The Native Forms of the Phycobilin Chromophores of Algal Biliproteins

A CLARIFICATION

Padraig O'CARRA,* Richard F. MURPHY† and S. Derek KILLILEA‡
Department of Biochemistry, University College, Galway, Ireland

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Pigments released from phycoerythrins and phycocyanins by treatment with hot methanol are currently regarded as equivalent to the native chromophores phycoerythrobilin and phycocyanobilin. However, evidence presented here confirms the original views of O'Carra & O'hEocha [(1966) *Phytochemistry* 5, 993–997] that these methanol-released pigments are artefacts differing in their chromophoric conjugated systems from the native protein-bound prosthetic groups. By contrast, the native spectral properties are retained in pigments released by careful acid treatment of the biliproteins and these acid-released phycobilins, rather than the methanol-released pigments, are therefore regarded as the protein-free forms of the native chromophores. The conclusion reached by Chapman, Cole & Siegelman [(1968) *J. Am. Chem. Soc.* 89, 3643–3645], that all the algal biliproteins contain only phycoerythrobilin and phycocyanobilin, is shown to be incorrect. The identification of a urobilinoid chromophore, phycourobilin, accompanying phycoerythrobilin in B- and R-phycoerythrins is confirmed and supported by more extensive evidence. The cryptomonad phycocyanins are shown to contain a phycobilin chromophore accompanying phycocyanobilin. This further phycobilin has the spectral properties of the class of bilins known as violins and the provisional name 'cryptoviolin' is proposed pending elucidation of its structure.

The algal biliproteins are a group of brilliantly coloured fluorescent proteins that function as photosynthetic pigments in red, blue-green and cryptomonad algae (O'hEocha, 1965, 1966). They are subdivided into two major categories on the basis of visual appearance: the phycoerythrins are red with a golden fluorescence; the phycocyanins are blue with a red fluorescence (O'hEocha, 1965, 1966). Several subtypes of these chromoproteins are distinguished on the basis of differences in absorption spectra. R-, B- and C-phycoerythrins and R-, C- and allo-phycocyanins are found in various members of the Rhodophyta (red algae) and Cyanophyta (blue-green algae). Cryptomonad algae contain a complex group of further variants [see O'hEocha *et al.* (1964) for a review of these].

The brilliant coloration of the phycoerythrins and

the phycocyanins is imparted by open-chain tetrapyrrole chromophores that are covalently attached to the apoproteins (O'hEocha, 1965, 1966). These chromophores are termed phycobilins. They have labile structures and are difficult to release from the apoproteins without alteration. This has greatly hindered the complete structural characterization of the pigments. We believe that the structures currently accepted for the major phycobilins, phycoerythrobilin and phycocyanobilin, apply to artefact derivatives of the native chromophores. Since the confusion of native and artefact forms has been extensive (Beuhler *et al.*, 1976; Chapman *et al.*, 1967, 1968; Cole *et al.*, 1967; Crespi & Katz, 1969; Crespi *et al.*, 1968; Glazer & Fang, 1973; Glazer & Hixson, 1975; Troxler *et al.*, 1978), we decided that it was appropriate to clarify this issue before proceeding to a consideration of the structures of the native chromophores [see the following paper (Killilea *et al.*, 1980)].

A further issue needing clarification is the question as to whether phycoerythrobilin and phycocyanobilin are the only phycobilins occurring in

* To whom reprint requests should be addressed.

† Present address: Department of Biochemistry, Medical Biology Centre, Queen's University, Belfast, Northern Ireland, U.K.

‡ Present address: Department of Biochemistry, North Dakota State University, Fargo, ND 58102, U.S.A.

algal biliproteins, as claimed by Chapman *et al.* (1968). O'hEocha & O'Carra (1961) and O'Carra *et al.* (1964) suggested the occurrence of a third type of phycobilin with urobilinoid spectral properties in R-phycoerythrin, and evidence presented here strongly supports this. In addition, spectral evidence suggest that, as well as containing phycocyanobilin, cryptomonad phycocyanins have a further type of phycobilin with violinoid spectral properties (O'hEocha *et al.*, 1964). We propose that these further phycobilins be termed respectively phyco-urobilin and cryptoviolin.

