Fluorescence Properties of the Envelope Membranes from Spinach Chloroplasts

DETECTION OF PROTOCHLOROPHYLLIDE*

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At 77 K, under excitation at 440 nm, two major fluorescence emission peaks were observed in envelope membranes from spinach chloroplasts at 636 and 680 nm. A narrow range of wavelengths around 440 nm and a wider range of wavelengths between 390 and 440 nm, respectively, were responsible for excitation of the 636 and 680 nm fluorescence emissions which, in marked contrast with thylakoid fluorescence emission, were devoid of any exciting components between 460 and 500 nm. In acetone extract of envelope membranes, two fluorescence emission peaks were observed at 635 and 675 nm. After extraction of the acetone solution by nonpolar solvents (petroleum ether or hexane), the 675 nm fluorescence emission was partitioned between the polar and nonpolar phases whereas the 635 nm fluorescence emission was solely recovered in the polar phase. All together, the results obtained suggest that envelope membranes contain low amounts of pigments having the absorption and fluorescence spectroscopic properties, together with the behavior in polar/nonpolar solvents, of protochlorophyllide and chlorophyllide. In addition, modulation of the level of fluorescence at 636 and 680 nm could be obtained by addition of NADPH to envelope membranes under illumination. The presence of protochlorophyllide in chloroplast envelope membranes together with its possible photoconversion into chlorophyllide could have major implication for the understanding of chlorophyll biosynthesis in mature chloroplasts.

The chloroplast envelope is a yellow membrane system with a unique carotenoid composition (1, 2); the major carotenoid present in both envelope membranes is violaxanthin (3) whereas thylakoids contain a much higher proportion of β-carotene (2). Douce et al. (1) have demonstrated that envelope membranes are devoid of chlorophylls, the presence of such pigments in envelope preparations reflecting contamination by small thylakoid fragments.

However, although devoid of the most conspicuous plastid pigment—chlorophyll—some nongreen plastids such as etioplasts contain chlorophyll precursors, for instance, protochlorophyllide (4). Since all of the different plastids present in plant cells share the same membrane structure, i.e. the envelope membranes, we decided to analyze further the pigment composition of the chloroplast envelope in order to determine whether this unique membrane system contains chlorophyll precursors.

In this article, we present detailed analyses of envelope membranes by fluorescence spectroscopy. The results obtained show fluorescence emission and excitation spectra different from those already known for thylakoids and suggest the occurrence of protochlorophyllide in envelope membranes from mature chloroplasts.

MATERIALS AND METHODS

Isolation of Purified Intact Chloroplasts—Chloroplasts were isolated from 3–4 kg of spinach leaves obtained from local markets. Deveined leaf sections were cut into chilled medium (2.5 liters/kg of leaves) containing 330 mM mannitol, 25 mM tetrasodium pyrophosphate, 0.1% bovine serum albumin; pH was adjusted to 7.8 with HCl. The leaves were homogenized for 2–3 s in a 4-liter Waring Blender. All of the operations were carried out at 0–5 °C. A crude chloroplast pellet was obtained as described by Douce et al. (1) and purified further by isopycnic centrifugation in Percoll gradients (5).

Purification of Envelope Membrane from Spinach Chloroplasts—Purified intact chloroplasts were lysed in hypotonic medium as described by Douce et al. (1). The total envelope membranes and thylakoids were purified from the lysate (swollen chloroplasts) by centrifugation through a step sucrose gradient as described by Douce et al. (1). In such a preparation, the contamination of thylakoids was reduced to a very low level. For instance, the chlorophyll content of the envelope membrane fraction was less than 0.2 μg/mg of envelope protein.

Spectrophotometry—Absorption spectra of isolated envelope membranes were recorded on an Amino DW-2 spectrophotometer, at a band width of 2 nm. All spectra were recorded at 77 K, using Pyrex glass cells of 1.5 nm optical length. Absorption spectra of solvent extracts of envelope membranes (see below) were recorded with Cary 14 (Varian) spectrophotometer equipped with a laboratory-made scale expansion accessory, at a band width of 2 nm. All species were recorded at room temperature, in glass cells of 1 cm optical length.

