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PHYTOCHROME MODELS

V*. CHROMATOGRAPHIC DETECTION OF SOLVENT ADDUCTS OF BILINOID PIGMENTS

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SUMMARY

The dimethyl esters of biliverdin, its XIIIα isomer (mesobiliverdin) and bilirubin and the 7,13-dimethyl-2,3,8,12,17,18-hexaethyl- and 2,3-dihydrooctaethylbilitrienes form strong adducts with solvents such as tetrahydrofuran, acetonitrile and ethyl lactate. These adducts are differently adsorbed on silica gel. They give rise to two peaks in high-performance liquid chromatography and are separated into two zones on preparative low-pressure column chromatography. As chromatographic methods are normally used to assess the purity of bilinoid pigments, the results represent a warning regarding the solvent systems chosen in high-performance liquid and thin-layer chromatographic analyses.

INTRODUCTION

In our investigation of the solvent-dependent spectroscopic properties of biliverdin dimethyl ester (I), its XIIIα isomer (II)[1,2] and bilirubin dimethyl ester (VI)[2–4], the purity of the samples used was examined by high-performance liquid chromatography (HPLC). Only one peak was observed for each of the compounds when injected in chloroform solution. This observation served as a basis for our standard purity analysis of the different samples used. When dissolved in other solvents [*e.g.*, tetrahydrofuran (THF) or acetonitrile], however, samples of I, II and VI were found to give more than one HPLC peak each, using the same column and mobile phase as for the chloroform probe.

We present here the results of a systematic study of this phenomenon, which is also observed with compounds III–V, but not, *e.g.*, with VII and the (*E,Z,Z*)- and (*Z,Z,E*)-isomers of I.

* For Part IV, see ref. 1.

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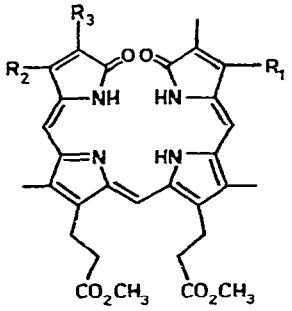
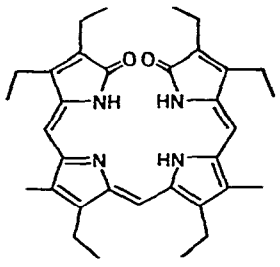


I $R_1 = R_3 = CH=CH_2$, $R_2 = CH_3$
II $R_1 = R_2 = CH=CH_2$, $R_3 = CH_3$
III $R_1 = R_3 = CH_2CH_3$, $R_2 = CH_3$



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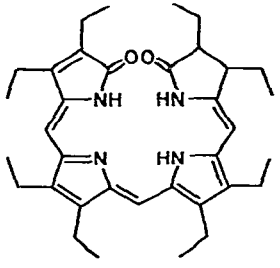

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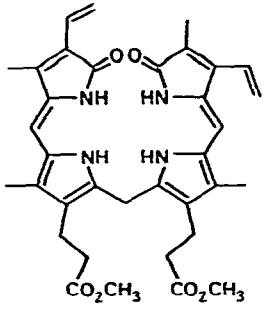
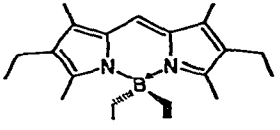


VI



VII

EXPERIMENTAL

Chemicals

The preparation and purification of compounds I, II and VI and the solvents were described earlier[2,5]. The (*E,Z,Z*)- and (*Z,Z,E*)-isomers of biliverdin dimethyl ester were prepared as described by Falk *et al.*[6]. Mesobiliverdin dimethyl ester (III), 7,13-dimethyl-2,3,8,12,17,18-hexaethylbilitriene (IV) and 2,3-dihydrooctaethylbilitriene (V) were used as received from Professor A. Gossauer, Professor D. A. Lightner, and Dr. H. Scheer, respectively. N,N'-Diethylboryl-3,3',5,5'-tetramethyl-4,4'-diethyl-2,2'-dipyrrylmethene (VII) was provided by Professor D. Dolphin and recrystallized from benzene–light petroleum.

All bilitrienes were handled in green light, and VI and VII in red light.