Experimental

Biliprotein preparations

R-phycoerythrin from the red alga *Rhodomenia palmata*, C-phycoerythrin from the red alga *Phormidium persicinum*, C-phycocyanin from the blue-green alga *Nostoc punctiforme* and R-phycocyanin from the red alga *Ceramium rubrum* were isolated and purified as by O'Carra (1965). Cryptomonad phycocyanin was isolated from *Hemiselmis virescens* as by O'hEocha *et al.* (1964).

Bilin preparations

Methanol-released 'purple pigment' from R-phycoerythrin and 'blue pigment' from C-phycocyanin were prepared as by O'Carra & O'hEocha (1966). Preparations of phycoerythrobilin and phycocyanobilin from R-phycoerythrin and C-phycocyanin, respectively, were obtained by incubating the biliproteins for successive periods of 15 min at 18°C in 11.5–12 M-HCl (O'Carra *et al.*, 1964; O'hEocha, 1963). Phycocyanobilin was similarly released from R-phycocyanin when most of the phycoerythrobilin had been first released from the protein by two successive incubations with HCl (O'Carra, 1962; Murphy, 1968). T.l.c. on silica-gel G was used for analysis of the free diacid forms of the phycobilin preparations and for purification of individual bilin constituents (Murphy, 1968). The solvent systems used were: carbon tetrachloride/acetic acid (2:1, v/v); carbon tetrachloride/acetone/acetic acid (4:8:1, by vol.); heptane/acetone/acetic acid (16:16:3, by vol.). Bilins were esterified (Murray, 1966) in a solution (1 ml) of BF₃ in methanol (14%, v/v; Sigma, Poole, Dorset, U.K.) for 25 min at 4°C. Ice-cold water (4 ml) was then added and the esterified pigment was extracted with three 2 ml portions of chloroform.

Tryptic digestion of biliproteins

Previous studies have shown that, under some conditions used to release phycoerythrobilin from phycoerythrins, random artefact covalent linkage of the bilin to the polypeptide chains of the apoprotein can take place and the chromophore is also converted into a urobilinoid artefact derivative

(O'Carra *et al.*, 1964; Rüdiger & O'Carra, 1969). Since the artefact attachment takes place by condensation of the vinyl-side-chain group of ring D of phycoerythrobilin with thiol groups on the polypeptide chains (O'Carra *et al.*, 1964; Rüdiger & O'Carra, 1969), blocking of the thiol groups by reduction and aminoethylation (Raftery & Cole, 1966) before tryptic digestion was carried out to eliminate this complication. To further protect the chromophores, all procedures used were carried out in the dark and under nitrogen. Tryptic digests were carried out as follows. Reduced and aminoethylated biliprotein (50 mg) was suspended in 10 ml of 0.2 M-sodium phosphate buffer, pH 7.0. A solution (0.1 ml) of diphenylcarbamoyl chloride-treated trypsin (2.5 mg; Sigma) in 1 mM-HCl was added. The mixture was incubated with stirring for 6 h at 25°C under nitrogen, and any insoluble material was removed by centrifugation. After the addition of a further portion (0.1 ml) of trypsin solution to the supernatant, digestion was continued for 16 h. Digests were stored at -20°C under nitrogen.

Starch-gel electrophoresis of tryptic digests of R-phycoerythrin

Electrophoresis of tryptic digests of R-phycoerythrin was carried out on horizontal starch gels by using 'Starch Hydrolysed' (Connaught Medical Research Laboratories, Toronto, Ont., Canada) and adopting the procedure of Smithies (1955). The buffer systems used were: glycine/HCl, pH 2.2; sodium acetate, pH 4.0, 4.5, 5.0 and 5.5; Tris/malate, pH 7.4 and 8.3. For the glycine/HCl and sodium acetate buffers, the gel buffer concentration was 0.025 M and the bridge buffer concentration was 0.05 M. For the Tris/malate buffers, the gel buffer concentration was 0.005 M and the bridge buffer concentration was 0.1 M. Electrophoresis was carried out at 4°C in the dark for 16 h at 7.5 V/cm. After electrophoresis the starch gels were soaked in ethanol/pyridine (1:1, v/v) for 10 min. The bathing fluid was replaced for each of two subsequent washings. The gels were then placed in a saturated solution of zinc acetate in ethanol for 10 min to convert the bilin chromophores into their zinc complexes. Although the peptide bands that contained bilin chromophores could be detected visually after electrophoresis, much greater sensitivity was achieved by viewing the fluorescent zinc complexes under u.v. light, when phycoerythrobilin and phycourobilin exhibit orange and green fluorescence respectively. At each pH value used for electrophoresis of the tryptic digest, only one peptide containing phycoerythrobilin and one containing phycourobilin could be detected. Similar results were obtained when the digests were subjected to electrophoresis on polyacrylamide disc gels by the method of Dietz & Lubrano (1967).