Spectrofluorometry—Fluorescence emission and excitation spectra were recorded either at room temperature or at 77 K on a laboratory-made spectrofluorimeter, as previously described by Fineau et al. (6). UV light was excluded by using a proper filter (J555, Métallisations et Traitement Optiques, Massy, France) which was placed between the 250-watts xenon bulb and the exciting monochromator (M25, Jobin et Yvon, Longjumeau, France). A cut-off filter (OG 590, Schott, Mainz, RFA) was placed between the sample and the analysis monochromator (M25, Jobin et Yvon). The sample (10 μl) was put on a filter paper disk at the bottom of a cylindrical microcell (diameter: 10 mm). The top of the microcell was covered with a two-arm light pipe: the first one coming from the exciting monochromator and the second one going to the analyzing monochromator equipped with a red-sensitive photomultiplier (RTC XP 2203 B, La Radiotechnique, U.S.A).
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France). The emission and excitation spectra were recorded with emission and excitation band widths of 3 and 9 nm, respectively. Excitation spectra were corrected for energy variations of the xenon light source. A linear relationship between the intensity of fluorescence emission and the amount of envelope suspension was observed for protein concentrations in the range of 1–3 mg/ml. Therefore, all spectra were recorded using envelope suspensions containing about 2 mg of protein/ml.

Partition of Envelope Membrane Pigments between Polar and Non-polar Solvents—Four volumes of a mixture containing acetone and ammonium hydroxide (0.1 M) (9:1, v/v) (7) were added to 1 volume of envelope membrane suspension (1.5 mg of protein/ml), and the mixture obtained was vigorously mixed and then centrifuged at 8000 rpm (Sorvall rotor SS 34) for 10 min to remove the proteins. The pellet was devoid of fluorescent signals and 2.5 ml of supernatant were added either to 0.9 ml of petroleum ether (b.p. 40–60 °C) according to Griffiths et al. (8) or to 1 volume (2.5 ml) of hexane according to Rebeiz et al. (7). In both cases, a two-phase separation of the solution was obtained, namely petroleum ether-extracted (or hexane-extracted) acetone phase or lower phase, containing polar molecules and petroleum ether (or hexane) phase or upper phase, containing nonpolar molecules such as carotenoids. Each phase was recovered and used for further analyses (spectroscopic or thin-layer chromatography analyses).

Thin-layer Chromatography—The two phases recovered from hexane extraction of acetone-soluble envelope material were applied to Silica Gel 60 precoated thin-layer chromatography plates (Merck). The developing solvent system used to separate the pigments was hexane-acetone-hexanol (9:20:10, v/v) (9), the well characterized envelope carotenoids (2) were easily located on TLC plates under dim light and were identified by comparing their Rf values with those of reference standards. Fluorescent molecules were located under UV light (360 nm).

Incubation of Envelope Membranes with NADPH—Purified envelope membranes were incubated for 1 h at room temperature under illumination with white light (0.15 watt/m²) in a medium containing 10 mM Tricine¹/NaOH (pH 7.8), 10% glycerol, and 2 mM NADPH (final protein concentration, 2.3 mg/ml). Control experiments were performed under illumination (in the absence of NADPH or in the presence of 2 mM NADH). After incubation, 10% of the sample was deposited on a filter paper disk and the fluorescence emission spectra were analyzed at 77 K as described above, under excitation at 440 nm.

Protein and Chlorophyll Determination—Protein concentration was determined according to Lowry et al. (10) (using bovine serum albumin as a standard). Chlorophyll concentration was measured according to Arnon (11).

RESULTS

Absorption Spectra of Isolated Envelope Membranes—The absorption spectra of purified envelope suspension shown in Fig. 1 is typical of chloroplast envelope membranes when examined at low (77 K) temperature; three main maxima were observed in the blue region at 429, 454, and 486 nm whereas essentially no absorption was observed above 540 nm. Such a spectrum is due to the presence of significant amounts of carotenoids and the lack of chlorophyll in purified envelope membranes (for reviews, see Refs. 12–14). However, in agreement with all the data published so far, a very weak signal was always detected in the red region, around 680 nm (Fig. 1A). By amplification of the absorbance scale in the red region, two maxima appeared distinctly at 632 and 677 nm (Fig. 1B). The 632 nm peak does not correspond to an absorption maximum of thylakoids (see Ref. 2, for example; therefore, in order to obtain more information about the pigments responsible for this absorption, we used the more sensitive spectrofluorometry.

