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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION AND ELECTROCHEMICAL DETECTION OF RETINOL AND ITS ISOMERS

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SUMMARY

Baseline separation of the isomers of retinol using reversed-phase high-performance liquid chromatography (HPLC) in less than 30 min is presented. A new approach to the detection of retinol using electrochemical detection is developed. The oxidative electrochemistry of retinol is studied at a glassy-carbon electrode using coulometry, ultraviolet–visible spectrophotometry and HPLC. Amperometric detection in HPLC for retinol provided a linear response from 0 to 1.5 μg/ml and a detection limit of 4.1 ng/ml. Electrochemical detection was compared to ultraviolet–visible absorbance detection for the determination of retinol in human serum extracts. Good agreement is found for the results obtained with the two detectors.

INTRODUCTION

All-*trans*-retinol (I) and its geometric isomers (II–VI), see Fig. 1, have well documented vitamin activity.

Recently, a number of laboratories [1–3] have begun studying the cancer chemopreventive properties of retinol and other retinoids (such as 13-*cis*-retinoic acid and pro-vitamin A, all-*trans*-β-carotene). As part of a National Cancer Institute-sponsored program, the National Bureau of Standards is evaluating the currently used methods for the determination of several fat-soluble vitamins. As part of this study, we are developing alternative methods of separating and detecting retinol and its isomers. In this paper, we will describe a reversed-phase separation of all of the mono-*cis* isomers and all-*trans*-retinol as well as present a new method of detection: oxidative electrochemistry.

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We became interested in separating and identifying all-*trans* and the *cis* isomers of retinol for several reasons. Careful work has shown that the 13-*cis* isomer has a somewhat different physiological activity, ca. 75% [4], from the all-*trans*, and the other isomers have even less activity. In our experience, the commercially available all-*trans*-retinol contains several percent of the 13-*cis* isomer. Also, a number of workers have found varying amounts of the mono-*cis* isomers in a number of foods [5–7]. Finally, we were concerned about the artifact formation of *cis* isomers in the sample preparation used for the extraction of retinol from serum. Thus, in order to fully characterize the retinol content of serum samples, separating and identifying the different isomers became an important goal.

Because not all of the retinol isomers are commercially available, we generated mixtures by the photolysis of retinal followed by reduction to the alcohol as described by Landers and Olson [8]. Identification of the resulting isomers was done by the known conversion ratios in several solvents of differing polarity and by the use of the available compounds.

Stancher and Zonta [5–7] and Paanakker and Groenendijk [9] have completely separated all of the mono-*cis*-retinol isomers using a normal-phase system, employing a silica stationary phase and mobile phases containing hexane with polar modifiers, such as aliphatic alcohols. Some, but not all isomers may be separated using reversed-phase high-performance liquid chromatography (HPLC), using an octadecylsilane (C_{18}) modified silica column with acetonitrile–water [10], methanol–water [11] and a cyano (CN) modified silica with hexane and two alcohols [12]. Since we were interested in examining electrochemical detection (ED) (with its requirement of conducting mobile phases), we chose to investigate the reversed-phase approach in more detail.

Little work has been done on the ED of retinol, with only bulk electrochemical studies for guidance [13]. Retinol is a pentaene and may undergo oxidation at glassy carbon electrodes by a similar mechanism to other conjugated double-bond systems [14]. Some investigation of the electro-oxidation reaction of retinol will be presented.

EXPERIMENTAL*

HPLC apparatus

The liquid chromatograph consisted of two Waters Assoc. (Milford, MA, U.S.A.) Model 510 solvent delivery pumps with a Model 680 gradient controller. Sample injections were made with a Waters Model 710 B autosampler. Data were collected using a Nelson Analytical (Cupertino, CA, U.S.A.) Model 4416 data system that allowed cursor-controlled integration of eluted peaks from two detectors. A Kratos (Ramsey, NJ, U.S.A.) Model 773 variable-wavelength UV–visible absorbance detector was used, with a spectral bandpass of 5 nm and time constant

*Certain commercial equipment, instruments, or materials are identified in this report to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

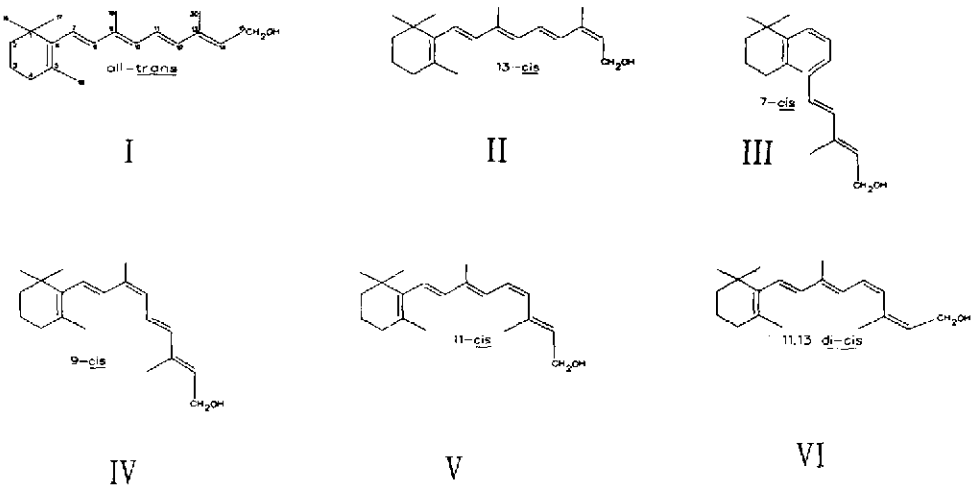

Fig. 1. Chemical structures of all-*trans*-retinol (I) and its geometric isomers (II–VI).

