Oligomerization Oscillations of L-Lactic Acid in Solution

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ABSTRACT: We employ high-performance liquid chromatography with diode array, evaporative light scattering, and mass spectrometric detection to monitor the oligomerization of L-lactic acid in pure acetonitrile and in 70% aqueous ethanol. The production of higher oligomers appears to proceed in an oscillatory fashion. A model is presented that involves the formation of aggregates (micelles), which catalyze the oligomerization.

INTRODUCTION

Low molecular weight profen drugs, amino acids, and hydroxy acids exhibit spontaneous in vitro oscillatory chiral conversion under appropriate conditions.† However, monitoring oscillatory chemical reactions in colorless organic solutions is a challenging experimental task. High-performance liquid chromatography (HPLC) with diode array detection (HPLC-DAD) is a promising tool to follow such phenomena when there are UV-absorbing species present. A bottleneck of the chromatographic approach is the time needed for a single analytical run, which makes continuous measurement of the relevant concentration changes virtually impossible, even if an autosampling device is available. One way to ameliorate this drawback is to minimize the time for a single analytical run, in that way obtaining a more nearly continuous series of quantitative results. Such an approach has been employed1 to study the oscillatory oligomerization of S(+)-ketoprofen based on HPLC data collected at 25 min intervals.

Detailed liquid chromatographic and mass spectrometric evidence has been presented for the spontaneous oscillatory peptidization of S-, R-α-phenylglycine dissolved in 70% aqueous ethanol and stored for lengthy intervals.3 Crude theoretical models have been proposed for these phenomena.1,3

L-Lactic acid is a compound of particular interest because of its biological importance. In humans and animals, the l-lactate ion is produced from the pyruvate ion through the enzymatic action of lactate dehydrogenase (LDH). It serves as a key intersection of several metabolic pathways that include carbohydrate digestion and energy transport to living cells.4 It is also produced on a large scale in industrial fermentation processes performed, e.g., by Lactobacillus bacteria.5

Despite the key role played by l-lactic acid in many biological systems, there have been only a handful of studies of its ability to undergo a spontaneous oscillatory in vitro chiral conversion.6,7 Lactic acid is poorly retained in HPLC systems8 and thereby causes considerable analytical difficulties. To solve this problem, an alternative enantioseparation method has been developed.9 A thin-layer chromatographic method is also available.10 More detailed investigation of the possibility that l-lactic acid can undergo spontaneous in vitro oscillatory oligomerization in either aqueous or nonaqueous solutions is clearly desirable.

Our earlier studies1 demonstrat that the spontaneous oscillatory oligomerization of low molecular weight carboxylic acids is often accompanied by oscillatory chiral conversion. In aqueous media, the conversion mechanism can be schematically described as11

\[ \text{L-lactic acid} \rightleftharpoons \text{enolate ion} \rightleftharpoons \text{D-lactic acid} \] (1)

In anhydrous media and in the presence of trace amounts of water, the probable mechanism of chiral conversion is12

\[ \text{l-lactic acid} \rightleftharpoons \text{enol} \rightleftharpoons \text{D-lactic acid} \] (2)

In this study, we present high-performance liquid chromatographic and mass spectroscopic experiments on the chemical transformations of l-lactic acid dissolved either in 70% aqueous ethanol or in pure acetonitrile. In order to gain deeper insight into the nature of these processes, we employ HPLC with three different detection methods: diode array (DAD), evaporative light scattering (ELSD), and mass spectrometric (MS) detection. Finally, we propose a theoretical model to describe the observed phenomena.

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Reagents. Analytical grade L-lactic acid (LA) was purchased from Fluka (Buchs, Switzerland; cat. #69771). For the spontaneous condensation experiments, 2.00 g L⁻¹ (2.22 × 10⁻² mol L⁻¹) of LA was dissolved in acetonitrile (ACN) or in 70% aqueous ethanol (EtOH). Both solvents were HPLC purity grade (Merck, Darmstadt, Germany); water was double distilled and deionized (Elx Advantage model Millipore system; Molsheim, France). Samples were stored in tightly stoppered colorless glass vials and

Figure 1. Sequences of three chromatographic concentration profiles of L-lactic acid in ACN registered at 22 °C after 0, 170, and 750 min with the DAD detector at (a) 220 nm and (b) 250 nm and (c) with the ELSD detector.