Fluorescence Emission and Excitation Spectra of Isolated Envelope Membrane—At room temperature, two broad emission peaks were observed at 642 and 684 nm, when purified envelope membranes were excited at 440 nm (Fig. 2A). The 642 nm fluorescence emission was excited preferentially at 440 nm whereas the 684 nm fluorescence emission was strongly excited by a wide range of wavelengths between 390 and 440 nm with a maximum at 438 nm (Fig. 2B). At low temperature, sharper fluorescence emission peaks at 634–636 nm and 678–680 nm were obtained under excitation at 440 nm and a weak peak (or in some cases only a shoulder) appeared at 737 nm (Fig. 3). Lowering the temperature to 77 K resulted first in a blue shift of the two maxima from 634 to 634–636 nm and from 684 to 678–680 nm, second in a strong enhancement of the fluorescence intensity for these two emissions, and third in the appearance of a weak 737 nm emission which was quenched at room temperature.

Obviously, the fluorescence emission spectrum obtained with envelope membrane vesicles was clearly different from the well-known fluorescence emission spectrum of thylakoids excited at 440 nm, as shown in Fig. 3. In fact, the thylakoids were characterized, at 77 K, by two small emission peaks near 685–690 nm and 695–700 nm and a major one at 738 nm, which correspond to several organization states for chlorophylls within thylakoids membranes (15).

The low temperature (77 K) excitation spectra for the 636 nm fluorescence emission of envelope membranes showed a main maximum at 440 nm with only two small components at 390 and 415 nm (Fig. 4). The 680 nm emission exhibited one excitation maximum at 438 nm together with significant peaks at 390 and 415 nm (Fig. 4). In fact, accurate analysis of the 680 nm emission peak showed that the blue side of the peak was more excited by 415 nm light than the red side of the peak (result not shown), thus indicating that the 680 nm emission was probably due to a mixture of molecules having neighboring fluorescence properties. The excitation spectrum for the small 737 nm fluorescence emission peak of the envelope membranes exhibited three distinct excitation maxima around 390, 415, and 438 nm and a reduced excitation level at 470 nm (Fig. 5). It thus appeared different from that of the 738 nm thylakoid emission which was characterized by two broad excitation maxima at 438 and 470 nm (Fig. 5).

All of these observations led us to the conclusion that envelope membranes from spinach chloroplasts have charac-

1 The abbreviation used is: Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
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Fig. 2. Room temperature fluorescence emission (A) and excitation (B) spectra of isolated envelope membranes from spinach chloroplasts. Fluorescence was recorded at room temperature using 10 μl of an envelope membrane suspension as described under "Materials and Methods." A, excitation light was set at 440 nm. Fluorescence emission intensity is expressed in arbitrary units. B, excitation spectra for the 642 nm (F642, ...) and for the 684 nm (F684, ...) fluorescence emissions. Fluorescence excitation energy is expressed in arbitrary units.

Fig. 3. Low temperature fluorescence emission spectra of isolated envelope membranes (A) and thylakoids (B) from spinach chloroplasts. The emission spectra of 10 μl of envelope membrane suspension (—, A) (2 mg of protein/ml) or 10 μl of thylakoid membrane suspension (-----, B) (20 μg of chlorophyll/ml) were recorded at 77 K. Excitation light: 440 nm. Fluorescence emission intensity is expressed in arbitrary units.

Fig. 4. Low temperature excitation spectra of the two main fluorescence emission (F636 and F680) of isolated envelope membranes from spinach chloroplasts. The experimental conditions were identical to those of Fig. 3A. Excitation spectra of the 636 nm (F636, ...) and the 680 nm (F680, ...) emissions. Fluorescence excitation energy is expressed in arbitrary units.

teristic fluorescence properties clearly different from those of thylakoids. The fluorescence emission maxima for the envelope membranes which were observed at 642 or 636 nm at room temperature or at 77 K, respectively, under excitation at 440 nm, closely resemble the spectroscopic properties of nonesterified protochlorophyllide (16, 17). To probe this hypothesis, we have followed the behavior of the envelope fluorescing molecules after partition in solvents able to distinguish between polar and nonpolar molecules.

