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Polarographic Investigation of Conjugated Fat-Soluble Vitamins

Abstract. *The half-wave potentials of all-*trans *β-carotene, all-*trans *retinol, 13-*cis *retinol, all-*trans *retinyl acetate, all-*trans *retinal, and vitamins D_2 and D_3 were related to the number of double bonds in conjugation. A minimum of three double bonds in conjugation was required before reduction took place at the dropping-mercury electrode. As the number of conjugated bonds increased in the fat-soluble vitamins, the initial reduction took place at a lower half-wave potential. All of the waves were linearly proportional to the concentration of the vitamins in the concentration range studied.*

The electrochemical reductions at the dropping-mercury electrode have been applied to a wide series of water-soluble vitamins. The mechanism of reduction or the conditions necessary for the determination of thiamine, riboflavin, pyridoxine, and vitamin B_{12}, as well as ascorbic acid, niacin, pantothenic acid, and folic acid, in biological samples have been reviewed (*1*). The fat-soluble vitamin E has been investigated by reduction of the quinone or the oxidation of the hydroquinone (*2*). As with vitamin E, vitamin K in the quinone form is reduced in aqueous (*3*) potassium chloride solution and nonaqueous solutions (*4*).

Vitamin A (*5*) and β-carotene (*6*) in plants were separated and then converted to the iodinated derivatives, which gave anodic waves in methanolic solutions. In a more direct method, Salah and Heyrovski (*7*) showed that no polarographic wave was given by vitamin A. However, the aldehyde at low *p*H gave two reduction waves, and in alkaline media, a third wave. Conjugated hydrocarbons are known to be reduced at the dropping-mercury electrode (*8–11*) in protonic solvents, which on addition of hydrogen ions or water shift the reduction to more negative potentials. Although 10 percent aqueous dioxane with tetra-butyl ammonium hydroxide would not be considered a protonic solvent, reproducible polarograms were obtained for all the conjugated fat-soluble vitamins in this solvent. In this report I describe the polarographic reduction and determination of various fat-soluble vitamins and give reasons for associating the increase in conjugation with greater ease in placing the first electrons to form a conjugated anion.

The polarograms were obtained by using a jacketed polarographic cell (*12*) maintained at a constant temperature of 25° ± 0.2°C; the inner compartment contained a 1.1 cm² mercury anode. The resistance of the 10 percent aqueous dioxane containing either 0.1*N* tetra-butyl ammonium hydroxide or the chloride, as measured by a conductivity bridge (*13*), was 9800 and 23,200 ohms, respectively. At −2.50 volts the capillary constant $m^{2/3}t^{1/6}$ in tetra-butyl ammonium hydroxide was 1.82, and in tetra-butyl ammonium chloride, 3.03 (*14*).

All the conjugated fat-soluble vitamins studied were purchased commercially (*15*) and considered to be of high chemical purity. Vitamins D_2 and D_3, all-*trans* retinyl acetate, all-*trans* retinol, 13-*cis* retinol, and all-*trans* retinal were soluble in 10 percent aqueous dioxane containing 0.1*N* tetra-butyl ammonium chloride or hydroxide as a supporting electrolyte at $10^{-3}M$ concentrations or higher. Limited solubility of β-carotene made it necessary to run polarograms starting at a maximum concentration of $10^{-4}M$ in tetra-butyl ammonium chloride.

As shown in Fig. 1, the polarograms of vitamin D_2, 13-*cis* retinol, and β-carotene gave stepwise reduction waves depending on the degree of conjugation in 10 percent aqueous dioxane containing 0.1*N* tetra-butyl ammonium hydroxide. The first reduction wave of each of these fat-soluble vitamins shows β-carotene to be the easiest to reduce, followed by 13-*cis* retinol and vitamin D_2. Similar polarographic reduction waves were found in tetra-butyl ammonium chloride with half-wave potentials slightly more negative. This is in agreement with the polarographic reduction of a series of increasingly conjugated polyenes (*9, 10*) where the first reduction wave appeared at more positive half-wave potentials.