Figure 2. Sequences of three chromatographic concentration profiles of L-lactic acid in 70% EtOH registered at 22 °C after 0, 70, and 80 min with the DAD detector at (a) 220 nm and (b) 250 nm and (c) with the ELSD detector.
allowed to age for 980 min (ACN) or 400 min (70% EtOH) at 22 °C.

High-Performance Liquid Chromatography with Diode Array Detection (HPLC-DAD) and Evaporative Light-Scattering Detection (HPLC-ELSD). High-performance liquid chromatographic analysis was carried out using a Varian model 920 liquid chromatograph (Varian, Harbor City, CA, USA) equipped with a Varian model 900-LC autosampler, a gradient pump, a Varian model 330 DAD detector, a Varian model 380-LC ELSD detector, and Galaxie software for data acquisition and processing. The analyses were performed on 20 μL aliquots of the l-lactic acid solutions in the isocratic mode, using a Pursuit 5 C18 (5 μm particle size) column (250 mm × 4.6 mm i.d.; Varian, Harbor City, CA, USA; cat. no. A3000250C046) with a methanol–water (5:5, v/v) mobile phase at a flow rate of 0.5 mL min⁻¹. The chromatographic column was thermostatted at 30 °C with a Varian Pro Star 510 column oven. These analyses were done at 10 min intervals for 980 min (ACN) and 400 min (70% EtOH), respectively.

High-Performance Liquid Chromatography with Mass Spectrometric Detection (LC-MS). Liquid chromatographic analysis with mass spectrometric detection (LC-MS) was carried out using an LC-MS System (Varian, Palo Alto, CA, USA) equipped with a Varian ProStar pump, Varian 100-MS mass spectrometer, and Varian MS Workstation, v. 6.9.1, software for data acquisition and processing.

The LC analyses were performed on 20 μL aliquots of the l-lactic acid solutions in the isocratic mode, using a Pursuit X RS 3-C18 column (50 mm × 2.0 mm i.d.; Merck KGaA, Darmstadt, Germany; cat. no. A6001050C020) and a methanol–water (5:5, v/v) mobile phase at a flow rate of 0.20 mL min⁻¹.

Mass spectrometric detection was carried out in the electrospray ionization (ESI) mode (full ESI-MS scan, positive ionization, spray chamber temperature 45 °C, drying gas temperature 200 °C, drying gas pressure 25 psi, capillary voltage 70 V, and needle voltage 5 kV).

### RESULTS

HPLC-DAD and HPLC-ELSD. In Figure 1, we present chromatograms of lactic acid dissolved in pure ACN, registered for the freshly prepared sample and after 170 and 750 min. These chromatograms were recorded with the DAD and ELSD detectors. In Figure 2, we give analogous results for lactic acid dissolved in 70% aqueous ethanol as the solvent, satisfactory (although not always baseline) separations were obtained (Figure 2) with the peaks for which the oscillatory plots were drawn. Thus, the chromatographic peak heights were considered to be proportional to the respective concentrations. With ACN as the solvent, for the sake of comparison we employed the same chromatogram registration time of 10 min and the same chromatographic system as in the case of 70% aqueous ethanol. These conditions did not allow full separation of the various oligomeric species. In this case, the time course of the peak heights was monitored only for the two peaks for which the respective maxima were clearly separable in the chromatographic profiles.
In order to probe in as much detail as possible of the evolution of the concentration profiles (fingerprints) of our samples, we employ two UV wavelengths (\(\lambda = 220 \text{ and } 250 \text{ nm}\)) with the DAD detector as well as utilizing ELSD detection. The fingerprints found in the ACN solution differ significantly from those seen in the 70% EtOH solution. The observed dissimilarities presumably arise from differences between the ability of ACN and of EtOH/H\(_2\)O to participate in hydrogen bonding with lactic acid. Because of the greater tendency of 70% EtOH to form H-bonded aggregates with LA, the concentration profiles found in this solvent exhibit more and much better resolved peaks attributable to oligomerization products than in ACN.

