440. *The Chemistry of the Bile Pigments. Part II.*[1] *The Preparation and Spectral Properties of Biliverdin.*

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The absorption spectrum, between 250 and 700 mμ, of pure biliverdin has been determined for methanol, chloroform, and methanolic hydrogen chloride solutions. The position and molecular extinction coefficients of the absorption maxima differ from those reported by other workers whose preparations of biliverdin were probably contaminated with violinoid pigments and residual bilirubin.

The absorption spectrum of biliverdin in neutral solutions is reported (see Table 1) to show a band between 380 and 400 mμ (ε 28,000) and a broad maximum at about 640—665 mμ (ε 15,000). In hydrochloric acid or in methanolic hydrogen chloride, hydrochloride formation increases absorption of both bands and causes a bathochromic shift of the maximum in the visible region, together with a slight hypsochromic shift of the maximum

[1] Part I, Gray and Nicholson, *J.*, 1958, 3085.

in the ultraviolet region. In the present study, the absorption spectrum of specially purified biliverdin in chloroform, methanol, and methanolic hydrogen chloride has been determined. The positions of the maxima, the molecular extinction coefficients at these maxima, and the ratio of these coefficients, as found by us, do not agree with those reported by others.

TABLE 1. *Previous spectroscopic data for biliverdin.*

$\lambda_{max.}$ (mμ)		E_2/E_1	$\lambda_{min.}$ (mμ)	Remarks	Ref.
			MeOH solutions		
640 (10·4)	392 (25·0)	2·43	480		*a*
640	390—400	2·46	500	Infl. 560 mμ	*b*
			$CHCl_3$ solutions		
640	380	2·44	500		*b*
640	390	2·73			*c*
		2·91			
660	380	3·95	480	Absorption increased below 350 mμ	*d*
665 (15·8)	384 (52·5)	3·31	490 (2·2)	Secondary max. 316 (24·0) & 282 mμ (19·5)	*e*
			Solutions in 5% HCl–MeOH		
680 (28·0)	377 (48·0)	1·72	510		*a*
680	375	2·89	495		*c*
680—690	370	3·91	470	Absorption below 350 mμ increases	*d*
665	384		490		*e*

Figures in parentheses give millimolar extinction coefficients where available; E_2/E_1 is the calculated ratio of the absorption at the two maxima, from reported data.

References: (*a*) Ref. 4. (*b*) Sakamoto, *Acta Med. Okayama*, 1956, **10**, 11. (*c*) *Idem, ibid.*, p. 47. (*d*) Fox and Milliott, *Experientia*, 1954, **10**, 185. (*e*) Tixier, *Ann. Inst. Oceanogr.*, 1945, **22**, 361, for the dimethyl ester (same figures obtained for dioxan solutions).

Biliverdin was obtained by the ferric chloride oxidation of bilirubin, Lemberg's method [2] being modified to facilitate the elimination of traces of residual bilirubin and oxidation products of biliverdin. This material gave the values shown in Tables 2 and 3.

During the first half-hour after the preparation of the solutions the maximum in the red region was broad and its position difficult to define: often two close maxima were detected. This was probably due, in spite of our attempts at complete purification, to the partial formation of a metal complex, the absorption maximum of which [3] is at 695 mμ, and near that of the free pigment. After a few hours in the dark, the intensity of absorption was unchanged but the maximum became more clearly defined at about 640 mμ, so that the

TABLE 2. *Our absorption data of biliverdin and its hydrochloride.*

Solvent	$\lambda_{max.}$ (mμ)	$10^{-3}\varepsilon$	$\lambda_{max.}$ (mμ)	$10^{-3}\varepsilon$	E_2/E_1
MeOH	640—650	12·9	375—380	40·5	3·13
$CHCl_3$	640—650	13·4	378—380	41·7	3·12
HCl–MeOH (5% w/v)	665—670	23·3	372·5	47·3	2·05
0·2N-HCl in MeOH	670	22·8	375	47·4	2·08
0·6N- ,, ...	660—670	23·2	375	46·2	2·00
1·1N- ,, ...	660—675	22·9	375	47·1	2·03
1·9N- ,, ...	665—670	22·9	375	45·6	1·99
2·8N- ,, ...	660—680	22·9	375	45·5	2·00
5·9N- ,, ...	670—685	23·0	375—380	45·2	1·98
Mean values for acidic solutions	660—680	23·0	370—380	46·35	2·02

wavelength readings were reproducible. The maximum at 375 mμ remained unchanged. Spectra of solutions with pH values between 4·4 and 6·9 were almost identical and showed no hydrochloride formation. At pH of about 2, most of the pigment was present as the hydrochloride, as was shown by the ratio of the optical density at 375 to that at 670 mμ. In more acid solutions (see Table 2) the hydrochloride was formed immediately; the molecular extinction coefficients were then independent of the concentration of the acid.