Results and Discussion

To simplify discussion, the phycobilins are considered individually.

Phycoerythrobilin

This red phycobilin imparts the characteristic red colour to the phycoerythrins. In 1958, O'hEocha reported that careful treatment of phycoerythrins with cold concentrated HCl releases a chloroform-soluble pigment from the proteins. The free bilin was considered to be closely related to the native protein-bound chromophore, and this was confirmed later by O'Carra *et al.* (1964). The acid treatment, however, resulted in many side reactions producing artefacts, so that yields of the acid-released pigment were low.

O'Carra & O'hEocha (1966) showed that treatment of phycoerythrins with hot methanol slowly released a more stable derivative of the chromophore. This methanol-released pigment was shown to be identical with a bilin extracted from algae with hot methanol and previously considered by Fujita & Hattori (1962, 1963) to be a biosynthetic precursor of phycoerythrobilin. The results of O'Carra & O'hEocha (1966) showed that the extracted pigment was a derivative rather than a precursor of phycoerythrobilin. O'Carra & O'hEocha (1966) considered the acid-released pigment to be much closer in properties to the native protein-bound pigment than the methanol-released 'purple pigment', which was regarded as a spectrally altered artefact. However, in much subsequent work by other authors this distinction has been disregarded. Since the methanol-releasing procedure offers a more convenient method of obtaining protein-free pigment, much of the recent structural work (Frackowiak & Skowron, 1978; Gossauer & Weller, 1978; Fu *et al.*, 1979) has concentrated on the methanol-released derivative (cf. e.g. Chapman *et al.*, 1967; Crespi *et al.*, 1968).

The spectral properties, summarized in Table 1, clearly demonstrate a distinction between the acid-

released and methanol-released pigment preparations. The two preparations retain their spectral distinctiveness through a variety of derivatization procedures, e.g. formation of the hydrochloride and zinc complex (Table 1), and also when converted into the dimethyl esters. The pigments as dimethyl esters travel with similar R_f values when subjected to t.l.c. on silica-gel G in some solvent systems, and this may have helped to create the impression that they were identical. In other solvent systems, however [benzene/light petroleum (b.p. 100–120°C)/methanol (9:5:1, by vol.); carbon tetrachloride/acetic acid (1:1, v/v) carbon tetrachloride/ethyl acetate (1:1, v/v)], the two pigment preparations are readily separated.

In Table 1 and Fig. 1 the spectral properties of the released pigments are compared with those of denatured C-phycoerythrin. Protein-denaturing conditions were used to ensure unfolding of the polypeptide chains of the phycoerythrin, thus exposing the covalent bound chromophore to the same solvent conditions as for the protein-free-pigment

Fig. 1. Absorption spectra in 0.1M HCl of (—) denatured C-phycoerythrin, (---) acid-released phycoerythrobilin and (····) methanol-released 'purple pigment' derivative of phycoerythrobilin

Table 1. Spectral absorption maxima of denatured phycoerythrins, phycoerythrin-derived bilins and *i*-urobilin. The major maxima are italicized and minor maxima or absorption shoulders are in parentheses. The absorption maximum at 278 nm is attributable to aromatic amino acids.