In order to visualize the evolution of a solution of lactic acid in ACN, we plot the peak heights for the two peaks with distinct maxima (even if not fully resolved) against the time after the preparation of the sample in Figure 3a–c. The plots in Figure 3a,b show the peak heights at retention times \(t_R\) of 4.61 and 5.26 min, as recorded with the DAD detector at 220 and 250 nm. The corresponding plots at \(t_R = 5.07\) and 5.57 min are shown in Figure 3c. In all cases, we observe irregular, low amplitude oscillations in the respective concentrations.

The signal corresponding to the peaks in Figure 3a–c at retention time \(t_R = 5.26\) min (DAD) and \(t_R = 5.57\) min (ELSD) undergoes a sharp drop in height and in the amplitude of its oscillation between about 360 and 460 min. We attribute this phenomenon to the temporary disappearance of the corresponding oligomerization product in the reaction medium, either due to the dissociation of this oligomer to a lower species or due to its consumption to produce a higher species. Similar phenomena have been observed in the analogous studies of the oscillatory polycondensation of \(S(+)\)-ketoprofen (ref 2; Figure 3) and the oscillatory peptidization of \(\alpha\)-phenylglycine and \(\alpha\)-phenylalanine (unpublished results).

To assess whether our signals contain a significant periodic component, we Fourier transform the data in Figure 3 and calculate the corresponding power spectra. All the power spectra contain a large peak at zero frequency, which we remove from our plots. In Figure 4, we show as an example the power spectrum calculated from the data for the peak at \(t_R = 4.61\) and \(\lambda = 250 \text{ nm}\) (Figure 3b). The two highest peaks in this power spectrum appear at frequencies just below and above 0.01 min\(^{-1}\), implying a periodicity of ca. 100 min.

Analogous results for lactic acid dissolved in 70% EtOH are given in Figure 5a–c. In this case, the peaks of the separated oligomers are much better resolved than in the ACN solution, and thus, the heights are nearly uncontaminated by peak overlap. Hence, they can be considered as proportional to the concentrations of distinct species. Examples of the corresponding power spectra calculated from these chromatographic data are shown in Figure 6a–d. The highest peak in Figure 6a occurs at 0.035 min\(^{-1}\). This frequency roughly corresponds to a periodicity of 30 min, which can be seen in the peak height data at \(t_R = 5.36\) min in Figure 5b. The power spectrum for the peak at \(t_R = 5.53\) min (Figure 6b) also displays a maximum at 0.035 min\(^{-1}\), though the highest peak in this plot is located at 0.005 min\(^{-1}\). Figure 6c,d also show peaks at a frequency ca. 0.035 min\(^{-1}\).

The results obtained with the ELSD detector (Figures 3c and 5c) should be a somewhat better indicator of the dynamics of the system than the DAD data. ELSD is a universal detector that is lacking in UV-absorbing chromophores, though the DAD detector may also respond to differences in the refractive index of the compounds in the effluent.

In Figure 7, we present spectochromatograms of freshly prepared samples of lactic acid in ACN and 70% EtOH (Figure 7a,c, respectively) and also after 980 min in ACN (Figure 7b) and 400 min in 70% EtOH (Figure 7d). These figures give evidence of significant changes in the composition of the solutions after several hours. In the 70% EtOH sample, we easily identify a

Figure 5. Time series of chromatographic peak heights for \(\alpha\)-lactic acid in 70% EtOH at 22 °C, for 400 min. (a) DAD detector at \(\lambda = 220 \text{ nm}\); (b) DAD detector at \(\lambda = 250 \text{ nm}\); and (c) ELSD detector. Plots in a and b are for peaks at \(t_R = 4.85, 5.01, 5.36\), and 5.53 min; plots in c are for \(t_R = 5.07, 5.20, 5.38\), and 5.42 min.
pronounced growth of one or more species with increased retention times and hence higher molecular weights.

