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Thin-layer chromatographic behaviour of urobilinoid pigments

Based on the observations of BIRCH[1], GRAY[2] proposed that the lactam structure was the most probable one for the bile pigments. He also suggested that the keto and enol forms of the urobilinoid molecule are in an equilibrium state under certain circumstances[3] and that their structure might vary according to their chemical environment.

According to KELLER[4], "if a substance exists in two forms which are interconverting and if the rate of conversion is slow relative to the time of development, then two spots can be obtained" by chromatography. In the experiments described here we made use of the above-mentioned fact. Urobilinoid pigments were fractionated into two main fractions by means of thin-layer chromatography thus strongly supporting GRAY's assumption.

Materials and methods

For thin-layer chromatography, chloroform solutions of crystalline *d*-urobilin, *i*-urobilin (urobilin IXα) and stercobilin were used. For comparison, stercobilin dimethyl ester, mesobiliviolin, mesobiliviolin dimethyl ester, bilirubin (Reanal) and bilirubin dimethyl ester were used.

Mesobiliviolin and mesobiliviolin dimethyl ester were prepared according to GRAY and coworkers[5]; the bulk of the product so obtained proved to be the dimethyl ester, and to obtain mesobiliviolin, a subsequent hydrolysis with 0.1 *M* potassium hydroxide in methanol at boiling point for 10 min was employed.

Stercobilin dimethyl ester, bilirubin dimethyl ester and biliverdin dimethyl ester were prepared according to FISCHER[6].

Glass plates 20 × 20 cm were coated with a 250 μ layer of silica gel (Kieselgel G, Merck), the proportion of gel to water being 1:1.5 and were activated for 1 h at 110°.

As solvent systems *n*-butanol–ethanol–water (3:1:1.5)[7], or *n*-butanol–pentanol–water (3.2:2:1.5) were used.

For two-dimensional chromatography the urobilinoids were applied as a spot 15 mm from the edges of the plate, and the solvent level was 10 mm from the edge of the plate. The development was performed at different temperatures (+2–+15°). The spots of urobilinoids were dissolved in chloroform containing 10% methanol and the solutions so obtained were evaporated to dryness. Just prior to measuring absorption spectra the dry residues were dissolved again in either chloroform containing 1% methanol, or 100% methanol, or 0.1 *N* HCl, or 0.1 *N* NaOH, or 0.1 *N* NaOH + 0.1 *N* NaCl.

U.V. absorption spectra were determined with a Beckman DU spectrophotometer, infra-red spectra with a Unicam SP. 200 spectrophotometer in potassium bromide discs.

Results

Chromatography was performed at 4° and when a relatively large quantity of urobilinoid had been applied the separation of the urobilinoids into two spots was clearly visible. When working at a higher temperature (15°) three to four smaller

spots instead of two were obtained on the chromatogram. The R_F values differed greatly from those of other bile pigments, which are supposed to be due to concomitants or byproducts resulting from chromatography (Fig. 1). If the development was not stopped after separation of the spots—in some instances, especially, if cooling was not sufficient—the spot which was nearer to the solvent front increased, and the spot nearer to the start line decreased and finally the two spots reunited. In two-dimensional chromatography, where the spots were separated in the second direction again, four spots were obtained on the chromatogram in all. All the urobilinoids (*i*-urobilin, *d*-urobilin and stercobilin) had the same above-mentioned chromatographic feature. The ratio by which the different urobilinoids separated in the same solvent system, however, was not the same. D-Urobilin separated first (Fig. 2).