Several fat-soluble vitamins with increasing unsaturation with known geometrical isomers were investigated in 10 percent aqueous dioxane containing tetra-butyl ammonium chloride and hydroxide as supporting electrolytes (Table 1). A minimum of three double bonds in conjugation was needed before a reduction wave could be observed. This minimum was confirmed by alkaline isomerization of linolenic acid (purity > 99 percent), which showed a reduction wave at half-wave potential of −1.95 volts in tetra-butyl ammonium hydroxide. Alkaline isomerized linoleic acid showed no reduction wave even at concentrations greater than $10^{-3}M$. In tetra-butyl ammonium chloride and hydroxide, a linear relationship existed between the diffusion current and the concentrations shown

Table 1. Observed half-wave potentials ($E_{\frac{1}{2}}$) for various fat-soluble vitamins in basic and neutral solvents.

Double bonds * (No.)	$E_{\frac{1}{2}}$ †	$\frac{i_d}{Cm^{\frac{2}{3}}t^{\frac{1}{6}}}$	$E_{\frac{1}{2}}$ ‡	$\frac{i_d}{Cm^{\frac{2}{3}}t^{\frac{1}{6}}}$
		Vitamin D_2		
3	−2.01	2.57	−2.25	1.50
		Vitamin D_3		
3	−2.01	2.76	−2.22	1.66
		Retinyl acetate (*all*-trans)		
5	−1.53	2.51	−1.44	1.87
	−1.71	1.65	−1.92	1.82
	−2.16	1.19	−2.39	1.07
		Retinol (*all*-trans)		
5	−1.53	2.64	−1.86	1.60
	−1.77	1.85	−2.10	0.99
	−2.34	1.58	−2.58	0.92
		Retinol (*13*-cis)		
	−1.50	2.51	−1.74	1.72
	−2.18	1.52	−2.37	1.19
		Retinal (*all*-trans)		
5	−0.78	1.31	−0.96	1.11
	−1.08	1.31	−1.26	1.11
	−1.65	0.26	−2.37	1.67
	−2.19	2.04		
		β-Carotene (*all*-trans)		
11	−1.14	1.58	−1.35	0.11‖
	−1.50	0.73	−1.74	0.10‖
	−1.77	1.50	−1.96	0.14‖
	−2.10	1.32	−2.22	—

* Number of double bonds in conjugation. † Half-wave potential with reference to mercury pool anode in 10 percent aqueous 0.1*N* tetra-butyl ammonium hydroxide as supporting electrolyte. ‡ Half-wave potential with reference to mercury pool anode in 10 percent aqueous 0.1*N* tetra-butyl ammonium chloride as supporting electrolyte. ‖ Apparent diffusion current calculated in the concentration range of 10^{-4} to $10^{-5}M$, due to limited solubility; all other vitamins 10^{-3} to $10^{-4}M$.

in Table 1 for the various vitamins, except for β-carotene in tetra-butyl ammonium chloride, where the reduction waves at half-wave potentials at −1.76 and −1.96 volts had to be combined before a linear relationship existed between diffusion current and concentration.

The observed half-wave potentials in Table 1 for the first reduction wave for all conjugated vitamins in tetra-butyl ammonium chloride, except retinyl acetate, were more negative by 0.2 to 0.3 volt than those in the tetra-butyl ammonium hydroxide supporting electrolyte. If the voltage drops across the cell in the two supporting electrolytes is calculated and then is compared, a value of 0.03 volt is obtained. When added to the relative difference in reference anodic potential between Cl- (0.1*N*) HgCl/Hg and OH^- (0.1*N*) HgO/Hg (*11*) (assuming relative potential are similar in 10 percent aqueous dioxane), this value would show that the difference in half-wave potential between the neutral and basic solvent media is −0.2 volt. With differences in half-wave potentials greater than −0.2 volt in the tetra-butyl ammonium chloride electrolyte, other factors are important, as suggested by the geometrical isomer in the case of the all-*trans* retinol and 13-*cis* retinol. With retinyl acetate the difference in the half-wave potential of the first wave is +0.09 volt. This reversal in the general observation that the half-wave potential is relatively more negative in tetra-butyl ammonium chloride than in tetra-butyl ammonium hydroxide is unexplained at present.