**LC-MS.** Combining liquid chromatography with mass spectrometry provides an independent method to monitor the processes of interest. We first performed initial separations of freshly prepared and aged samples on an LC column. We then recorded the mass spectra of the column effluents. The respective chromatograms are shown in Figure 8a–d. For each chromatogram, insets show the mass spectra of the effluents corresponding to the key peaks.

With the fresh lactic acid sample in ACN (Figure 8a), there is only one major peak in the chromatogram. The predominant signal in the corresponding mass spectrum is at $m/z$ 258.34, which is consistent with the following structure: $[\text{LA trimer} + \text{Na} + \text{He}]^+$. This $m/z$ 258.34 signal is essentially absent in the effluents collected after 980 min (Figure 8b). In that case, however, in each mass spectrum a characteristic group of signals can be seen with the distribution of percent yields resembling a normal distribution centered around $m/z$ 627. This signal is consistent with the structure $[\text{LA octamer} + \text{Na} + \text{He}]^+$. The concentration profile of the fresh lactic acid sample in 70% EtOH (Figure 8c) is richer than that of the analogous sample prepared in ACN. We attribute the greater variety of entities present to the increased capacity for hydrogen bonding between the lactic acid species and the EtOH and H$_2$O in the solution. Nevertheless, the mass spectra corresponding to the highest peaks in this chromatogram are relatively simple and quite similar to one another. The predominant signals in the three recorded mass spectra are at $m/z$ 306 or 319. The signal at $m/z$ 306 corresponds to an LA tetramer. Mass spectra recorded after 400 min (Figure 8d) suggest a greater abundance of higher aggregation products than in the fresh sample. Again, in each mass spectrum a characteristic group of signals can be seen with a near-normal distribution of percentage yields and centered at $m/z$ values between 650 and 800. There are also significant low-level signals at $m/z$ values above 1000.

Thus, all three techniques employed, HPLC-DAD, HPLC-ELSD, and LC-MS, point to the spontaneous oscillatory oligomerization of L-lactic acid in both an aqueous (70% EtOH) and a nonaqueous (ACN) solvent.

**Theoretical Model.** In an effort to gain a further understanding of our experimental results, we constructed a model of an oscillatory oligomerization process. The model contains only three species: a precursor, P (e.g., lactic acid), a short oligomer, E, derived from the precursor, and an aggregate (possibly a micelle), M, that is formed from several molecules of E. The key assumption is that M can form either via an uncatalyzed pathway or by an autocatalytic pathway in which M serves as a template for the formation of more M. The reactions are

$$n_1P \rightarrow E \quad \text{rate} = k_0P \quad \text{(oligomerization)}$$

$$n_2E \rightarrow M \quad \text{rate} = k_0E \quad \text{(uncatalyzed aggregation)}$$

$$2M + n_2E \rightarrow 3M \quad \text{rate} = k_2M^2E \quad \text{(catalyzed aggregation)}$$

$$M \rightarrow \text{products} \quad \text{rate} = k_bM \quad \text{(decomposition)}$$

A more detailed model would contain a sequence of aggregation steps of the form

$$E + E \rightarrow E_2 \quad k_1$$

$$E_2 + E \rightarrow E_3 \quad k_2$$

$$\ldots$$

$$E_i + E \rightarrow E_{i+1} \quad k_i$$

$$\ldots$$

and similarly for the formation of E from P.
Since we have no information about the individual aggregation rate constants, we instead employ a coarse-graining process analogous to that proposed by Coveney and Wattis and group these steps into a single aggregation step with an effective aggregation number, \( n_2 \), and an effective rate constant, \( k_u \). We adopt a similar approach to the formation of the reactive species, \( E \), where \( n_1 \) might be 1 if a rate-determining enolization suffices to activate the molecules for further rapid aggregation, \( n_1 = 2 \) if dimerization is required, etc. Again, if \( n_1 > 2 \), the model equation is presumed to capture a sequence of initiation processes. The model, which is equivalent to the Gray–Scott model with precursor decay augmented with the coarse-graining parameters \( n_1 \) and \( n_2 \), gives oscillations with values of \( n_1 \) at least as high as 20 and with \( 1 \leq n_2 \leq 8 \). The number of oscillations decreases with both increasing \( n_1 \) and \( n_2 \), i.e., oscillations are more robust with smaller aggregates. In Figure 9, we show a simulation with \( n_1 = 5 \) and \( n_2 = 8 \). The amplitude (about 1.4 mmol/L) and period (about 30 min) of the oscillations are in reasonable agreement with our experiments.