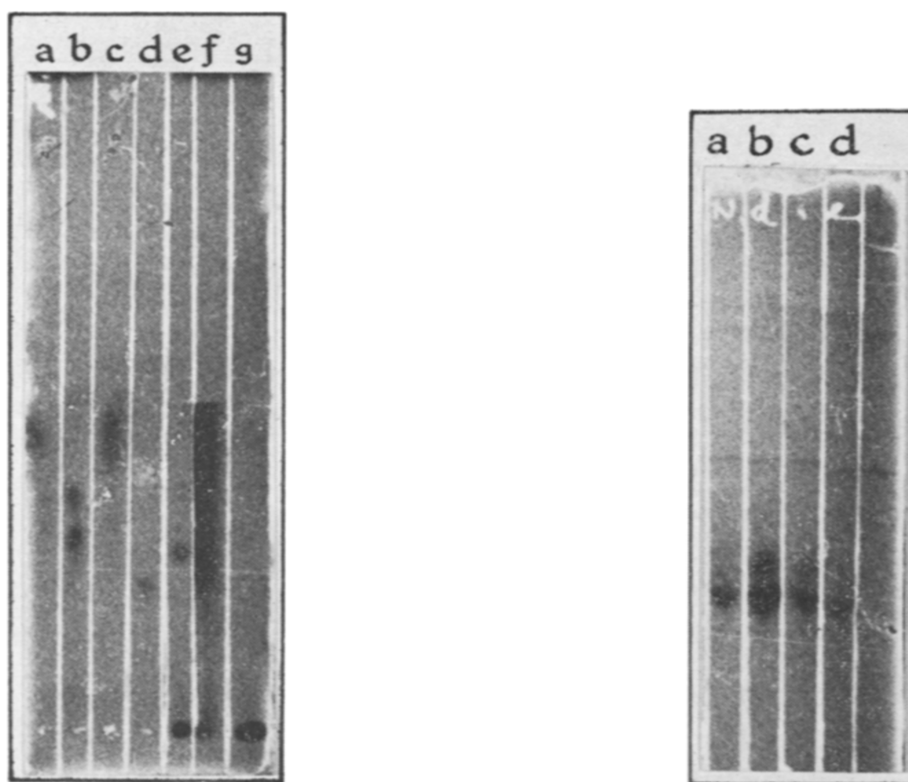

Fig. 1. Thin-layer chromatogram of: (a) mesobiliviolin dimethyl ester; (b) stercobilin; (c) stercobilin dimethyl ester; (d) mesobiliviolin; (e) bilirubin and biliverdin; (f) bilirubin dimethyl ester; (g) bilirubin. Solvent: *n*-propanol–ethanol–water (3:1:1.5).

Fig. 2. Thin-layer chromatogram of: (a) urobilinoid isolated from faeces (further identification was not made); (b) *d*-urobilin; (c) *i*-urobilin; (d) stercobilin. Solvent: *n*-propanol–ethanol–water (3:1:1.5).

The separated spots were isolated in a test tube and the urobilinoids were dissolved from the chromatographic medium in the solvents mentioned. The U.V. absorption spectra of the two spots showed no difference in either solvent. (The spectra in chloroform solution correspond to those referred to in the literature.) Infrared spectra of both spots were determined in KBr discs with a Unicam SP 200 infra-red spectrophotometer (Fig. 3).