Bilin or denatured phycoerythrin	Chromophore as hydrochloride				Chromophore as zinc complex in 8 M urea, pH 7.0, containing 1% (w/v) zinc acetate			
	In 0.1 M-HCl		In acidified chloroform					
Denatured C-phycoerythrin	287,	307,	556	Insoluble	278,	321,	(542),	586
Phycoerythrobilin (acid-released)		307,	556	312, 576		321,	(540),	586
'Purple pigment' derivative of phycoerythrobilin		326,	590	329, 602		340,	(564),	609
Denatured R-phycoerythrin	287,	307,	498, 556	Insoluble	278,	321,	510, (545),	586
<i>i</i> -Urobilin			495	499			510	

preparations. In the native proteins the chromophores are buried in the interior by the folding of the polypeptide chains and lie in environment(s) different from the external medium (O'hEocha & O'Carra, 1961; Lavorel & Moniot, 1962; O'Carra & O'hEocha, 1965; Murphy & O'Carra, 1970). That the denaturing conditions cause no covalent alteration of the bound chromophore is shown by the fact that C-phycoerythrin readily renatures when the denaturing conditions, low pH or 8M urea, are removed. The spectral properties of the native protein then return in a rapid spontaneous process.

It can be seen that the protein-bound chromophore is almost identical in spectral properties with the acid-released phycoerythrobilin preparations and differs considerably from the methanol-released pigment. Muckle & Rüdiger (1977) also emphasized that the methanol-released pigment exhibits spectral properties, including absorption coefficients, different from those of protein-bound chromophore.

Phycocyanobilin

The situation regarding phycocyanobilin and the blue pigment released from C-phycocyanin by hot methanol is analogous to that outlined above for phycoerythrobilin and its methanol-released 'purple pigment' derivative. The 'blue pigment' released from blue-green algae by methanol treatment was originally considered by Fujita & Hattori (1962, 1963) to be a biosynthetic precursor of phycocyanobilin, but was shown by O'Carra & O'hEocha (1966) to be an artefact derived from protein-bound phycocyanobilin. As can be seen from the spectral properties recorded in Table 2, this 'blue pigment' differs spectrally from protein-bound phycocyanobilin. When C-phycocyanin was treated by the method of O'hEocha (1963) with 12M-HCl, a phycobilin preparation that absorbed maximally at 630–645nm in acidified chloroform was obtained. By t.l.c. analysis this preparation was shown to contain a mixture of pigments. The predominant component was a blue-green bilin with an absorp-

tion maximum at 630nm, and this is referred to as 'phycobilin-630'. It could be further resolved on chromatograms into two closely spaced components that were identical by other criteria, including absorption spectra, acid/base properties, isomerizations in acids and alkali, chemical reactions and degradation products (Rüdiger & O'Carra, 1969). The two forms of phycobilin-630 may reflect spatial isomerism, which does not involve any rearrangement of the double-bond system. The other bilin components were a violinoid pigment, which may be a hydrated derivative of phycobilin-630 (Murphy, 1968), and a bilin, which was chromatographically and spectrally identical with the methanol-released blue pigment. The proportion of the components extracted into chloroform varied with the concentration of HCl used to treat the C-phycocyanin. Time-course studies with C-phycocyanin and with chromatographically purified phycobilin-630 in HCl at various concentrations between 10M and more than 13M, however, showed that the blue-green phycobilin-630 was the bilin initially released. In 10–12M-HCl it was converted into the blue pigment together with some violin, but, as the concentration of HCl was increased to over 13M, the conversion into violin became the major transformation. These observations were confirmed with acid-released phycobilin-630 from R-phycocyanin.

Spectral comparison of denatured C-phycocyanin with the chromatographically purified phycobilin-630 shows that this pigment closely resembles the native protein-bound chromophore (Fig. 2, Table 2).

As for phycoerythrobilin, the structure and properties currently accepted (Brown & Troxler, 1977; Troxler *et al.*, 1978; Frackowiak & Skowron, 1978; Gossauer & Hinze, 1978) for phycocyanobilin (Cole *et al.*, 1967; Crespi *et al.*, 1968) relate to the methanol-released 'blue pigment'. This is now seen to be identical with a spectrally altered artefact derived from the native phycocyanobilin chromophore. Because of the differences between the

Table 2. *Spectral absorption maxima of denatured C-phycocyanin and its released bilins*

The major maxima are italicized. To form the zinc complex of the chromophore in C-phycocyanin, the biliprotein was dissolved in 8M-urea containing 1% (w/v) zinc acetate. The zinc complex of protein-free bilins was formed in chloroform, which contained a drop of ethanolic zinc acetate and a trace of pyridine to neutralize protons. The absorption maximum at 278nm is attributable to aromatic amino acid residues.