[D_8]Tetrahydrofuran (TDF) and $CDCl_3$ were obtained from Merck, Sharp and Dohme (Munich, G.F.R.). Prior to use, TDF was passed through molecular sieve 4A, and $CDCl_3$ and chloroform through Woelm basic alumina. [1-^{14}C]acetonitrile (9.7 μCi/mmol) was purchased from Amersham Buchler (Düsseldorf, G.F.R.) and diluted five times before use.

Instrumentation and procedure

HPLC was performed at room temperature with a Perkin-Elmer 3000 chroma-

tograph equipped with an LC 55 single-beam variable-wavelength spectrophotometer and a stop-flow valve, which made possible the recording of UV–visible absorption spectra of eluted peaks during a chromatographic run (spectral resolution *ca.* 5 nm). The volume ratio of injected sample solution to injection port loop was *ca.* 1:17. The column was 7-μm Si 60 (20 × 0.46 cm I.D.) and the mobile phase was chloroform–*n*-heptane (4:1) either alone or containing 1.5% THF or 5% methanol at 1–2 ml/min and 40–80 atm.

The highly coloured substances were visible in the spots and zones following thin-layer chromatography (TLC) and preparative column chromatography without further treatment. Merck silica gel plates and high-performance TLC (HPTLC) plates (Merck) were used for TLC analysis. Chloroform–*n*-heptane (4:1) containing 0.5% of methanol served as the mobile phase for chromatography on Merck ready-made columns (2 atm), LiChroprep SI 60, Type A. For the preparation of samples for the UV–visible and nuclear magnetic resonance (NMR) measurements of the chromatographic fractions adsorbed on silica gel, a dialysis tube, filled with silica gel 60 (Merck; 0.06–0.20 nm), was used instead. After the chromatographic development of a sample solution of I in THF, column sections containing one fraction each, were cut out and transferred as a whole into appropriate cells. The similarity of the refractive indicies of the SiO_2 particles, of chloroform and of chloroform containing up to 70% of THF rendered the sections, in the absence of I, transparent in the UV–visible range studied. For the scintillation analysis of similar column sections resulting from chromatography of I when introduced in [1-^{14}C]acetonitrile and using the above-mentioned mobile phase, the following procedure was chosen. The dialysis tube serving as a chromatographic column was surrounded by a moveable glass tube with a window through which localized scintillation counts could be carried out by means of a Geiger–Müller counter (FHZ 15 Endfensterzählrohr, Laboratorium Professor Berthold, Wildbad, G.F.R.).

^{1}H NMR spectra were measured on a Bruker WH 270 spectrometer, FT mode, and UV–visible absorption spectra were recorded on Cary 17, Cary 219 and Perkin-Elmer 554 spectrophotometers.

RESULTS AND DISCUSSION

The HPLC of sample solutions of I–VI in chloroform gave only one peak each, independent of the chromatographic parameters such as flow-rate, injection volume and mobile phase composition (Table I and Fig. 1a). The two constitutional biliverdin isomers I and II were well separated[5].

We have now found that I–VI in some solvents give rise to more than one HPLC peak. On the other hand, the (*E*,*Z*,*Z*)- and (*Z*,*Z*,*E*)-isomers of I (in chloroform and THF) and the diethylboryl complex VII (in any of the solvents) gave only one HPLC peak. This rules out perturbations arising from the difference between the mobile phase and the injected solvent as the cause of the appearance of two detection signals. Rather, it must reflect a particular property of compounds I–VI.

This phenomenon was studied in greater detail with THF solutions of I which produced two peaks, P_A and P_B (Fig. 1b). A third intermediate peak, P_C, appeared when solutions of I in, *e.g.*, THF–chloroform (1:4), were injected. The intensity of P_C was proportional to the amount of chloroform in the solution injected (Fig. 1c;

TABLE I

HPLC RETENTION VOLUMES (R_v) FOR ELUTION OF COMPOUNDS I–VII AND THE (*E,Z,Z*)- AND (*Z,Z,E*)-ISOMERS OF I

Compound	R_v *(ml), when injected in*			
	$CHCl_3$*	*THF***,***		
		P_A		P_B
Biliverdin dimethyl ester (I)§,§§	5.5	4.6		6.5
Biliverdin XIIIα dimethyl ester (II)§	7.0	4.8		7.6
(*E,Z,Z*)-Biliverdin dimethyl ester§	40.6		40.6	
(*Z,Z,E*)-Biliverdin dimethyl ester§	51.6		51.6	
Mesobiliverdin dimethyl ester (III)§	5.2	4.2		7.2
7,13-Dimethyl-2,3,8,12,17,18-hexaethyl-bilitriene (IV)§	7.9	6.9		8.2
2,3-Dihydrooctaethylbilitriene (V)§§§	4.4	4.3		5.8
Bilirubin dimethyl ester (VI)†	4.6	4.0		5.4
N,N′-Diethylboryl-3,3′,5,5′-tetramethyl-4,4′-diethyl-2,2′-dipyrrylmethene (VII)††,†††	3.1		3.1	

* One peak was also obtained for I and II when injected in ethanol, toluene, furfuryl alcohol, and pyridine.