of 2 s. For ED, a thin-layer flow-by cell, Model Tl-5A (Bioanalytical Systems, West Lafayette, IN, U.S.A.), was modified to use a 1.2-mm glassy carbon electrode. All applied potentials are referenced to an Ag/AgCl, (0.1 mol/l potassium chloride in 70% methanol) electrode. The methanolic filling solution for the reference was employed to prevent significant daily volume changes observed with totally aqueous reference solutions. The potential was applied with a commercial potentiostat using a 2-s time constant. The two chromatographic columns that were tested for the separation of the retinol isomers were both octadecylsilyl (C_{18}) modified silica with 5-μm particle size, packed in 25×0.46 cm beds: a Vydac 201 TP (The Separations Group, Hesperia, CA, U.S.A.) and a Zorbax ODS (Dupont, Wilmington, DE, U.S.A.).

Photolysis

The retinol isomer mixtures were prepared by the photolysis of all-*trans*-retinal dissolved in hexane, ethanol, and acetonitrile. The solutions were irradiated under an incandescent lamp for 2 h while cooled in an ice bath. The ratio of the *cis* isomers at equilibrium is controlled by the relative thermodynamic stability of each isomer in solvents of a given polarity, and has been measured [6,15]. Following photo-isomerization, the retinal was then reduced by the addition of solid sodium borohydride [7] to an ethanolic solution. Using the known ratios and the published absorbance maxima [7] for the mono-*cis* isomers, the identity of the chromatographically separated peaks could be found by injection of the three mixtures in the different solvents, at several detector wavelengths. A sample of 13-*cis*-retinol was obtained as a gift (J.N. Thompson) and 9-*cis*-retinol was prepared by the reduction of the commercially available aldehyde (Sigma, St. Louis, MO, U.S.A.).

Coulometry

In order to investigate the electro-oxidation of retinol at the glassy carbon electrode, a small-volume coulometry cell was employed (see Fig. 2). The cell con-

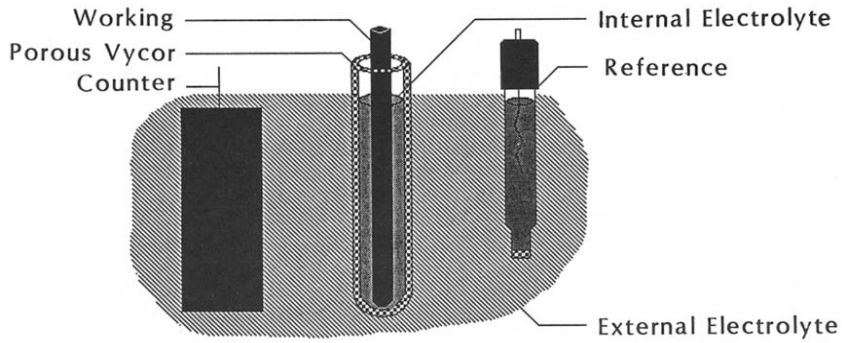

Fig. 2. Mini-volume coulometric cell. Components: 7 mm O.D. porous Vycor tube, 3.0 mm diameter glassy carbon working electrode, Ag/AgCl (0.1 mol/l potassium chloride in 70% methanol) reference electrode, platinum flag counter electrode.

sisted of a 5.5 mm I.D. porous Vycor (Corning, Corning, NY, U.S.A.) tube, heat-sealed at one end. Test electrode (500 μl) was added to the tube. The working electrode consisted of a 3 mm diameter glassy carbon rod (Atomergic, Anaheim, CA, U.S.A.), centered in the Vycor tube. This provided a relatively small distance for the reactant to diffuse (about 1.2 mm). The Vycor tube was then suspended in an external electrolyte containing a counter electrode (0.5×4 cm platinum foil) and reference electrode. Because of the small distance of diffusion and low resistance of this cell design, complete electrolysis of a reversible analyte takes place in under 0.5 h, yet sufficient volume was provided for HPLC analysis of the products. Some loss of the analyte occurs by diffusion through the large area of the porous tube, but only 5% of an unelectrolyzed solution was lost in 0.5 h.

Reagents

All-*trans*-retinol (synthetic, crystalline), all-*trans*-retinal and 9-*cis*-retinal were obtained from Sigma. Solvents used for the photo-isomerization were spectroscopic grade. For the HPLC separations, methanol and *n*-butanol were of HPLC grade, and the water was purified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.). Supporting electrolyte was prepared from Suprapur-grade acetic acid and ammonium hydroxide (Merck, Darmstadt, F.R.G.), the resulting buffer pH was adjusted using a commercial glass pH electrode. All stated pH values refer to the aqueous buffer before dilution to prepare the mobile phase.