Bilin or biliprotein	Chromophore as hydrochloride				Chromophore as zinc complex in 8M-urea or chloroform
	In aq. methanolic 0.1 M-HCl			In acidified chloroform	
	278,	~350,	655–660	Insoluble	
Denatured C-phycocyanin					278, 375, 630
Phycocyanobilin (acid-released phycobilin-630)		~360,	655	357, 630	375, 630
'Blue pigment' derivative of phycocyanobilin		374,	684	680	378, 665

Fig. 2. Absorption spectra in 0.1M HCl of (—) denatured cryptomonad phycocyanin, (----) denatured C-phycocyanin and (....) acid-released phycocyanobilin (phycobilin-630)

spectral properties of the methanol-released pigment and those of native phycocyanobilin bound to the protein, Muckle & Rüdiger (1977) state that the use of the absorption coefficient of the methanol-released pigment to calculate the chromophore content of phycocyanin can give erroneous results. The phycobilin-630 released by brief acid treatment consists predominantly of a pigment very close in properties to the native chromophore.

Phycourobilin

The grounds on which Chapman *et al.* (1968) have disputed the suggestions that R-phycoerythrin contains a urobilinoid chromophore (phycourobilin) accompanying phycoerythrobilin may be summarized as follows. (1) The spectral properties of phycoerythrobilin are distorted by the interaction of the chromophore with the native protein environment and thus the peak at 495–598 nm might be attributable to such non-covalent modulation of the properties of phycoerythrobilin in the native protein. (2) Free phycoerythrobilin readily undergoes covalent conversion into urobilinoid artefacts both by isomerization and by oxidation reactions, and the appearance of urobilinoid material in denatured R-phycoerythrin might be attributable to such artefact material formed while the chromophore is still attached. (3) Refluxing R-phycoerythrin in methanol releases only the 'purple pigment' equated

Fig. 3. Absorption spectra in 0.1M HCl of (—) denatured R-phycoerythrin, (----) acid-released phycoerythrobilin and (....) *i*-urobilin

with phycoerythrobilin by Chapman *et al.* (1967, 1968), and no urobilin is released.

In order to check the first two objections, the spectral data shown in Table 1 and Fig. 3 were obtained with fully denatured R-phycoerythrin in which the effects of the native protein environment are eliminated by unfolding of the polypeptide chains. The possibility that the procedures used for obtaining these spectra might cause covalent modification of phycoerythrobilin was investigated by subjecting purified acid-released phycoerythrobilin and denatured C-phycoerythrin to exactly the same procedures. Their spectral properties are compared in Table 1 and Fig. 1. No significant amount of urobilinoid material was formed in either case.

As Table 1 and Fig. 3 show, a distinctive peak, characteristic of urobilins, persists in the spectrum of denatured R-phycoerythrin. Quantitative subtraction of the absorption spectrum of phycoerythrobilin, by using an adjusted spectrum of denatured C-phycoerythrin, leaves a spectrum almost identical with that of *i*-urobilin. Addition of Zn^{2+} causes the characteristic shift of the urobilinoid absorption peak (Table 1) associated with the formation of the zinc complex. The fluorescence spectrum of the zinc-complexed denatured R-phycoerythrin shows two fluorescence bands, one (λ_{max} , 520 nm) characteristic of urobilin-zinc and the other (λ_{max} , 600 nm) attributable to phycoerythrobilin-zinc.

To further eliminate any influence of the protein environment, denatured R-phycoerythrin was digested with trypsin. The digestions were carried out under carefully controlled conditions (neutral pH, exclusion of oxygen) designed to minimize any isomerization or oxidative conversion of phycoerythrobilin units into artefact urobilinoid material. Again, the procedures used were fully checked by

Fig. 4. Elution profiles of (●) the phycourobilin- and (○) the phycoerythrobilin-containing chromopeptides in a tryptic digest of R-phycoerythrin from a Sephadex G-50 column