** Two peaks were also obtained for I–III, V and VI when injected in acetonitrile (Fig. 2d), dioxan and ethyl lactate.

*** Three well defined peaks were observed for I and II under the following conditions: (1) injection in THF–$CHCl_3$ (1:4) solutions§, (2) injection in THF, mobile phase $CHCl_3$–*n*-heptane (4:1) + 1.5–4% THF (R_v = 4.2, 5.6 and 6.2 ml for I). Only one peak was obtained, however, when $CHCl_3$ solutions of I and II were analysed with this mobile phase.

§ Mobile phase: $CHCl_3$–*n*-heptane (4:1) + 1% CH_3OH.

§§ See also Fig. 2a–c.

§§§ Mobile phase: $CHCl_3$–*n*-heptane (4:1) + 0.5% CH_3OH.

† Mobile phase: $CHCl_3$–*n*-heptane (4:1) + 3% CH_3OH.

†† Mobile phase: $CHCl_3$–*n*-heptane (1:1) + 1% CH_3OH.

††† One peak was also obtained when injected in acetonitrile, dioxan and ethyl lactate.

see also Table I, third footnote). Evaporation of each of the two major fractions and re-injection of chloroform solutions of the residues always produced the "chloroform peak" (Fig. 1a). However, re-injection of THF solutions of the same residues gave the two-peak pattern P_{A+B}, which resulted also from the injection of a THF solution of I (Fig. 1b).

The separation efficiency of TLC was not sufficient to resolve the spot of I into components. However, the R_F values of the spots obtained on both silica and HPTLC plates varied with the solvent used for deposition of the sample on the plate (Fig. 2), even though the sample spots were carefully dried before running the chromatograms.

The electronic absorption and ^1H NMR spectra of the chromatographic fractions of I were identical when measured after collecting them as homogeneous solutions. However, they differed from each other while the materials were still adsorbed on silica gel. Thus, the UV–visible spectra of the HPLC peaks P_A and P_B (Fig. 1b) [and of the corresponding peaks obtained when THF was added to the mobile phase (see the third footnote in Table I)] were all of the same characteristic biliverdin type when measured by the stop-flow technique immediately after elution (Fig. 3a and b).

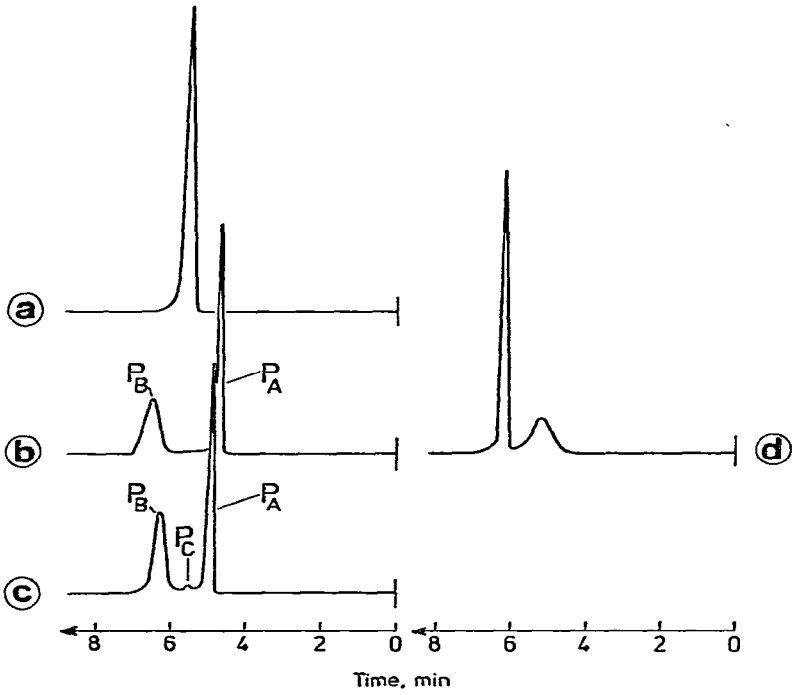


Fig. 1. HPLC of I when injected in (a) $CHCl_3$, (b) THF, (c) $CHCl_3$–THF (1:4), and (d) CH_3CN; mobile phase, $CHCl_3$–*n*-heptane (4:1) + 1% CH_3OH.