RESULTS AND DISCUSSION

Separation of retinol isomers

Two columns were evaluated for the separation of the photo-generated retinol isomer mixtures: a wide-pore (300 Å) silica with a polymeric C_{18} phase (Vydac) and a small-pore (80 Å) silica with a monomeric C_{18} phase (Zorbax). Both columns were tested using methanol–water and acetonitrile–water mixtures. We found that the methanol–water combination worked best, in agreement with other work on reversed-phase separations of retinoids [3,11,16–24]. Fig. 3 shows the

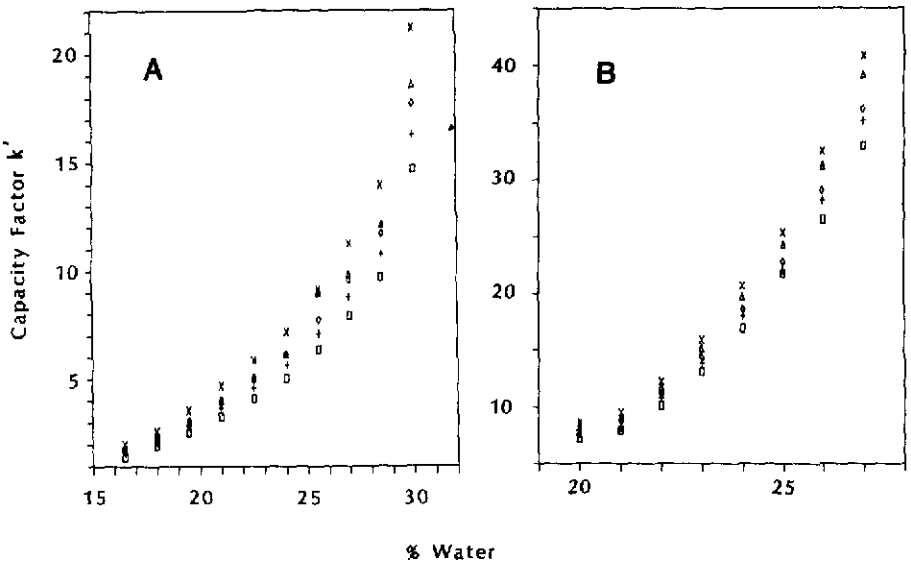

Fig. 3. Capacity factors of retinol isomers on C_{18} columns. (A) Zorbax ODS: (B) Vydac 201 TP. Flow-rate 1.0 ml/min. □, di-*cis*; +, 11-*cis*; ◇, 9-*cis*; △, 13-*cis*; ×, all *trans*.

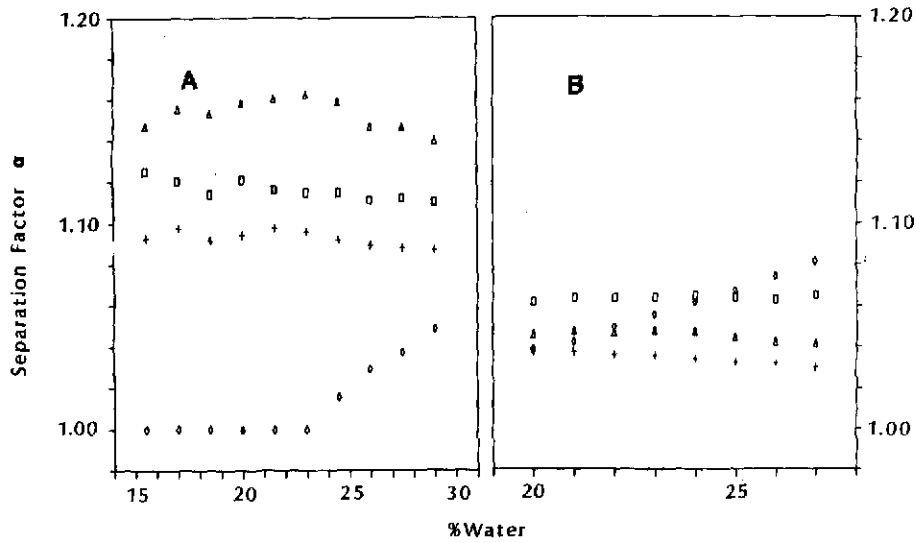

Fig. 4. Separation factor of isomers on C_{18} columns. (A) Vydac 201 TP; (B) Zorbax ODS. □, 11-*cis*/di-*cis*; +, 9-*cis*/11-*cis*; ◇, 13-*cis*/9-*cis*; △, all-*trans*/13-*cis*.

capacity factors for the various isomers on the Zorbax and Vydac columns using methanol–water mobile phases. For a given percentage water the Zorbax provides higher retention. The order of elution for the reversed-phase separations is somewhat different from that observed for normal phase. In normal phase the order of increasing retention is all-*trans* > 9-*cis* > 11-*cis* > 13-*cis* [5,6,9], but in reversed-phase, the 13-*cis* is situated between the 9-*cis* and 7-*cis* (Vydac), with the order of elution being all-*trans* > 7-*cis* > 13-*cis*, > 9-*cis* > 11-*cis*. The retention with the