A sample (3 ml) of tryptic digest of R-phycoerythrin was applied to the column (67.5 cm $\times$ 2.1 cm) and equilibrated with phenol/acetic acid/water (1:1:1, by vol.) that had been flushed with nitrogen. Owing to the highly aromatic nature of the bilin chromophores, the tryptic chromopeptides were adsorbed on to Sephadex when subjected to gel filtration in conventional aqueous buffers and were eluted much later than the internal volume of the column. Such adsorption of aromatic compounds had been reported previously and eliminated with the above solvent systems (Carnegie, 1965; Janson, 1967). Gel filtration was carried out in the dark and the flow rate was 12 ml/h. Fractions (2 ml) were collected and made 0.1 M with respect to HCl. When the absorption spectrum was determined, the A_{495} was corrected by subtracting any absorbance in this region due to phycoerythrobilin, calculated as $A_{555} \times 0.244$. This factor was determined from the absorption spectrum of phycoerythrobilin in acid-denatured C-phycoerythrin. Since phycourobilin does not absorb at 555 nm, no reciprocal correction was necessary.

control experiments in which R-phycoerythrin was replaced with C-phycoerythrin, and negligible conversion of phycoerythrobilin, the only chromophore of C-phycoerythrin, into a urobilin was observed. The tryptic digests of R-phycoerythrin retained the same spectral properties as the denatured biliprotein, and when the peptide mixture was fractionated on the basis of a number of independent properties (e.g. size or charge characteristics), a clear-cut separation of the phycoerythrobilin-containing and phycourobilin-containing peptides was observed (Figs. 4 and 5). The results show that the two chromophores reside in quite distinct sections of the polypeptide sequence,

Fig. 5. Effect of pH on the electrophoretic mobility of (●) the phycourobilin- and (○) the phycoerythrobilin-containing chromopeptides in tryptic digests of R-phycoerythrin

Tryptic digests of R-phycoerythrin were subjected to starch-gel electrophoresis at several pH values as described in the Experimental section.

further eliminating the possibility that the urobilin might be an artefact form of phycoerythrobilin.

Refluxing the phycourobilin-containing peptide fraction, isolated by gel-filtration, with methanolic KOH for 5 min released the chromophore and converted it rapidly into a green product that was identified as the bilin mesobiliverdin. This result confirms that phycourobilin is an open-chain linear tetrapyrrole.

Treatment of the phycourobilin-containing peptide with hot methanol did not release the pigment. Structural studies show that the attachment of phycourobilin to the apoprotein is much stronger than that of phycoerythrobilin (P. O'Carra & S. D. Killilea, unpublished work). Thus the failure of Chapman *et al.* (1968) to release urobilinoid material by hot methanol is readily explained.

Although the experiments described here provide strong evidence that phycourobilin is a discrete and natural chromophore of R-phycoerythrin, this concept now also receives acceptance by some other groups (Frackowiak & Skowron, 1978; Glazer & Hixson, 1977).

Cryptoviolin

All the cryptomonad phycocyanins in the native state exhibited an absorption peak at about 588 nm in addition to varied spectral characteristics at longer wavelengths, usually manifested by a peak at either 615, 630 or 645 nm (O'hEocha *et al.*, 1964).

When these phycocyanins are unfolded by denaturation, the longer-wavelength absorption in all cases collapses to a spectral peak characteristic of phycocyanobilin. The shorter-wavelength peak also shifts, but remains clearly distinct from the phycocyanobilin spectrum. Fig. 2 shows the spectrum of a typical denatured cryptomonad phycocyanin in dilute acid; all cryptomonad phycocyanins give almost identical spectra under such denaturing conditions. The short-wavelength absorption peak is at 610 nm under these conditions, but if the spectrum is analysed by subtracting out the phycocyanobilin absorption spectrum with an adjusted spectrum of acid-denatured C-phycocyanin, the true absorption maximum of the peak is found to be about 590 nm. This maximum and the shape of the analysed peak coincides exactly with the absorption characteristics of the class of bilins known as violins; the absorption maximum of mesobiliviolin in dilute acid is at 590–600 nm.

The bilin nature of the extra chromophore in cryptomonad phycocyanin was indicated by the observation that both it and the phycocyanobilin chromophore were converted into mesobiliverdin when samples of the phycocyanin were refluxed in methanolic 1 M-KOH for 30 min.

The extra chromophore seems clearly to have a violinoid conjugated system and it is tempting to suggest the term phycoviolin, which would be in line with the terminology of the other phycobilins. A similar term, phycobiliviolin, has been used by Rüdiger (1971) for the 'purple pigment' derivative of phycoerythrobilin, so, to avoid confusion, it is suggested that the violinoid chromophore of cryptomonad phycocyanin be termed cryptoviolin.

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