**DISCUSSION AND CONCLUSIONS**

We have employed a variety of chromatographic techniques to demonstrate that l-lactic acid generates higher oligomers during prolonged storage in both aqueous and nonaqueous media. This oligomerization process takes place in an irregular, oscillatory fashion. The key biological role played by lactic acid and its derivatives lends significance to these observations, though it is difficult to extrapolate the present results to physiological conditions. We note that similar phenomena have been observed in an amino acid, phenylglycine. We propose a simple model involving activation/oligomerization of the monomer followed by aggregation of oligomers to form a micelle or similar species. This aggregate then serves as a template for further aggregation. Such a scheme, perhaps augmented by surface-assisted aggregation and/or catalysis, might be helpful in understanding the origins of some of the earliest biomolecules.

We note that the data that we have presented are quite noisy and irregular and that, at least in the case of ACN, the chromatographic peaks are not sufficiently well separated to allow for identification of individual peaks with particular molecular species. What we can say definitively is that the observed oscillations of the peak heights result from chemical processes in the samples rather than from experimental noise. To confirm this, we carried out analogous measurements under similar conditions on stable analytes that do not undergo any structural transformation such as chiral inversion and/or oligomerization. In these control experiments, all samples showed (constant) peak height reproducibility of ±3% relative error or better, a deviation considerably smaller than the variations observed in our lactic acid samples.

![Figure 7. Spectrochromatograms of l-lactic acid solution registered with the DAD detector after (a) 0 min and (b) 980 min storage in ACN and after (c) 0 min and (d) 400 min storage in 70% EtOH; \( T = 22 ^\circ C \). The colors represent peak heights; red corresponds to the highest peaks, blue to the lowest.](image-url)
if our inability to resolve the peaks means that some peaks may have contributions from more than one species, the long duration of the oscillations implies that at least one species in each oscillatory peak must exhibit oscillatory behavior.

Figure 8. Continued
Our experiments, thus, demonstrate qualitatively the presence of oscillatory oligomerization of lactic acid. Our model simulations are similarly limited in that we have no data about the rate constants of individual steps nor about the identity of the oligomeric species. We must, therefore, be satisfied at this stage with a qualitative demonstration of the feasibility of oscillatory oligomerization, which our simulations provide. Further progress will require the development and application of techniques that make it possible to identify and monitor the individual components of this important system.

Figure 8. Chromatogram of an L-lactic acid solution registered with the LC-MS system after (a) 0 min and (b) 980 min in ACN and after (c) 0 min and (d) 400 min in 70% EtOH; T = 22 °C. Insets show mass spectra of the effluents collected at the maxima of the respective peaks.

Figure 9. Simulated oscillations. Initial concentration of P = 0.02 M. Parameter values: $n_1 = 5$, $n_2 = 8$, $k_0 = 1.5 \times 10^{-5}$ s$^{-1}$, $k_a = 5 \times 10^{-5}$ s$^{-1}$, $k_a = 2.5 \times 10^7$ M$^{-2}$ s$^{-1}$, $k_a = 5 \times 10^{-5}$ s$^{-1}$.

Our experiments, thus, demonstrate qualitatively the presence of oscillatory oligomerization of lactic acid. Our model simulations are similarly limited in that we have no data about the rate constants of individual steps nor about the identity of the oligomeric species. We must, therefore, be satisfied at this stage with a qualitative demonstration of the feasibility of oscillatory oligomerization, which our simulations provide. Further progress will require the development and application of techniques that make it possible to identify and monitor the individual components of this important system.

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