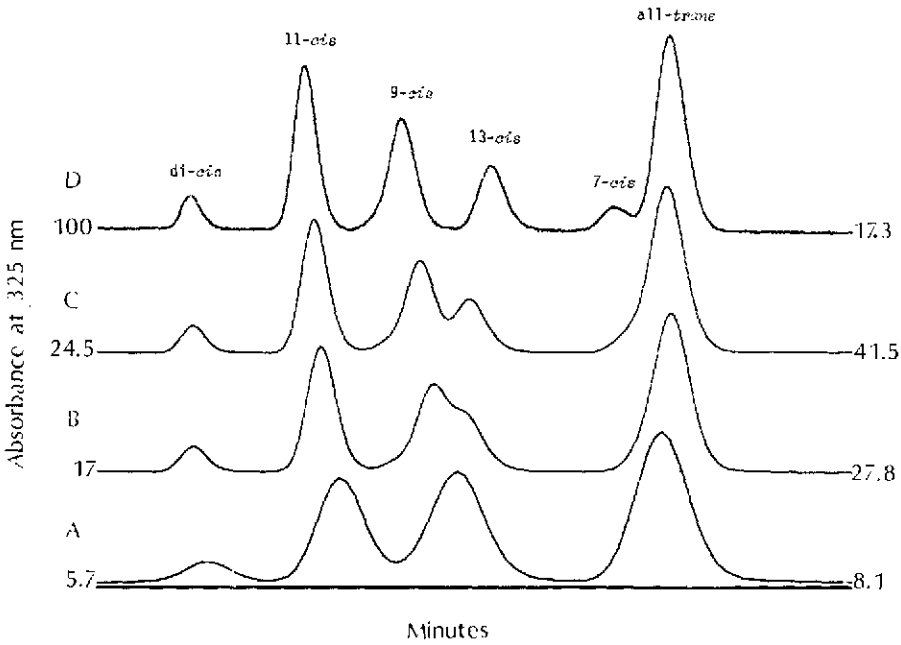

Fig. 5. Separation of retinol isomers at increasing percentage water. Conditions: column, Vydac 201 TP; flow-rate, 1.0 ml/min; sample, photolysis/reduction product from acetonitrile solvent. (A) 16.5% water; (B) 25.5% water; (C) 28.5% water, and (D) 30.0% water in methanol. Start/stop times of each chromatogram as marked.

Zorbax column for the 13-*cis* is even greater relative to the other isomers with the order being all-*trans* > 13-*cis* > 7-*cis* > 9-*cis* > 11-*cis*. Fig. 4 shows the comparison of the separation factor ($\alpha = k_2'/k_1'$) for the two columns as a function of the percentage water in the mobile phase. For the separation of the retinol isomers the wide-pore polymeric column provides greater selectivity, allowing baseline separation of all of the isomers. Fig. 5 shows the change in the selectivity as the water content was increased from 16.5% (A) to 30.0% (D). Note the more pronounced effect of the percentage water on the retention of the 9-*cis* isomer, allowing the complete separation of the 9-*cis*/13-*cis* pair at higher water content.

Having found a stationary phase that would allow the separation all of the retinol isomers, we decided to investigate different solvent systems to decrease the time needed for the analysis. We first tried the use of argentation chromatography, but were not successful, as also found by De Ruyter and De Leenheer [25]. A number of workers have noted the improved selectivity that can be achieved by the use of a third solvent in HPLC that has the properties of both a non-polar and a hydrophilic end, i.e., tetrahydrofuran, methyl *tert.*-butylether and various alkanols [12,26,27]. These solvents may be able to wet the C_{18} phase, changing the three-dimensional structure of the stationary phase. Based on a recommendation of Thompson et al. [27] we evaluated the use of *n*-butanol as a selectivity-enhancing third solvent. We found that as we replaced a part of the methanol in

