CHEMISTRY LETTERS, pp. 1411 - 1414, 1984.

EPIMERIZATION IN THE PHEOPHYTIN a/a' SYSTEM[1)]

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By means of a recently developed high-performance liquid chromatographic procedure with high resolution, a kinetic aspect of the epimeric interconversion between pheophytin a and a' has been investigated for the first time.

Each of the four chlorophyll (Chl) derivatives [Chl a and b, and Mg-free pheophytin (Pheo) a and b] exists as a pair of epimers (e.g., Chl a and a') distinguished from each other by the steric position of H and $COOCH_3$ attached to carbon 10 in the cyclopentanone ring.[2-12)] Except for the UV-visible absorption characteristics, the a- and a'-form behave quite differently in many respects (NMR,[4,8,9,12)] circular dichroism,[2,5,6,10)] solubility,[9)] chemical reactivity,[9)] rate of pheophytinization,[9,13)] intermolecular aggregation,[4,7-9,13)] etc.). For a reliable in vitro characterization of Chl derivatives, it is hence essential to have a correct knowledge on the rate of epimeric interconversion and the equilibrium composition in a given environment. However, due mainly to the lack of an effective method for rapid determination of the epimer pairs, the knowledge on these fundamental aspects has been very sparce[4,9,11)] and sometimes controversial.[4,9)] An improved high-performance liquid chromatographic (HPLC) procedure, which we have developed recently,[2)] is useful to clarify these aspects. This Letter describes a first part of our systematic investigations on the epimerization of Chl derivatives in organic solvents.

The a-form is converted to the a'-form, and vice versa, via a common enol (cf. Fig. 1). This conversion is promoted by a nucleophilic attack of a solvent molecule to carbon 9.[4,8,9)] For Chl derivatives having a Mg^{2+} ion in the chlorin macrocycle, the Mg^{2+} ion is also attacked by nucleophilic molecules and by the carbonyl moiety of a neighboring Chl molecule; this tends to introduce some complication in

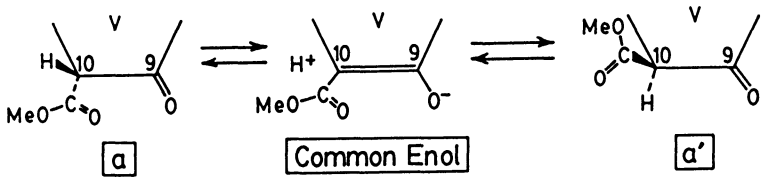

Fig. 1. Scheme for epimerization of Chl derivatives. See Ref. 2 for the ring and carbon numberings.

the kinetics of epimerization.[13] In view of this, we chose the Mg-free Pheo a/a' system to initiate a series of epimerization studies.

Pheo a and a' were prepared as in a previous work.[2] Their epimeric purities were 97.5% and 94.8%, respectively. Impurities other than the corresponding epimers were not detected at a 0.1-% level. Each pigment was dissolved in deoxygenated pyridine at a concentration of ca. 5 mmol dm^{-3}. The pyridine solutions, thermostated at 40 °C in darkness, were sampled at an appropriate time interval and analyzed by the HPLC specified in Ref. 2.

The kinetics of epimerization are treated as follows. Denoting Pheo a and Pheo a' by a and a', respectively, the epimerization is expressed by

$$\underline{a} \underset{k'}{\overset{k}{\rightleftharpoons}} \underline{a}' \qquad (1)$$

where k and k' are first-order rate constants. In what follows, $[\underline{a}']_t$, $[\underline{a}']_0$, and $[\underline{a}']_\infty$ represent the mole fraction of a' (ratio of the concentration of a' to the sum of the concentrations of a and a') at time t, at the start of measurement, and at equilibrium, respectively. Equation 1 is equivalent to the following kinetic formula:

$$[\underline{a}']_t = [\underline{a}']_\infty + ([\underline{a}']_0 - [\underline{a}']_\infty)\, e^{-(k+k')t} \qquad (2)$$

with

$$[\underline{a}']_\infty = k/(k+k') \qquad (3)$$

Thus the values of k and k' could be obtained by analyzing a single $[\underline{a}']_t$ vs. t curve, starting from a sample with any value of $[\underline{a}']_0$, as long as the latter has