Absorption and Fluorescence Analyses of Solvent-extracted Envelope Membranes—The room temperature absorption spectrum of alkaline acetone extract of envelope membranes (see "Material and Methods") is shown in Fig. 6. In agreement with the data presented in Fig. 1, the acetone phase presented only a weak absorption in the red region which was resolved after a high amplification in a maximum absorption peak at 664 nm and a small peak at 628 nm with a shoulder at 615 nm (Fig. 6A). Obviously, the 628 nm absorption peak corresponds to the 632 nm maxima observed in isolated envelope membranes (see Fig. 1).

Partition of the pigments on the basis of their solubility in polar or nonpolar solvents, such as acetone or petroleum ether, respectively, according to Griffiths et al. (8), resulted in the total extraction of the yellow pigments from the alkaline acetone solution by petroleum ether. However, the 628 nm absorption peak was recovered only in the petroleum ether-extracted acetone phase (Fig. 6, B and C), thus suggesting that the molecules responsible for this absorption are polar.

The 77 K fluorescence emission spectra of the three fractions obtained as described above are shown in Fig. 7A. When excited at 440 nm, the alkaline acetone phase was characterized by two maxima at 635 and 675 nm (Fig. 7A). After extraction of the acetone solution by petroleum ether, the 635 nm fluorescence emission peak remained in the acetone phase (Fig. 7A) whereas the 675 nm fluorescence emission peak was clearly partitioned between the petroleum ether-extracted acetone phase and the petroleum ether phase.

Similar results were obtained when the acetone solution was extracted with hexane, as described by Rebeiz et al. (16). For instance, the results obtained by spectrofluorometry (Fig. 7B) clearly demonstrate that the 634–635 nm fluorescence emission peak present in the acetone solution of the envelope membrane pigments could not be extracted by hexane, whereas the 675–677 nm emission maximum was partitioned between the hexane-extracted acetone phase and the hexane phase.
The acetone extraction of the envelope membrane pigments does not induce a significant shift in the 636 nm emission maximum present in the native envelope membranes. In addition, the excitation spectra (not shown) of the 634-635 nm emission present in the hexane-extracted or petroleum ether-extracted acetone phase were very similar to that of the 636 nm emission of the envelope membrane suspension (see Fig. 4).

The observation that a 634-635 nm fluorescence emission peak was obtained after excitation at 440 nm of a hexane-extracted (or petroleum-extracted) acetone phase is good evidence for the presence of protochlorophyllide in native envelope membranes from mature spinach chloroplasts. Further evidence for such a hypothesis was provided by thin-layer chromatography of the different phases obtained after solvent extraction of envelope membranes. A clean separation of the envelope carotenoids extracted by hexane from an acetic solution was obtained in the TLC system described by Seliskar (9). In addition, no significant amount of chlorophyll, as judged by the lack of green spots on TLC plates, was found in the hexane phase, and the colorless hexane-extracted acetone phase was entirely devoid of carotenoids.

When observed under UV light (360 nm), two fluorescent spots were observed in the first acetone extract. After extraction with hexane, one of them (RF value 0.75) was present only in the hexane phase whereas the other (RF value 0.2) was found only in the hexane-extracted acetone phase. Since the latter fluorescent spot behaved like protochlorophyllide or chlorophyllide in the chromatographic system used, such a result provides further evidence for the presence of such chlorophyll precursor(s) in envelope membranes from mature spinach chloroplasts.

These results prompted us to question whether the presence of protochlorophyllide could have any physiological significance. Therefore, we looked for an effect of illumination and NADPH on the fluorescence properties of isolated envelope membranes.