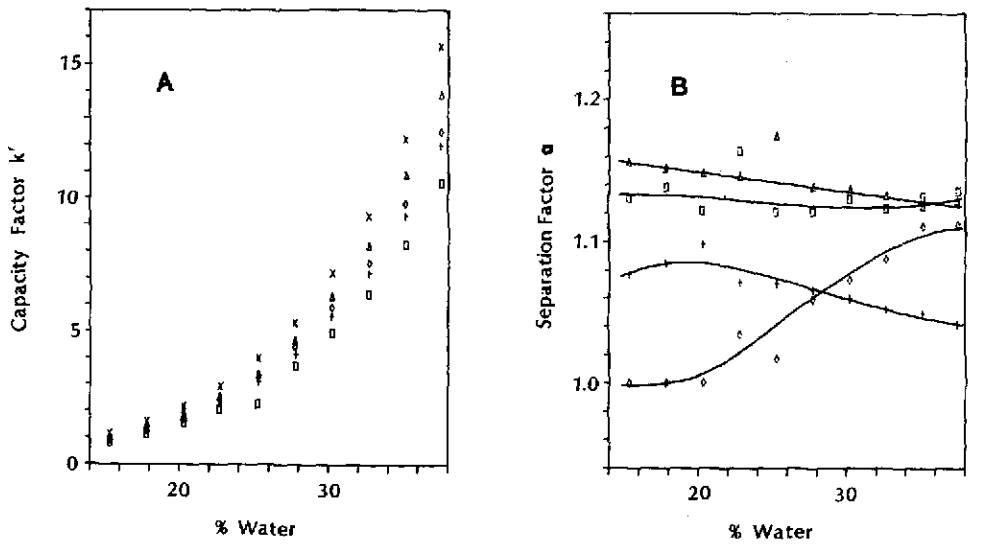

Fig. 6. (A) *Capacity and* (B) *separation factors of isomers on Vydac with n-butanol solvent system.* (A) □, di-*cis*; +, 11-*cis*; ◇, 9-*cis*; △, 13-*cis*; ×, all-*trans*. (B) □, 11-*cis*/di-*cis*; +, 9-*cis*/11-*cis*; ◇, 13-*cis*/9-*cis*; △, all-*trans*/13-*cis*.

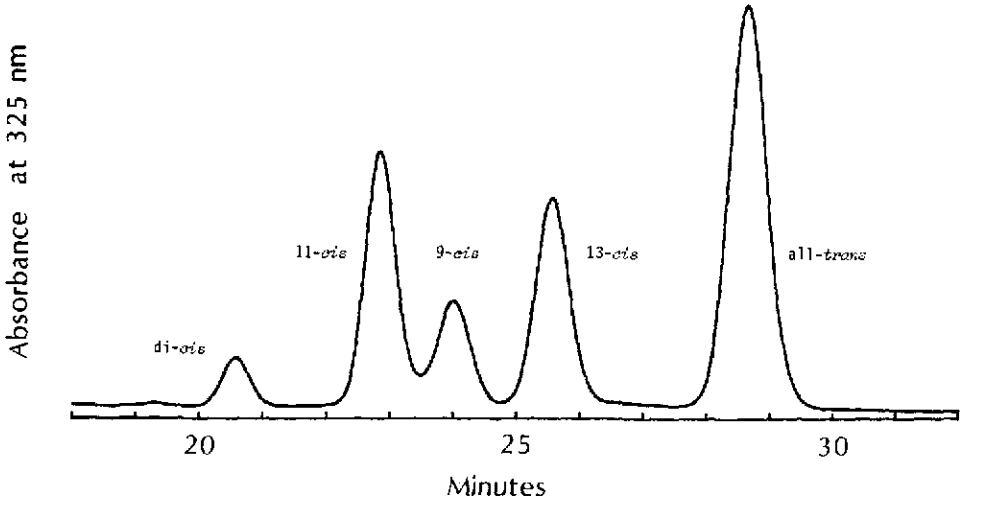

Fig. 7. Separation of retinol isomers with Vydac/*n*-butanol system. Conditions: column, Vydac 201 TP; solvent, methanol-*n*-butanol-water (65:10:25), containing 0.01 mol/l ammonium acetate, pH 3.2; flow-rate, 1.0 ml/min.

the mobile phase with *n*-butanol, the retention was sharply decreased but the separation of the isomers was maintained. Fig. 6A shows the retention of the isomers as a function of the water content using a mobile phase with a constant *n*-butanol content of 10%. This amount of *n*-butanol was selected so that the useful effect was achieved without significantly increasing the mobile phase viscosity. A plot of the separation factors is given in Fig. 6B. Fig. 7 shows the complete separation of the isomers in only 30 min as opposed to more than 1 h required

for the methanol–water system. This separation allows the measurement of the individual isomers in a reasonable time frame and is compatible with the requirements of ED.

Electrochemistry of retinol

Only one study has been made of the electro-oxidation of retinol [13]. Since retinol is a conjugated pentaene, the oxidation should follow a sequence of reactions similar to that found for other di and trienes [14]. The expected reactions for alkyl dienes consist of an initial formation of a radical cation from the loss of a single electron of one double bond. This unstable intermediate can undergo several reactions, depending on the nature of the solvent (protic or aprotic) and the geometric substitution around the double bond. One frequent reaction of the radical cation is to attack an available double bond of an unoxidized molecule to form a dimer. The remaining dimer may react with the solvent with the expulsion of a proton, leaving a radical that can undergo a further one-electron oxidation and further reaction with the solvent. In this way, a diene such as 1,3-butadiene may be oxidized at carbon electrodes in methanol to form dimethoxyoctadienes [14]. The second major course of the reaction is the direct reaction of the radical cation with the solvent followed by the loss of a proton. The remaining radical may undergo a second one-electron oxidation to a cation, again followed by reaction with the solvent and loss of a proton. Following this path, for example, 1,3-butadiene oxidized in methanol will give 1,3-dimethoxybutene [14]. In aqueous solvents, either the dihydroxy derivative may be formed or the epoxide may result. In carefully dried dimethylformamide, Mairanovskii et al. [28] found a much different sequence: the simultaneous transfer of two electrons for a retinol-like compound, axerophtene. In cyclic voltammetric experiments [28], a reversible oxidation of the conjugated double-bond system was found. In the mixed protic solvent systems that we might use for the reversed-phase HPLC–ED of retinol, many possible reaction pathways exist, particularly when taking into account the fact that the molecule has five conjugated double bonds that are potentially oxidizable.

