CHYMOTRYPSIN

Sir:

We wish to report from this Laboratory experimental evidence which indicates that the enzymes trypsin and chymotrypsin possess a functional basic group with a pK in the vicinity of four. The existence of this group in chymotrypsin, concluded from an investigation of the hydrolysis of 2,4-dinitrophenyl acetate (DNPA), has been discussed previously.[1] This work has been verified under different experimental conditions and extended to trypsin using a specific substrate, N-benzoyl-L-arginine ethyl ester (BAEE), with the same result.

The hydrogen-ion inhibition constants, which may be used to help identify functional basic groups in trypsin and chymotrypsin, are given in Table I as pK values. They were determined by studying the effect of hydrogen-ion on the apparent rate constants related to the three-step mechanism presented in Fig. 1. The pK values are tabulated according to the rate constants from which they were evaluated and the enzyme or intermediate to which they correspond, *i.e.*, pK_1, the enzyme; pK_2, the Michaelis complex; and pK_3, the acyl-enzyme.

The pK_1 and pK_2 for chymotrypsin with DNPA were calculated by plotting the apparent values of K_m and $1/k_2$, respectively, against the hydrogen ion concentration. These apparent constants were obtained at several pH values between 3.6 and 7.1 by investigating the acetylation kinetics employing excess substrate in 10% isopropyl alcohol and using excess enzyme in water. At low pH, it was possible to use conventional mixing techniques, since the acetylation rate constant k_2 becomes small below pH 6.7.[6] The release of 2,4-dinitrophenoxide ion, which exists at low pH, was followed spectrophotometrically and a sensitivity of 1×10^{-7} mole/l. was attained. The results were treated theoretically by methods similar to those used for *p*-nitrophenyl acetate with chymotrypsin[5,7] and trypsin,[8] and DNPA with chymotrypsin.[4]

The apparent values of K'_m[2] and k_3 for trypsin with BAEE were determined by steady-state methods at several pH values, to values as low as 3.6. The pH dependence of these constants was used to evaluate pK_1 or pK_2, and pK_3, respectively. The rates of the hydrolyses were recorded by automatic titration using a Radiometer TTTI control unit and a titration assembly, which was designed in this Laboratory, with a sensitivity of $>5 \times 10^{-5}$ ml. With this sensitivity, it was possible to follow the hydrolysis at low pH, even though the rate is diminished considerably below pH 5.[9] No abnormal behavior was found at low pH as indicated by

TABLE I

pK VALUES FOR TRYPSIN AND CHYMOTRYPSIN SYSTEMS

System	Related rate constants[a]	Hydrogen-ion inhibition constants pK_i values	Acid-base equilibrium assignments: Related pK_A values	Acid-base equilibrium assignments: Form ionizing
Trypsin–BAEE	k_3	6.25	pK_3	Acyl-enzyme
	K'_m	3.9	...	...
	$K'_m/k_3 = K_m/k_2$	3.9 and 6–6.5	pK_1 and pK_2	Enzyme and Michaelis complex
Chymotrypsin–DNPA	k_3	6.56[a]	pK_3	Acyl-enzyme
	k_2	6.76[b]	pK_2	Michaelis complex
	K_m	4.37[b]	pK_1	Enzyme
	K_m	3.8[c]	pK_1	Enzyme
Chymotrypsin–CI[3]	K_{eq}/k_2	4.4[b] and 6.8[b]	pK_1, pK_2 and pK_3	Enzyme, Michaelis complex and/or substrate

[a] 10% acetonitrile. [b] 1.6% acetonitrile or water, excess enzyme method.[4,5] [c] 10% isopropyl alcohol, excess substrate method.[1]

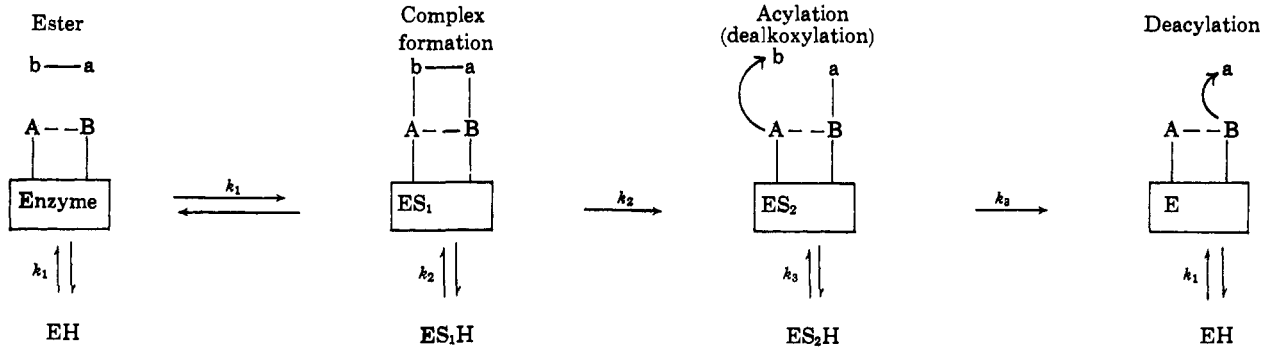

Fig. 1.—Interchange mechanism for esterases: A, carboxyl (aspartyl); B, hydroxyl (seryl); a, acyl, b, alkoxyl or aryloxy; E, enzyme; ES_1, Michaelis complex; and ES_2, acyl-enzyme.

(1) R. A. Dickie and J. A. Stewart, Abstracts of 140th National Meeting of the American Chemical Society, Chicago, Ill., Sept., 1961, p. 10C. In this abstract the reactive group was referred to as an acid; here, it will be referred to as a base, which is more explicit, since it is an electron donor in the pH region of interest.

(2) L. Ouellet and J. A. Stewart, *Can. J. Chem.* **37**, 737 (1959), these authors show that for deacylation $K'_m = [(k_3)/(k_2 + k_3)]\,[(k_{-1} + k_2)/(k_1)]$, which becomes $K'_m = (k_3/k_2)K_m$ when $k_2 > k_3$, so that $K'_m/k_3 = K_m/k_2$ where $K_m = (k_{-1} + k_2)/(k_1)$.

(3) M. L. Bender, G. R. Schonbaum and B. Zerner, *J. Am. Chem. Soc.*, **84**, 2562 (1962).

(4) G. W. Pepple, Senior Research Thesis, University of North Dakota, 1962.

(5) F. J. Kezdy and M. L. Bender, *Biochem.*, **1**, 1097 (1962).

(6) H. Gutfreund and J. M. Sturtevant, *Proc. Natl. Acad. Sci. U. S.*, **42**, 719 (1956).

(7) H. Gutfreund and J. M. Sturtevant, *Biochem. J.*, **63**, 656 (1956).

(8) J. A. Stewart and L. Ouellet, *Can. J. Chem.*, **37**, 751 (1959).

(9) H. Gutfreund, *Trans. Faraday Soc.*, **51**, 441 (1955).