**Modulation of the Fluorescence Emission of Isolated Envelope Membranes**—Illumination of an envelope suspension for 1 h under white light (0.15 watt/m²) induced a limited photodegradation of the pigments responsible for fluorescence emission. For instance, when recorded at 77 K, the fluorescence emission spectra obtained after excitation at 440 nm show a nonspecific decrease of all fluorescence peaks at 636, 680, and 737 nm, when compared to what was obtained in the control conditions (i.e. after 1 h in the dark) (Fig. 8). A very different phenomenon was observed when NADPH was added to the suspension prior to incubation for 1 h under white light (0.15 watt/m²) and at room temperature: as shown in Fig. 8, a decrease in the amplitude of the 636 nm fluorescence emission peak was observed together with a parallel increase of the 680 nm fluorescence emission peak, thus suggesting a phototransformation of the pigments responsible for the 636 nm emission into those responsible for the 680 nm emission. In addition, such transformations could not be observed when isolated envelope membranes were incubated either with NADPH in darkness or with NADH in darkness or under illumination (results not shown).
Fig. 7. Low temperature emission spectra of solvent extracts of envelope membranes from spinach chloroplasts. The experimental conditions for acetone extraction and partition between polar and nonpolar solvents were identical to those of Fig. 6. Each fluorescence emission spectrum was recorded at 77 K under 440 nm light excitation. Fluorescence emission is expressed in arbitrary units. A, partition between acetone and petroleum ether ether phase; — , petroleum ether-extracted acetone phase; — , petroleum ether phase. B, partition between acetone and hexane of acetone-extracted acetone phase; — , hexane-extracted acetone phase.

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The results presented above show several lines of evidence for the occurrence of protochlorophyllide in envelope membranes isolated from mature chloroplasts. First, when analyzed by spectrophotometry at 77 K, isolated envelope membranes show a discrete absorption peak at 632 nm. Second, when analyzed by spectrofluorimetry under excitation at 440 nm, envelope membranes show a characteristic fluorescence emission at 634-636 nm (at 77 K), a value which is in agreement with the spectroscopic properties of protochlorophyllide (16, 18, 19). In addition, this 636 nm fluorescence emission has been detected in osmotically lysed, intact chloroplasts as a very weak signal, but not in purified thylakoid or chloroplastic subfractions other than envelope membranes. Thus, such a fluorescence emission appears to be related to the presence of envelope membranes in the analyzed sample. Third, the 635 nm fluorescence emission observed in acetone extracts of envelope membranes was not removed by addition of nonpolar solvents, such as petroleum ether or hexane, as previously shown for protochlorophyllide (16), and has a low \( R_F \) value after chromatography on silica gel TLC plates as shown for protocholorophyllide by Seliskar (9).

It is, however, likely that the protochlorophyllide pool present in envelope membranes is heterogeneous. For instance, the excitation maximum changed from 440 to 446 nm when fluorescence emission was recorded on the blue side or on the red side of the 636 nm emission peak. In addition, we have observed a shift of the fluorescence emission maximum (from 642 to 636 nm) when the temperature was lowered from room temperature to 77 K. This shift could be due to variations of the relative fluorescence yield of single components occurring with temperature changes. In fact, such a heterogeneity is not surprising since Cohen and Rebeiz (17) have characterized in situ, in etiolated bean leaves and etiolated cucumber cotyledons, the presence of five distinct fluorescing forms of protochlorophyllide having emission and excitation maxima, respectively, in the range of 630-657 nm and 440-447 nm. Belanger and Rebeiz (21) have shown in solvent extracts of etiolated or greening tissues that the protochlorophyllide pool is composed of nonvinyl and divinyl protochlorophyllide having different excitation maxima (in ether at 77 K: 436-437 nm and 443-444 nm, respectively).

Characterization of the molecules responsible for the fluorescence emission at 680 and 737 nm (in isolated envelope membranes, Fig. 3) or 675-677 nm (in solvent-extracted envelope membranes, Fig. 6) obtained after excitation at 440 nm (and recorded at 77 K) is more difficult. We have shown that a significant part of the 680 nm fluorescence emission peak was not extracted by nonpolar solvents such as petroleum ether or hexane and remained in the acetone extract of envelope membranes. The spectral properties and solubility in polar solvents closely resemble those of chlorophyllide (7, 16, 17). Furthermore, the differences in the excitation spectra of the blue and red sides of the 680 nm emission obtained in
envelope membranes suggest that this emission proceeds from a mixture of closely related molecules.

The small 737 nm fluorescence emission peak is probably related to chlorophyll and therefore could reflect a very small contamination of envelope membranes by small thylakoid pieces. However, the differences observed in the excitation spectra of the 737 nm peak in thylakoids and in envelope membranes suggest that this contamination, if it occurs, is probably not due to bulk thylakoids, but more likely to a small discrete subthylakoidal pieces.