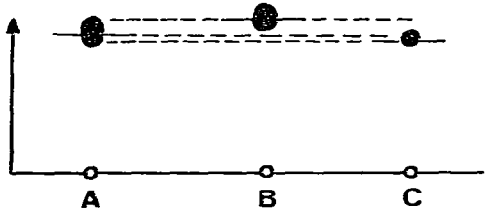


Fig. 2. HPTLC of I when deposited on the plate at (A) in $CHCl_3$, (B) in THF, and (C) in CH_3CN, and dried in a warm stream of nitrogen for 2 min before chromatography. The whole procedure was carried out under exclusion of air to avoid decomposition. Mobile phase: CH_2Cl_2–*n*-heptane (4:1) + 2% CH_3OH.

The central peak P_C (Fig. 1c) could not be separated in sufficient concentration for its spectrum to be recorded.

Distinctly different spectral data resulted after the chromatography of a THF sample solution of I on a silica gel column at 2 atm using the same mobile phase as in the HPLC experiments. Two well defined zones, Z_A and Z_B, were obtained in this way. The absorption spectra of these zones, *i.e.*, of silica gel sections containing the adsorbed fractions, are shown in Fig. 4. They were different when the spectra were taken immediately after the two zones had just separated on the column. The absorption of the faster moving Z_A (Fig. 4a) was qualitatively similar to the absorption of THF solutions of I with (Fig. 5a) and without added silica gel (Fig. 3d). When Z_A was

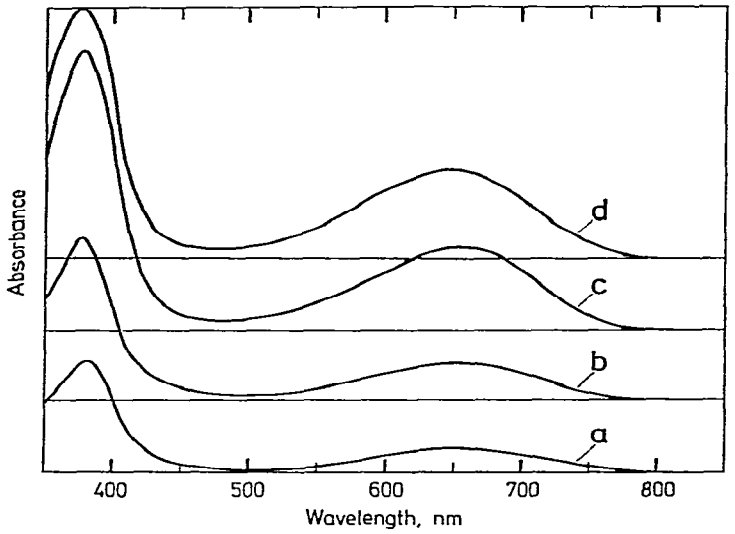


Fig. 3. Absorption spectra of the HPLC peaks P_A (a) and P_B (b), measured immediately after elution (mobile phase as in Fig. 1), and of I in $CHCl_3$ (c) and THF (d).