As calculated in Table 1, the similarity in apparent diffusion coefficient between all-*trans* retinyl acetate, 13-*cis* retinol, and all-*trans* retinol for the first reduction wave in tetra-butyl ammonium hydroxide indicates the same value for *n* in the Ilkovic equation,

$$i_d = 607\ n\ D^{\frac{1}{2}} C m^{\frac{2}{3}} t^{\frac{1}{6}}$$

where *n* is the number of electrons exchanged at the dropping mercury electrode; i_d is the diffusion current in microamperes; *D* is the diffusion coefficient, $cm^2\ sec^{-1}$; *C* is the concentration in millimoles per liter; *m* represents mg mercury sec^{-1}; and *t* is the drop time in seconds. In tetra-butyl ammonium chloride the value for *n* is possibly the same for all-*trans* retinyl acetate, 13-*cis* retinol, and all-*trans* retinol but not necessarily the same as would be found in tetra-butyl ammonium hydroxide.

The first reduction wave has been reported (*8, 9*) for a series of unsaturated polyene aromatic hydrocarbons in dioxane to be the addition of two consecutive electrons with the subsequent waves being related to a higher rate of protonation, as shown in the general equation,

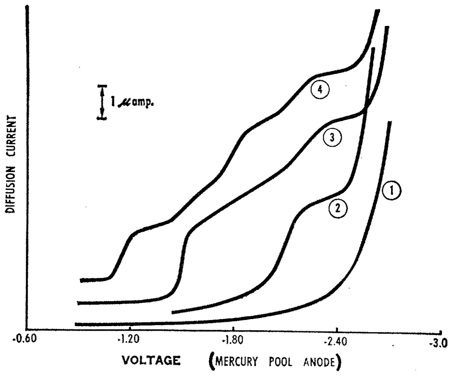

Fig. 1. Polarographic reduction in 10 percent aqueous dioxane containing 0.1*N* tetra-butyl ammonium hydroxide. *1*, Control, solvent media; *2*, vitamin D_2, 5 × 10^{-4}*M*; *3*, 13-*cis* retinol, 5 × 10^{-4}*M*; and *4*, β-carotene (all-*trans*), 5 × 10^{-4}*M*.

$$R + e \rightarrow R^- + e \rightarrow R^= + H\cdot \rightarrow RH^- + H\cdot \rightarrow RH_2$$

If R is a polyene unsaturated hydrocarbon, no reference could be found that the half-wave potential of the first reduction wave was also a function of the geometrical isomers of the polyene. In Table 1, the first reduction wave in the neutral supporting electrolyte for 13-*cis* retinol is −1.74 volts, for all-*trans* retinol, −1.86 volts. This indicates greater ease in adding the first electrons to the 13-*cis* retinol than to the all-*trans* retinol. The similarity in the half-wave potential in the alkali media of the first reduction wave for 13-*cis* retinol and all-*trans* retinol may possibly be due to alkaline rearrangement of 13-*cis* or all-*trans* retinol.

The half-wave potential of the first reduction wave in tetra-butyl ammonium chloride for all-*trans* retinal is in close agreement with the value Fields and Blout (*16*) obtained for 2,4,6,8,10 decapentaenal in 50 percent buffered dioxane, *p*H 5.05, when a correction is made for the differences in anodic electrode (*9*). The explanation they offered for the first wave in the polyene aldehyde was a one-electron, possibly reversible, reduction followed by dimerization of the free radical. Since no mention was made of the second reduction wave, it can be assumed that the aldehyde group is reduced. The reduction was for all-*trans* retinal observed at half-wave potential of −2.19 and −2.37 volts in tetra-butyl ammonium hydroxide and tetra-butyl ammonium chloride, respectively, was associated with the reduction of the aldehyde group. Evidence for reduction of the aldehyde group was obtained from polarograms of equal molar concentrations of all-*trans* retinal and heptaldehyde (5 × 10^{-4}*M*) in both supporting electrolytes. The polarograms had half-wave potentials almost identical to those in Table 1 with the only significant difference in the apparent diffusion coefficient at a half-wave potential of −2.35 volts in tetra-butyl ammonium chloride and −2.15 volts in tetra-butyl ammonium hydroxide which increased by a factor of approximately 2.

Current studies (*10*) for retinol and retinyl acetate in acetonitrile-benzene mixture indicate a diffusion controlled first reduction wave which is reversible with the concurrent addition of two electrons. The dropping mercury electrode adds one electron on each end of the conjugated double bonds to form a structure containing four double bonds in conjugation. At the second reduction wave two more electrons are added to give a more saturated retinol containing three double bonds in conjugation. Finally, the third wave is related to retinol with only two double bonds in conjugation.

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