We began the electrochemical study with cyclic voltammetry at a 1.0-mm glassy carbon disk in methanol–water (99:1) with a sodium perchlorate supporting electrolyte. A totally irreversible oxidation consisting of a small peak with $E_p = +0.80$ V and a larger peak at +1.20 V was found with this technique. Under the conditions of our experiment, we did not observe the passivation of the electrode found in tetrahydrofuran [13]. To measure the number of electrons transferred and to generate oxidation product for analysis, we used a small-volume coulometric apparatus (see Fig. 2). This cell allowed electrolysis of a sufficient volume of solution (500 μl) that aliquots could be withdrawn (25 μl) periodically for HPLC analysis. A 3-mm glassy carbon rod was used as the working electrode and was polished with 6-μm diamond paste and wiped with methanol before use. In some previous work using this coulometric cell, we found that only 60 min were required for the complete electrolysis of a reversibly oxidized compound [29]. However, when we performed electrolysis on 0.1 mmol/l solutions of retinol, the current only decreased to about 25% of the maximum value in 60 min. Thus, for the purpose of measuring the charge as a function of applied potential (Fig. 8), we

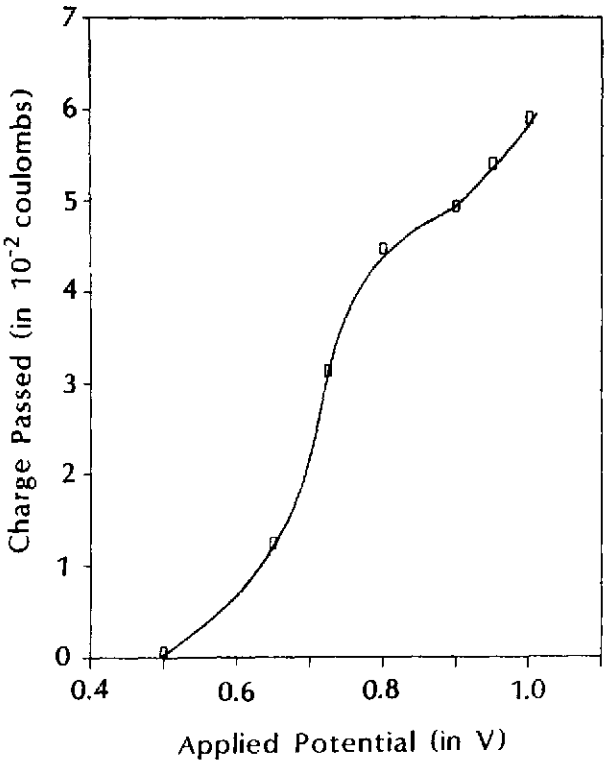

Fig. 8. Charge versus applied potential for the oxidation of retinol.

chose an arbitrary electrolysis time of 70 min. In a methanol–water (99:1) solvent system, primarily one wave is observed at $E_{1/2} = +0.71$ V, with the beginning of a second wave at higher potential. The potential of the waves found in the coulometry correspond reasonably well with the two waves found in the cyclic voltammetry. The charge produced in the oxidation at an applied potential of +0.9 V was 4.7 electrons per retinol molecule. More charge was produced at higher potential or longer electrolysis times. In an attempt to limit the number of electrons transferred, electrolysis was performed at an applied potential of +0.725 V, which corresponded to the first oxidation wave. The *n* value here was still 3.0 electrons per retinol molecule. Cyclic voltammetry performed on the glassy carbon disk, inserted into the electrolyzed solution, showed the complete disappearance of the first oxidation wave but little difference in the second wave.

UV–visible spectrophotometry was also run on the collected products at various times in the electrolysis using a supporting electrolyte of 0.1 mol/l sodium perchlorate in methanol with 1% water. The primary absorbance band of the retinol ($\lambda_{max} = 325$ nm) decreased with the appearance of several new bands. Two bands ($\lambda_{max} = 312$ and 295 nm) increase in intensity in the early phase (< 5 min) of the electrolysis. As the electrolysis proceeded, these bands decreased and a new band at 277 nm increased. Late (> 60 min) in the electrolysis a final band at 234 nm appeared. The dramatic hypsochromic shifts of the wavelength maxima can be correlated to the absorbance of the remaining unoxidized double bonds. For