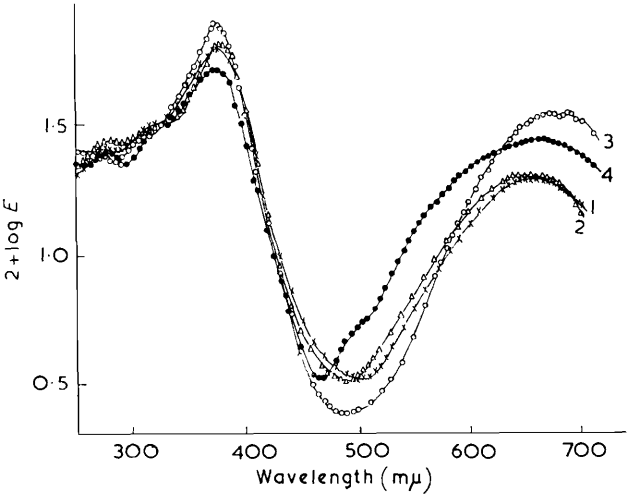
[2] Lemberg, *Annalen*, 1932, **499**, 25.
[3] Part III, following paper.

TABLE 3. *Absorption of biliverdin in weakly acidic methanolic solutions after different times.*

pH of solvent	pH of soln.	$\lambda_{max.}$ (mμ)	E_1	$\lambda_{max.}$ (mμ)	E_2	E_2/E_1
		0·25 *Hr. after dissolution*				
7·0	6·9	670	0·238	377·5	0·731	3·08
6·0	5·4	685	0·255	377·5	0·765	3·00
5·0	5·6	675	0·249	375·0	0·726	2·99
4·0	5·0	660	0·246	375·0	0·734	2·99
3·0	4·4	670	0·241	375·0	0·724	3·00
2·0	2·1	670	0·427	372·5	0·914	2·14
		16 *Hr. after dissolution.*				
7·0	6·8	640	0·242	377·5	0·730	3·07
6·0	6·2	640	0·243	377·5	0·714	2·94
5·0	6·0	665	0·244	377·5	0·712	2·92
4·0	5·5	665	0·238	375·0	0·712	2·94
3·0	5·1	640	0·241	375·0	0·674	2·79
2·0	2·4	675	0·412	372·5	0·919	2·23
		40 *Hr. after dissolution*				
7·0	6·8	635	0·279	372·5	0·604	2·17
6·0	6·3	640	0·256	375·0	0·691	2·70
5·0	6·3	635	0·284	375·0	0·617	2·18
4·0	6·7	640	0·246	375·0	0·670	2·72
3·0	5·3	635	0·281	375·0	0·614	2·19
2·0	2·2	680	0·417	372·5	0·905	2·17

The values for the spectroscopic constants of biliverdin (see Table 1) have been reported previously for 5% methanolic hydrochloric acid solutions but it is not clear whether anhydrous hydrogen chloride or concentrated hydrochloric acid was used. The light absorption

Absorption spectra of biliverdin: **1**, *in* MeOH; **2**, *in* $CHCl_3$; **3**, *in* MeOH–HCl; **4**, *in* MeOH–HCl *after solution had been kept for* 2 *hr. in sunlight.*



was, therefore, also measured in methanol containing 5% by weight of concentrated hydrochloric acid and was the same as that of a solution of methanol containing 5% (w/v) hydrogen chloride. Fig. 1 shows the absorption curves of free biliverdin in methanol and in chloroform, and of the hydrochloride in methanolic hydrogen chloride. Even when the vessels were stoppered and stored in the dark, oxidation of biliverdin occurred (see Table 3). The oxidation was accelerated by exposure to light and air and occurred more readily in acid methanol than in chloroform. Visually the solution appeared more

blue: spectroscopically there was a decrease of absorption at 375 mμ, the maximum at 670 mμ decreasing less and being shifted to lower wavelength. There was an increase of absorption in the 470—630 mμ region and new maxima appeared at ~270 and 315 mμ. The minimum at 480—500 mμ shifted to 470—480 mμ. The ratio of the intensity of absorption at the two main maxima (E_{375}/E_{670}) decreased and approached the value calculated from previously published data (Table 1). These changes are shown in curves 3 and 4 of Fig. 1.

Lemberg's absorption curves for biliverdin in methanol [4] reveal the presence of bilirubin in his pigment, for there is increased absorption at 490—530 mμ and the lower absorption band is at 392 mμ instead of 370—380 mμ. Traces of bilirubin in biliverdin hydrochloride are readily oxidised to the latter and catalyse the further oxidation of biliverdin to violinoid pigments.[5] Such impurities in Lemberg's hydrochloride are revealed by a ratio for E_{375}/E_{670} of 1·71 instead of 2·2. These changes in the spectra of ageing solutions of biliverdin are paralleled by similar changes in the spectra of its zinc complex (see Part III [3]). The effect of change in pH on the spectra of verdins is reported in Part IV of this series.[6]

Experimental

Chloroform was freed from acid by distillation over potassium carbonate. Peroxide-free ether was used. Oxygen-free nitrogen was obtained by passing the gas through three lots of alkaline sodium dithionite solution.[7]