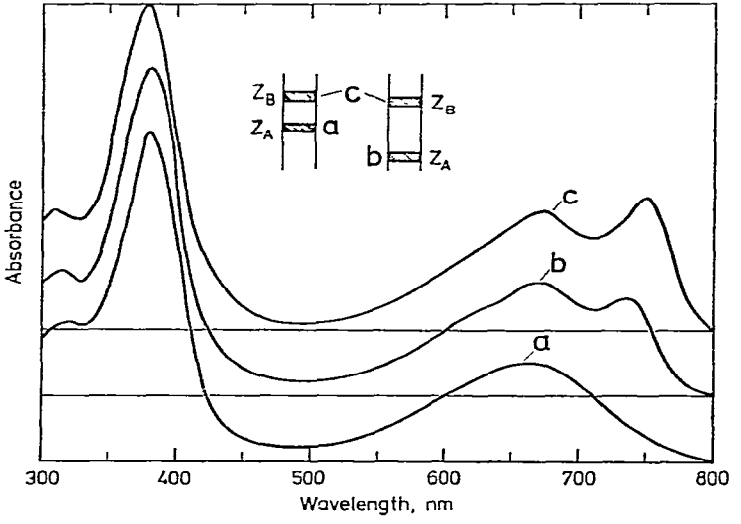


Fig. 4. Absorption spectra of column sections after chromatographic separation when I had been added in THF to a silica gel column and $CHCl_3$–*n*-heptane (4:1) +0.5% CH_3OH had been used as the mobile phase. The spectra are of the silica gel slurries containing (a) the faster moving zone Z_A immediately after chromatographic separation, (b) Z_A after the zones had separated at least 15 cm, and (c) the slower moving zone Z_B.

measured after the column had run for a longer period and the two zones were at least 15 cm apart, the features of the absorption (Fig. 4b) had changed. They were now similar to those of Z_B (Fig. 4c). The Z_B spectra in turn exhibit the pattern shown also by the spectrum of I in chloroform solution added to silica gel (Fig. 5b), which in turn is different from the absorption of I in homogeneous chloroform solution (Fig. 3c)[1].

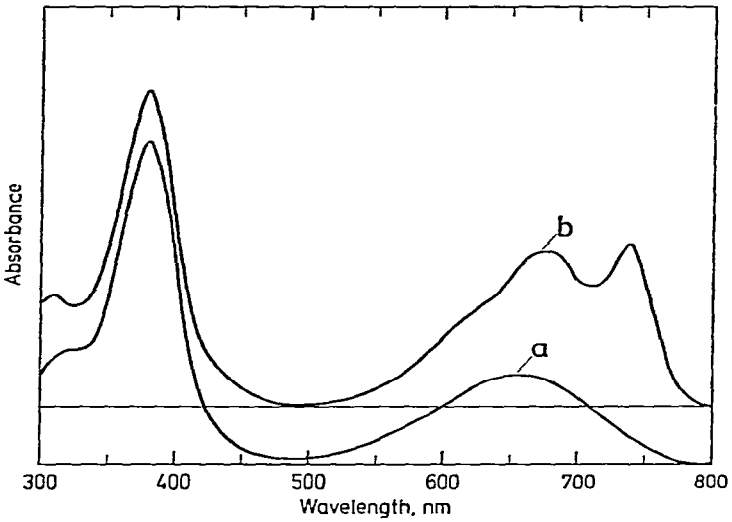
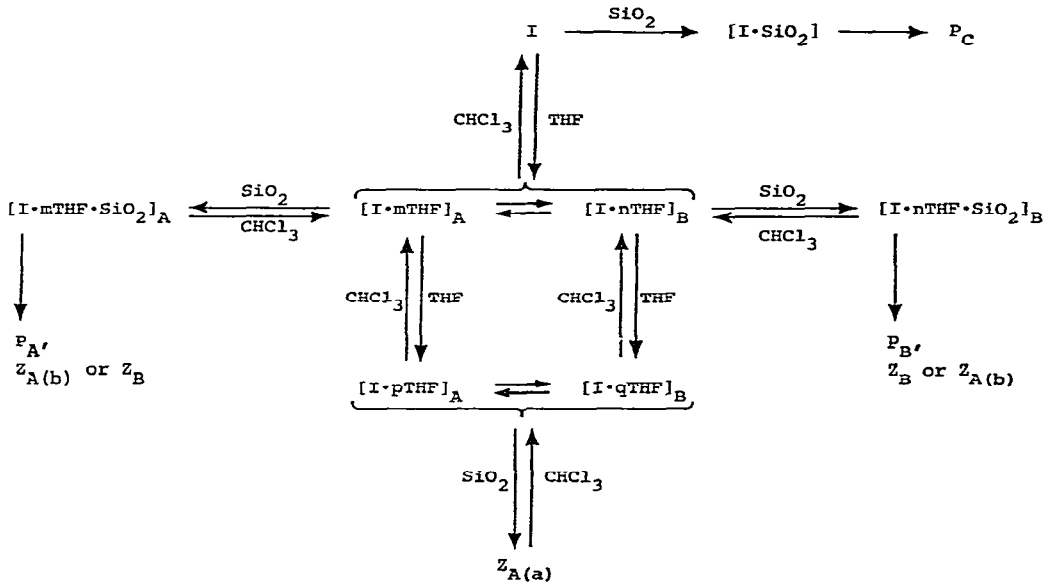


Fig. 5. Absorption spectra of a mixture of I and silica gel in (a) THF (with 30% $CHCl_3$ added to improve transparency), and (b) $CHCl_3$. The same spectrum (a) is obtained when I is dissolved in THF without silica gel (Fig. 3d).