conjugated enes, a hypsochromic shift of 30 nm is expected for the loss of each single double bond. Thus, the band at 295 nm (325 minus 30) should correspond to four remaining conjugated double bonds. Some insight can be gained on the identity of the other absorbance bands from the excellent work by Mairanovskii et al. [30] on the reduction of retinol. In this paper, the reduction of the alcohol moiety was found to occur first, followed by the reduction of the number 7 double bond. The reaction of the number 13 bond gave rise to a third reduction wave. Compounds with identical conjugation of the remaining double bonds were synthesized and found to have an absorbance maximum at 278 nm after the reduction of the 7 bond (corresponding to three remaining bonds) and at 237 nm after the reduction of the 7 and 13 bonds (corresponding to two remaining bonds). Although the actual products will be different for the oxidation of retinol, the remaining conjugation of the double bonds, and hence the absorbance maxima, should be similar. We must conclude from these UV results that the oxidation of retinol at the glassy carbon electrode shows much less preference for the selective reaction of specific double bonds (i.e., the number 7) than was found for the reduction [30]. Products corresponding to four (band at 295 nm) and three (band at 277 nm) remaining conjugated double bonds are formed early in the electrolysis, and those with two (band at 277 nm) are formed later. The decreased absorbance of the products that we observed is also consistent with the markedly decreased conjugation that oxidation of the double bonds would produce. No evidence was found for the formation of retinal ($\lambda_{max}=338$ nm [7]) from the oxidation of the alcohol moiety.

We also examined the electrolysis products using reversed-phase HPLC with UV detection. Fig. 9 shows the separation and detection at 325 nm of samples of the electrolyzed retinol as a function of time using the dry methanol–sodium perchlorate electrolyte at an applied potential of +0.9 V. The starting material ($t_R=41.7$ min) is nearly completely oxidized after only 4 min of electrolysis, yet significant current was still flowing after 60 min, showing that many of the remaining double bonds may be oxidized. Two classes of products were formed: those with shorter retention (t_R between 12 and 40 min) than retinol and those with longer retention ($t_R>52$ min). We feel that the shorter-retained products represent the formation of the methoxy derivatives of the oxidized double bonds. The number of products indicates that multiple bonds are oxidized, and that there are several double bonds that do not differ strongly in the difficulty of oxidation, producing many products. In electrolysis experiments in which 1% water is added to the electrolyte, some different products, with even shorter retention times than the suspected methoxy adducts, are formed. These may be dihydroxy and/or epoxy products. We also ran the HPLC–UV experiments at three different wavelengths: 325, 280, and 240 nm. After 5 min of electrolysis, the 1% water system showed most peaks with stronger absorbance at 325 and 240 nm. This is consistent with oxidations leaving four and two conjugated double bonds, respectively. In both the 100% methanol and the 1% water in methanol electrolytes, the long-retained products probably arise from the coupling of the electrogenerated radicals with the starting material. These dimers are prominent in the early phase

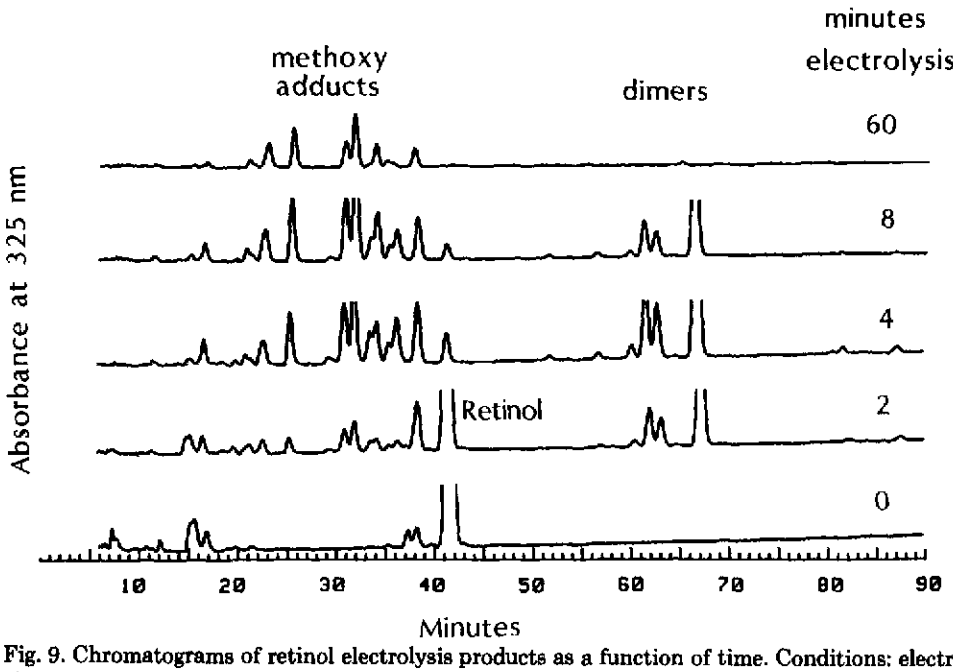

Fig. 9. Chromatograms of retinol electrolysis products as a function of time. Conditions: electrode, glassy carbon; applied potential, +0.90 V (vs. $AgCl/Cl^-$); electrolyte, 100% methanol–0.1 mol/l sodium perchlorate; retinol concentration, 0.217 mmol/l.