Preparation of Pure Bilirubin.—Bilirubin from two sources was used. (*a*) Fresh pig's bile (2 l.) was mixed with aqueous 0·1N-sodium hydroxide (3 l.) and left for 24 hr. for hydrolysis of conjugates of bilirubin. After acidification to Congo Red with 11N-hydrochloric acid, the mixture was extracted with chloroform (15 × 500 ml.). The extracts were washed with water and dried by filtration. Removal of the solvent under reduced pressure afforded crude bilirubin (1·6 g.), which was continuously washed with ether and then with methanol in a Soxhlet extractor until the washings were colourless. The pigment was then washed with methanol, extracted into chloroform, recovered, and crystallised. Continuous extraction with ether and methanol and crystallisation from chloroform were then repeated three times. Pure bilirubin was thus obtained in excellent yield (418 mg.). Recorded yields are 8 mg.[8] and 13·2 mg.[9]/100 ml. of pig's bile and 16·5 mg./100 ml. of human bile.[10]

(*b*) Commercial bilirubin (from Messrs. Light & Co.), although of high molecular extinction coefficient, contained impurities of the meso-type, for ethylmethylmaleinimide was obtained on oxidation by nitric acid. These impurities were eliminated by washing the material with ether and methanol successively in a Soxhlet apparatus. The final product was crystallised from chloroform.

The two bilirubin preparations showed ε 55,600 at 450 mμ in chloroform and behaved identically; both were shown to be free from mesobilirubin and other meso-pigments by demonstrating the absence of ethylmethylmaleinimide in the products of oxidation by nitric acid (see Part III [3]).

Preparation of Biliverdin.—The purified bilirubin (150 mg.) in methanol (200 ml.) was refluxed with a 20% solution of ferric chloride in 10N-hydrochloric acid (10 ml.) on a water-bath for 0·5 hr. The solution was cooled, filtered from residual bilirubin, and neutralised by addition of saturated aqueous sodium acetate. The mixture was extracted with ether (3 × 100 ml.), and the combined extracts were washed with 2N-sodium acetate and with water and were extracted with N-hydrochloric acid (3 × 100 ml.). The pigment present in the acid was removed in chloroform (3 × 100 ml.), which was then washed with water (5 × 100 ml.) to liberate the free pigment. The chloroform solution was dried by filtration and evaporated to

[4] See Lemberg and Legge, "Haematin Compounds and Bile Pigments," Interscience Publ. Inc., New York, 1949, p. 117.
[5] Kulczycka, unpublished observations.
[6] Part IV, Gray, Kulczycka, and Nicholson, *J.*, 1961, 2276.
[7] Linstead, Elvidge, and Whalley, "Modern Techniques of Organic Chemistry," Butterworths, London, 1955, p. 139.
[8] Gibson and Lowe, *J. Biol. Chem.*, 1938, **124**, xii.
[9] U.S.P. 2,386,716/1945.
[10] Libowitzky, *Z. physiol. Chem.*, 1940, **263**, 267.

dryness under reduced pressure. The biliverdin was dissolved in the smallest possible quantity of methanol (10 ml.), leaving a residue of bilirubin. The biliverdin obtained by evaporation of the methanol solution was washed with light petroleum (b. p. 40—60°; 2 × 50 ml.) to remove traces of oxidation products. The residue was washed with ether and crystallised from methanol under nitrogen, to give pure biliverdin (48 mg.). Chromatography of a portion on chalk and elution with 20 : 1 light petroleum (b. p. 40—60°)–chloroform proved its homogeneity and this was confirmed spectroscopically.

To increase the yield it is possible to extract additional crude biliverdin from the ether solution by further extraction with 1N- and 2·8N-hydrochloric acid.

Rapidity of work, protection from air and strong light, and exclusion of oxygen from reagents are necessary in the above preparation if the production of further oxidation products is to be avoided. When these conditions are fulfilled, the proportion of pigment requiring extraction with 2·8N-hydrochloric acid is negligible.

Measurements of Absorption (with Z. PETRYKA).—" AnalaR " methanol (pH 8·05) was used for the preparation of all solutions. Solutions of hydrogen chloride in methanol were prepared by dilution of a saturated methanolic solution of gas with methanol. Normality of these solutions was determined by titration against standard alkali. The pH of the weakly acid solutions was measured on an E.I.L. 23A type of pH meter, standardised with 0·05M-potassium hydrogen phthalate (pH 4·00) and 0·05M-sodium tetraborate (pH 9·07). A standard solution of biliverdin in chloroform was prepared. 0·3488 mg. quantities of biliverdin were obtained by evaporation of appropriate volumes of the standard solution and were stored under nitrogen. Approximately 0·5 hr. before spectrophotometry each residue was dissolved in 25 ml. of appropriate solvent, and the vessel was stoppered and stored in the dark. Measurements were carried out in 1 cm. stoppered quartz cells in a Hilger spectrophotometer (type H 700). Wavelength settings and optical-density readings were checked with a calibrated rhodiumised quartz filter, and the former were confirmed by using a chloroform solution of stercobilin ($\lambda_{max.}$ 496 mμ) and a calibrated didymium filter. The pH's of the dilute acid solutions were also measured after spectrophotometry on the pH-meter. Within the narrow range of concentrations used the solutions obeyed Beer's law (E 0·2—1·0).

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