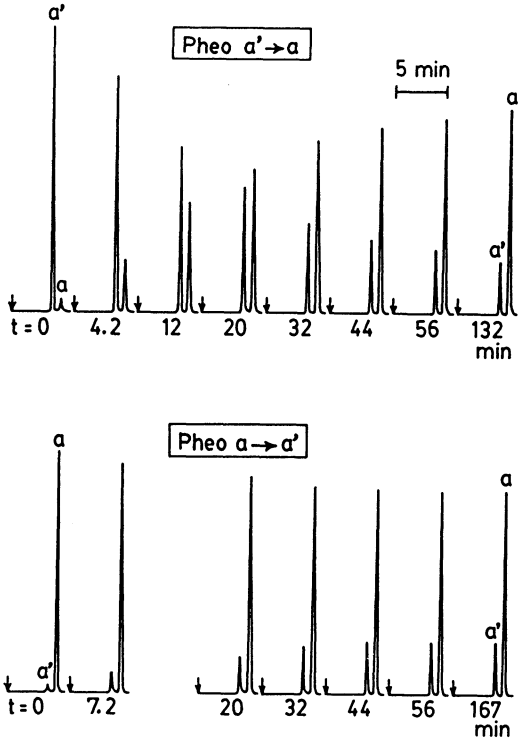

Fig. 2. HPLC charts showing the temporal change in the Pheo a/a' compositions in pyridine at 40 °C, starting from 94.8-% Pheo a' (top) and 97.5-% Pheo a (bottom). Each chromatogram has been normalized to give the same total peak area. Eluent, hexane/2-propanol (98.4/1.6, v/v); sample size, 3 μL.

been off-equilibrium. To see if this prediction is valid, the measurements have been conducted here on 97.5-% Pheo a and 94.8-% Pheo a' as starting materials.

A series of HPLC charts, showing the temporal evolution of the Pheo a/a' composition, are displayed in Fig. 2. The a/a' pair can be separated clearly in an elution time of about 5 min. The chromatograms at t = 0 correspond to the pigments dissolved in a hexane/2-propanol (98.4/1.6, v/v) mixture, employed here as the HPLC eluent, in which the epimerization proceeds much more slowly than in pyridine.[13] It is seen that each sample, initially rich in a or a', approaches a similar composition in the course of time. Both samples became practically indistinguishable at times longer than 100 min. Since the a- and a'-form have a common molar absorption coefficient,[2] the relative abundance of each form can be evaluated simply by integration of the corresponding peaks. No molecular transformations (e.g., allomerization and solvolysis) other than epimerization were noticed up to 30 h.

The HPLC analytical results are summarized in Fig. 3 as two $[a']_t$ vs. t profiles. The solid curves are those simulated by Eq. 2, using a common combination of forward and backward rate constants: $k = 1.65 \times 10^{-4}$ s^{-1} and $k' = 7.61 \times 10^{-4}$ s^{-1}. The equilibrium fraction of the a'-form, 0.178 ± 0.002, corresponds to an equilibrium constant $K = [a']_\infty / [a]_\infty = k / k' = 0.217$, which in turn gives a Gibbs energy of the reaction of 3.98 kJ mol^{-1} or 41.2 meV per molecule.

The present result ensures the validity of Eqs. 1-3 in studying the kinetics of epimerization. No previous works concerning epimerization of Chl derivatives have yielded information on the rate constants for interconversion. To our knowledge, even no attempts had been made to measure the temporal evolution of epimeric composition in solvents until the end of 1983, when Omata and Murata[11] pursued Chl a ⟶ a' and Chl b ⟶ b' conversions in a DEAE-Sepharose CL-6B column.

The equilibrium fraction of a', 0.178, which is attained in a fairly short time under the present experimental conditions, is sufficiently high and should not be neglected in in vitro characterization of Pheo a. A preliminary study[13] demonstrated that the equilibrium fraction of the a'-form in Pheo a/a' and Chl a/a' sys-

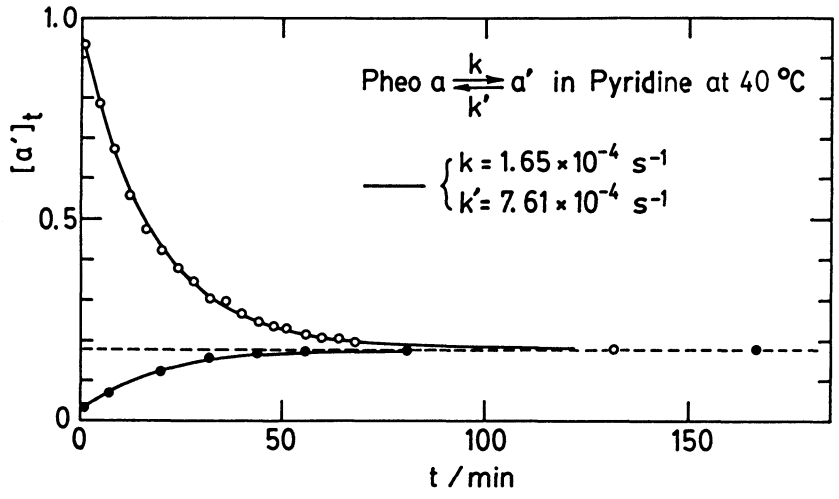

Fig. 3. Time courses of Pheo a/a' epimerization starting from 94.8-% Pheo a' (o) and 97.5-% Pheo a (●). The solid curves are theoretical ones according to Eq. 2.

tems ranges from 0.13 to 0.25, depending on the nature of the solvent and temperature. Further works are in progress on the kinetics as well as thermodynamics of epimerization of Chl derivatives.

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(Received June 7, 1984)