The ^1H NMR results showed a similar effect. The spectra of the HPLC fractions P_A and P_B from a $CDCl_3$–TDF sample solution of I, eluted with $CDCl_3$, were alike and identical with the NMR spectrum of I in $CDCl_3$[5]. However, when the above low-pressure chromatographic procedure was repeated with deuterated solvents, the ^1H NMR spectra of the two fractions adsorbed on silica gel (Z_A and Z_B) were, although poorly resolved, again distinctly different.

These results are explicable if it is assumed that I forms strong solvent adducts of varied nature and stoichiometry with, *e.g.*, THF, but not with chloroform, and that I and its solvent adducts are all adsorbed differently on silica gel. The most compelling evidence in favour of the formation of solvent adducts was obtained when in column chromatography samples of I in a radioactively labelled solvent were employed as described below.

An acetonitrile solution of I gave rise to two HPLC peaks, reminiscent of the results with THF (Fig. 1d and the second footnote in Table I). However, the retention times observed with these two solvents, acetonitrile and THF, and with chloroform all differed (Fig. 2). When a solution of I in [1-^{14}C]acetonitrile was subjected to low-pressure chromatography on a silica gel column [mobile phase chloroform–*n*-heptane (4:1) + 0.5% methanol], two zones similar to Z_A and Z_B developed. Scintillation assays over the entire length of the column were carried out at several time intervals from the beginning of separation until the zones were *ca.* 25 cm apart. The areas above, between and below the zones persistently gave zero counts, whereas both zones exhibited similarly strong radioactivities throughout. This unambiguously demonstrates that the biliverdin diester entities in these zones retained acetonitrile in one form or other. The difference between the two may reside in any of several properties of these adducts, *e.g.*, different stoichiometry, different conformational or tautomeric forms of the bilitriene within the solvent adduct. Scheme 1 may



Scheme 1. $m,n < p,q$.

account for the overall experimental observations. Note that the adducts with subscript A necessarily differ at least in structural terms from the adducts with subscript B.

In Scheme 1, the HPLC peaks P_A and P_B (Fig. 1b and c) originate from the solvent adducts $[I \cdot m\text{THF}]_A$ and $[I \cdot n\text{THF}]_B$, which adsorb differently on silica gel. On elution with chloroform, I is liberated again from the desorbed adducts. The third HPLC peak, P_C, appears both in the absence of THF and in competition with P_A and P_B, when chloroform is added in sufficient excess to THF samples. We suggest that P_C results from adsorption of THF-free compound, $[I \cdot SiO_2]$.

When the THF:I molar ratio is increased by adding THF also to the chloroform mobile phase, the solvation spheres of $[I \cdot m\text{THF}]_A$ and $[I \cdot n\text{THF}]_B$ become saturated with complexing solvent molecules. The interaction of the resulting new adducts containing more THF, $[I \cdot p\text{THF}]_A$ and $[I \cdot q\text{THF}]_B$, with silica gel is smaller, hence the similarity of the absorption spectra of THF solutions of I with (Fig. 5a) and without added adsorbent (Fig. 3d). The situation is different with chloroform solutions. Here the interaction of I with solvent is smaller and the adsorption, giving rise to $[I \cdot SiO_2]$, is sufficiently strong to render the absorption spectrum of a chloroform solution with added silica gel (Fig. 5b) different from that without adsorbent (Fig. 3c).