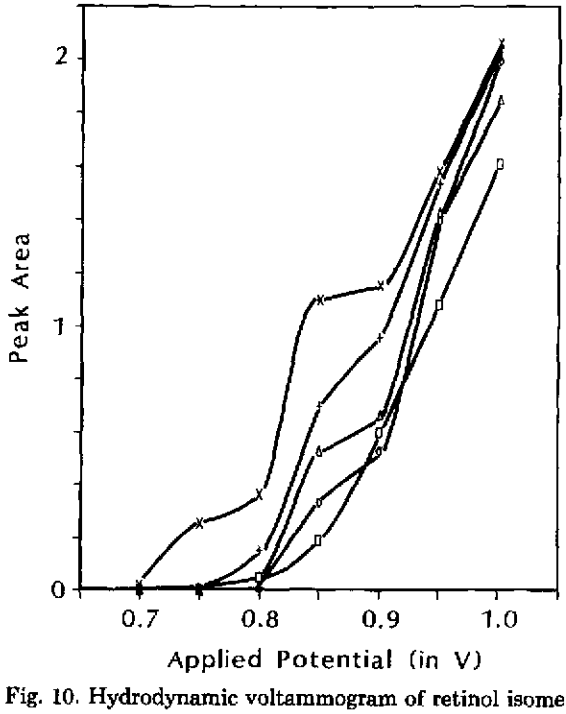

Fig. 10. Hydrodynamic voltammogram of retinol isomers. Conditions: electrode, 1.2 mm diameter glassy carbon; solvent, methanol–water (69:31), containing 0.01 mol/l buffer pH 3.2. □, di-*cis*; +, 11-*cis*; ◇, 9-*cis*; △, 13-*cis*; ×, all-*trans*.

of the electrolysis, but since they retain much of the original retinol structure, they may undergo further electrolysis and thus are depleted after 60 min of oxidation.

Based on the results of the HPLC and UV analysis of the coulometric retinol oxidation, the following conclusions can be made about the reaction. The oxidation is a multistep process, with the oxidation of more than one of the double bonds of retinol. Judging from the HPLC–UV analysis of the products, there is not a strong preference for which double bonds are oxidized first. After the formation of the initial cation radical, several follow-up reactions may occur: coupling with another retinol molecule to form dimeric products and addition of the solvent to produce dimethoxy (in pure methanol) and dihydroxy and/or epoxy (with small amounts of water) compounds. Several subsequent oxidations also occur to the remaining double bonds, generating many products even for short electrolyses.

Finally, in order to use ED for HPLC determinations, we examined the hydrodynamic voltammogram of all-*trans* and the *cis* isomers of retinol. Using the published absorptivities of the isomers [7] to provide corrected peak areas in the UV detector, the data in Fig. 10 show the corrected relative electrochemical signal strength as a function of the applied potential. All-*trans* has two waves ($E_{plateau}$ = +0.75 V and $E_{plateau}$ = +0.85 V). The 9-*cis*, 11-*cis* and 13-*cis* do not show the first wave and show decreased current for the second wave. The di-*cis* compound (probably 9,13) does not show the second wave at all. All of the isomers show the increase of current at potentials above +1.0 V which probably represents a third wave. Although more current could be measured at the higher potentials, the residual current noise from the oxidation of the methanol–water solvent used for the HPLC becomes too large. Thus, for routine detection of all-*trans*-retinol, we have chosen a potential of +0.90 V, which rests on the plateau of the second wave.

Determination of retinol

Despite the complex, multistep nature of the oxidation process for retinol, quantitation of the HPLC eluent using the electrochemical detector proved to be quite feasible. Calibration curves in the concentration range of 0–1.5 μg/ml proved to be linear (R^2=0.9992, *y*-intercept=0.011 μg/ml). We did not study the linearity of the calibration at higher concentrations because these values were out of the normal physiological range. The detection limit, defined as the concentration that provides a signal twice the peak-to-peak background noise, was found to be 4.1 ng/ml for a 20-μl injection (0.082 ng absolute) at the plateau potential of +0.90 V. This result is very similar to the detection limit found for the absorbance detector of 6.0 ng/ml (0.12 ng absolute).

We have compared the electrochemical detector to the UV–visible absorbance detector for the reversed-phase determination of extracts of human sera. Following a conventional hexane extraction of the fat-soluble material from the serum [31], the retinol was determined using a solvent [water–methanol–*n*-butanol (15:75:10 with 0.01 mol/l ammonium acetate buffer, pH 3.2] that provided a retention on the Vydac C_{18} column of 5 min. These conditions allowed the base-

TABLE I

COMPARISON OF THE DETERMINATION OF RETINOL IN SERA USING ELECTROCHEMICAL AND UV DETECTION

Each result is the average of two injections.

Serum sample No.	Retinol concentration (ng/ml)	
	UV detection at 325 nm	Electrochemical detection at +0.9 V
31	768, 780	780, 776
32	425	417
34	364, 363, 344	345, 379, 373
44	370	384

line separation of the all-*trans*- from the 13-*cis*-retinol and appears to provide good selectivity from other serum components in both the absorbance and electrochemical detectors. Table I provides comparative data on the determination of retinol using the two detectors. Results obtained with the electrochemical detector are very comparable to those obtained with the accepted UV–visible absorbance detector.

The electrochemical detector provides a useful alternative detection approach to absorbance detection. One disadvantage of the use of ED was the need to calibrate the response to external retinol standards at least twice daily, to allow for changes in sensitivity. This type of drift was not observed when using the absorbance detector.

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