All of the data discussed above lead to the conclusion that envelope membranes isolated from mature spinach chloroplasts, although devoid of contaminating thylakoids, contain small pools of chlorophyllide and protochlorophyllide. Because of the molecular diversity of these molecules (17, 20, 21), further investigation is necessary to determine their exact chemical nature.

An important question raised by our data is the physiological significance of the presence of protochlorophyllide and chlorophyllide in envelope membranes. These molecules are in fact precursors of chlorophyll which usually accumulate either in dark-grown etiolated plants or during dark phases of photoperiodically grown plants whereas illumination of these plants induced a rapid decrease in protochlorophyllide content and a parallel increase of chlorophylls (19, 22). Thus, the presence of small amounts of protochlorophyllide in envelope membranes from mature chloroplasts could be relevant to some steps of chlorophyll biosynthesis in this membrane system. The sequence of the reactions involved in chlorophyll biosynthesis has been thoroughly studied (for a review, see Ref. 23), but little is known about compartmentation of the involved enzymes within chloroplasts. The first steps in chlorophyll biosynthesis, namely the formation of protoporphyrin IX, occur in the soluble phase of chloroplasts (24), but all of the subsequent steps in protoporphyrin IX transformation are catalyzed by membrane-bound enzymes (23). This is the case of the enzyme involved in the transformation of protochlorophyllide into chlorophyllide (i.e. NADPH-protochlorophyllide oxidoreductase); this enzyme has been found to be membrane-bound in etioplasts (25-27) as well as in chloroplasts (8). Although NADPH-protochlorophyllide oxidoreductase is likely concentrated in prokaryotic bodies of etioplasts, the exact nature of the plastid membrane(s) containing this enzyme together with protochlorophyllide is not really clear (25-30). In the chloroplast, the localization of such an enzyme in envelope membranes cannot be ruled out. In fact, we have shown that modulation of the relative levels of the 636 and 680 nm fluorescence emission of the envelope membranes can be obtained in vitro by addition of NADPH under illumination (Fig. 8). Thus, this experiment might mimic some aspects of the photoconversion of protochlorophyllide into chlorophyllide as reported by Griffiths and co-workers in etioplasts (31) as well as in chloroplasts (8).

The involvement of envelope membranes in chlorophyll synthesis is also suggested by some experimental data: for instance, Fuesler et al. (32) reported that in isolated intact chloroplasts, Mg-chelatase was accessible to molecules unable to pass through the inner envelope membranes, thus suggesting that Mg-chelatase could be present in envelope membranes. Furthermore, in light/dark synchronized Chlamydomonas reinhardtii, Johannesmeier and Howell (33) have shown that the level of some chlorophyll precursors, such as Mg-protoporphyrin methyl ester, is able to regulate the light-induced cytosolic light-harvesting chlorophyll a/b binding protein mRNA accumulation. Consequently, the authors have postulated that such intermediates in chlorophyll synthesis could be located in the chloroplast envelope.

In this membrane system, fluorescent signals are not obscured by those of chlorophylls and, thus, spectrulfluorometric analysis could be extended to the analysis of other fluorescent intermediates which are known to occur between Mg-protoporphyrin and chlorophyllide (7).

If the envelope membranes are indeed involved in the synthesis of some chlorophyll precursors, we must imagine a system that provides chlorophyllide to growing thylakoids since: first, assembly of chlorophyllide with geranylgeranyl-PP to form geranylgeranyl-chlorophyllide and further reduction into chlorophyll occurs only in thylakoids (34-36), and second, chlorophyll accumulates only in thylakoids. In fact, a transport system is also required for the numerous plastid components such as polar lipids (galactolipids, phosphatidylglycerol) or prenylquinones (α-tocopherol, plastoquinone-9) that are synthesized on the inner envelope membrane from mature chloroplasts and accumulate in thylakoids (for reviews, see Refs. 12-14). Experiments to analyze further this major problem in chloroplast biogenesis are in progress.

REFERENCES

35. Block, M. A. (1985) Thèses de Doctorat d’État, Université Scientifique et Médicale de Grenoble, France