The spectrum of the first zone (Z_A), taken immediately after separation on the column (Fig. 4a), would consequently correspond to that of saturated solvent adduct, $[I \cdot p\text{THF}]_A$ and $[I \cdot q\text{THF}]_B$, [Scheme 1, $Z_{A(a)}$] and the spectrum of Z_B (Fig. 4c) to that of $[I \cdot m\text{THF}]_A$ or $[I \cdot n\text{THF}]_B$. The absorption spectrum of Z_A, taken some time after the zones had separated [Fig. 4b; Scheme 1 $Z_{A(b)}$], may also be ascribed to $[I \cdot m\text{THF} \cdot SiO_2]_A$ or $[I \cdot n\text{THF} \cdot SiO_2]_B$, if one considers that the differences between this spectrum and that of $[I \cdot SiO_2]$ (Fig. 5b) are sufficiently significant.

The strong interaction between bilirubin dimethyl ester (VI) and THF can be

deduced in a similar way by observing the absorption spectra taken in the absence and presence of silica gel (Fig. 6B, a and b). The spectra are similar, whereas those of VI measured in chloroform without or with silica gel (Fig. 6A, a and b) show significant differences, in accord with the postulate that also in this instance the interaction of VI with silica gel is stronger than with chloroform, but weaker than with THF.

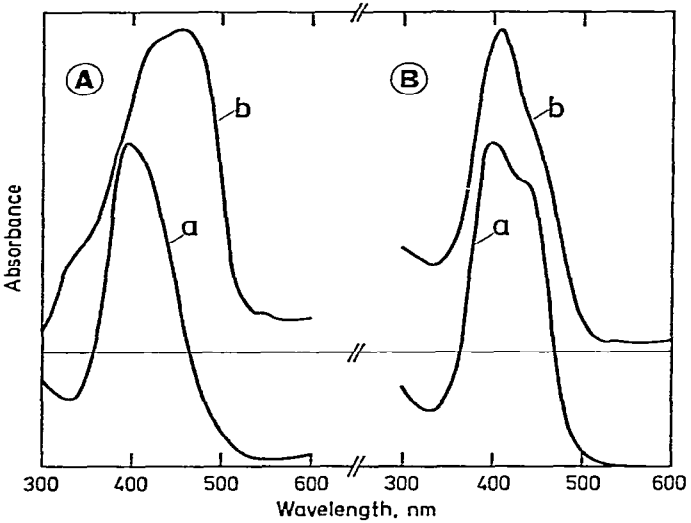


Fig. 6. Absorption spectra of bilirubin dimethyl ester (VI), (A) in (a) $CHCl_3$ and (b) $CHCl_3$, plus silica gel, and (B) in (a) THF and (b) THF plus silica gel (with 30% $CHCl_3$ added to improve transparency).

It is difficult at present to assert whether the several adducts detected for compounds I and VI by HPLC correspond to the different species coexisting in solutions of I[1] and of VI[3,4], as determined by absorption, emission and solvent-induced circular dichroism analysis. As no low-temperature splitting of the ^1H NMR signals could be detected for I in TDF down to 183°K, the conclusion would be that either the two solvent-saturated adducts $[I \cdot p\text{THF}]_A$ and $[I \cdot q\text{THF}]_B$ do not differ in their symmetry, or the position of the equilibrium is largely shifted towards one of them in homogeneous solution, rendering the other one undetectable on NMR analysis. This latter explanation together with an SiO_2-induced shift in the equilibrium between the two adducts seems the most reasonable one.

The results do not allow any more precise description of the observed adducts between I–VI and the solvents, except that they appear to reflect a property common to the ring skeletons of I–VI. Neither the vinyl nor the propionic ester side chains of I–III and VI are responsible for the observed adduct formation, as mesobiliverdin dimethyl ester (III), 7,13-dimethyl-2,3,8,12,17,18-hexaethylbilitriene (IV) and 2,3-dihydrooctaethylbilitriene (V) showed the same behaviour as I, II, and VI (see Table I). There is also no *Z–E* isomerism at C-5 and C-15 involved with this class of compounds. Thus, in contrast to biliverdin dimethyl ester (I), its (*E,Z,Z*)- and (*Z,Z,E*)-isomers gave only one broad HPLC peak (see Table I) at longer retention times than those of I.

CONCLUSION

The observation of more than one peak upon HPLC analysis of I–VI in different solvents may serve as a warning with regard to the criteria used to establish the purity of bilitrienes and bilidienes in general, including TLC methods. In addition, it provides further support to the claim that these compounds interact with their surroundings to form adducts strong enough to be reflected in their optical properties. This ability is of obvious importance, *e.g.*, with bilitrienes embedded in a highly reactive protein environment.

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