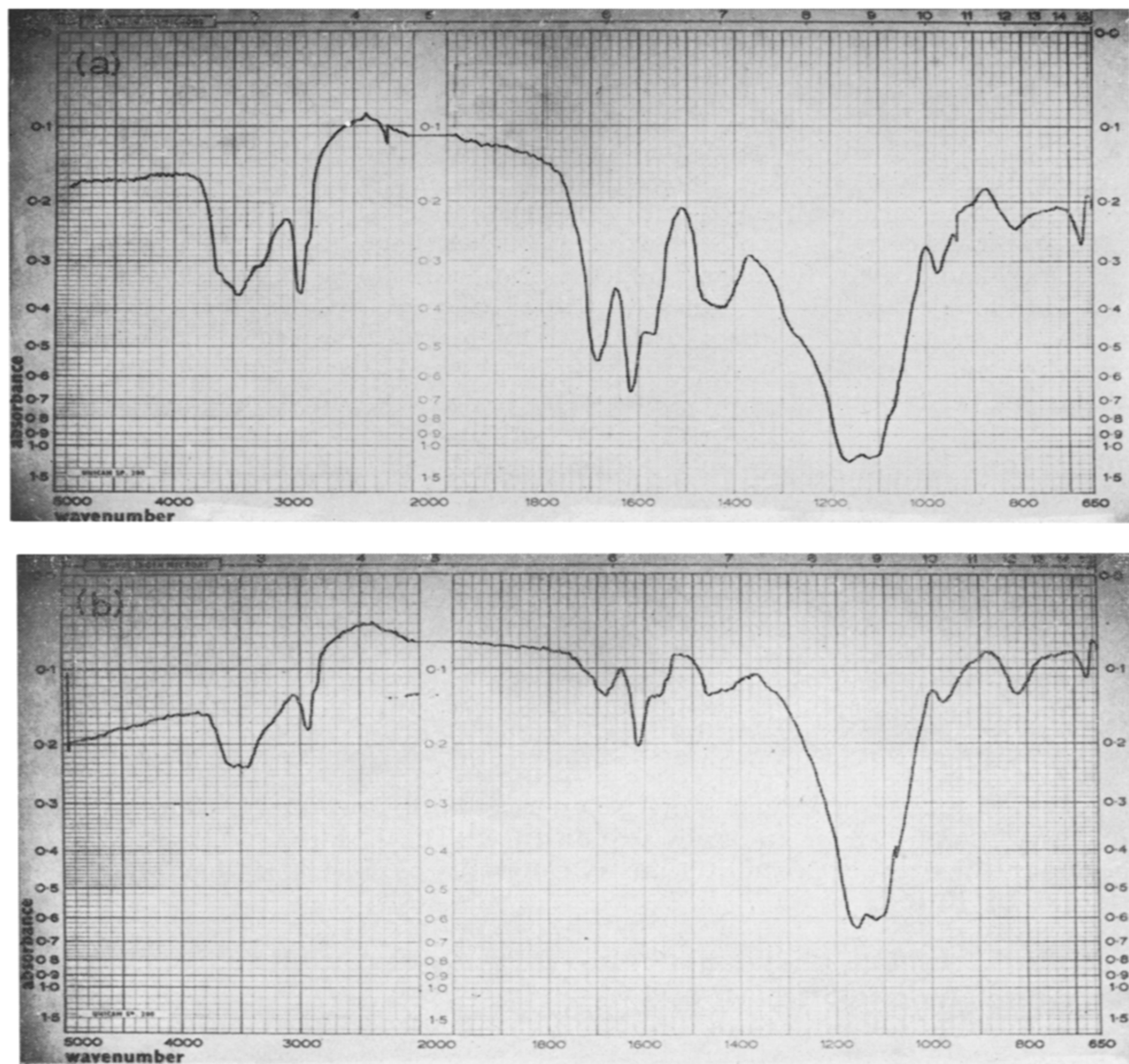

Fig. 3. Infra-red spectra of urobilinoid spots, nearer the start line (a) and nearer the solvent front (b).

Discussion

system used. Up till now no solvent has been found in which the two separate spots would not interconvert into the equilibrium state. MENDIOROZ[8] described a similar phenomenon while chromatographing bilirubin in chloroform containing ethanol.

The infra-red spectrum of the material nearer to the start line shows a shoulder at about 1270 cm^{-1} on the higher frequency side of the double band between 1200–1100 cm^{-1}, and a weak band at 3280 cm^{-1}, which are absent for the spot nearer to the solvent front. According to several authors[9–11] the amide III band occurs in secondary amides in the range 1304–1200 cm^{-1} which is weaker than either of the amide I or II bands and almost certainly due to a mixed vibration involving OCN and N-H modes.

The double band itself between 1200–1100 cm^{-1}, which is present in both spectra, is assigned to either ring vibrations or hydrogen deformation modes (*cf.* those of pyridine and derivatives[12]). Regarding the weak band at 3280 cm^{-1} the frequency, together with that of the stronger one at 3450 cm^{-1} is much too low to correspond to hydroxyl absorption (*cf.* refs. 13–15), thus both bands may be regarded as strong evidence for a keto-structure. On the other hand the absence of these bands would prove the enolic structure. We may mention that in this case a band differentiation can be observed at about 3600 cm^{-1} which can be assigned to OH stretching vibrations supporting the enolic character of the material nearer to the solvent front. Both spectra show the band at 1600 cm^{-1} which is due to CO absorption but it is greatly reduced in intensity in the latter case.

We may thus conclude that the spot nearer to the solvent front contains the enolic compound mixed to some extent with the keto form. On the other hand the spectrum of the keto form also shows a shoulder at about 3700–3600 cm^{-1}, thus the keto form itself contains a small quantity of the enolic substance.

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