The Photodecomposition of Bilirubin and Other Bile Pigments

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The photodecomposition of bilirubin in chloroform was studied by spectrophotometry and chromatography. A fraction of the pigment decomposed with retention of the tetrapyrrolic structure, giving biliverdin, chrysin, and choletelin. The rest of the bilirubin decomposed to propentdyopents, imides, a pyrrolic acid, and simple aliphatic acids. Verdins, mesobiliviolin, and i-urobilin treated similarly retained the tetrapyrrolic structure for a longer time.

BILE pigments, apart from stercobilin, are unstable compounds. Bilirubin [1] and d-urobilin [2] readily undergo isomerisation to other pigments, and the decomposition of these pigments, especially bilirubin, to simpler substances complicates their isolation, purification, and estimation. Bilirubin in alkali successively undergoes hydration, dehydrogenation, and fission at the a- and c-bridge carbon atoms.[3] The present work concerns the photodecomposition of bilirubin and (in less detail) of other pigments.

METHODS AND RESULTS

Decomposition of Bilirubin in Chloroform.—Disappearance of bilirubin. In four experiments a standard solution of bilirubin was exposed for up to 500 days at room temperature to air and diffuse daylight. At intervals the concentrations of bilirubin, biliverdin, and pentdyopents were determined, and the solutions were examined by paper chromatography for the presence of imides, pyrroles, propentdyopents, pyrrolecarboxylic acids, and other acidic compounds. Bilirubin disappeared completely within 12—25 days according to the intensity of the daylight, but the pattern of bilirubin decomposition was independent of light intensity. Bilirubin disappeared in a succession of rapid phases (A, C, and E) alternating with slow phases (B and D) (Figure 1). About 35 and 70% of the bilirubin had disappeared by the end of phases B and C, respectively.

Formation of Biliverdin, Purpurins, and Other Pyrrolic Products.—Biliverdin (never more than 13% of the initial bilirubin) was present sometimes as early as the first day, but had disappeared 40—50 days after the experiment was begun (Figure 1). Small amounts of purpurins were detected a few days after the appearance of biliverdin. After 100 days the chloroform solution had become almost colourless. A portion of the solution was evaporated to a small volume, giving a yellow solution with a broad absorption, $\lambda_{max.}$ 270, 420infl, and 520infl nm. Addition of zinc acetate caused green fluorescence in u.v. light, changed the position of the inflection at 420 to 460 nm and enhanced the absorption at 500 nm.

Formation of Propentdyopents and Other Colourless Products.—Propentdyopents could be detected sometimes

[1] C. H. Gray, A. Kulczycka, and D. C. Nicholson, *J. Chem. Soc.*, 1961, 2268.

[2] C. H. Gray and D. C. Nicholson, *J. Chem. Soc.*, 1958, 3085.

[3] J. D. Ostrow, D. C. Nicholson, and M. S. Stoll, *Gastroenterology*, 1970, **60**, 186.

in stage A, and then throughout the experiment. They were separated into 'water-extractable' and 'alkali-extractable' fractions, the former appearing earlier than the latter, which was formed in greater amount (Figure 2). The pentdyopents formed from these treated alkaline extract (*i.e.* of the pentdyopents formed from the total propentdyopents). If the extraction of propentdyopent is quantitative and the formation of pentdyopent is stoicheiometric, the two curves should parallel one another. That they do not

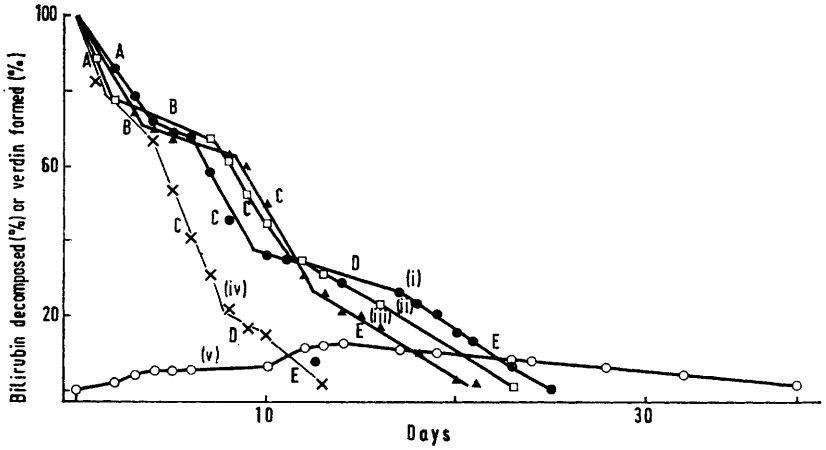


FIGURE 1 Course of disappearance of bilirubin in chloroform solution [four experiments (i)—(iv)] and formation of biliverdin in daylight (v)

fractions by dithionite treatment had distinct absorption maxima at 523—524 (the 'water-extractable' fraction) and 527—529 nm (the 'alkali-extractable' fraction). Propentdyopents were roughly estimated from the intensity of the absorption at 280 nm, but could not be determined quantitatively in this way because of interference of other products extractable from chloroform into water and dilute alkali.

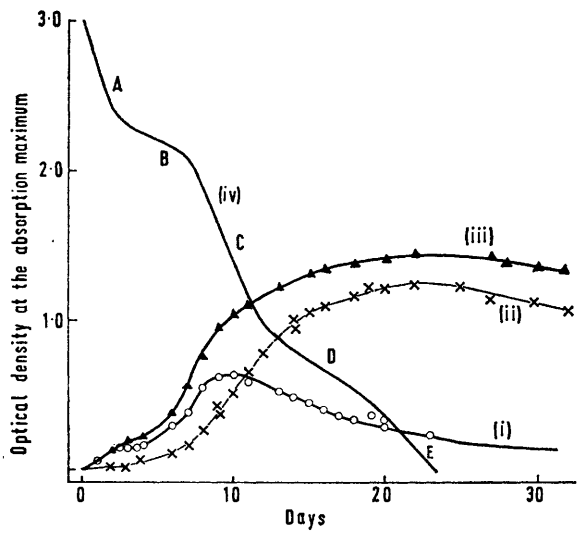


FIGURE 2 Formation of 'aqueous' (i), 'alkaline' (ii), and 'total' (iii) propentdyopents throughout one experiment. The form of the bilirubin decomposition (iv) is included for comparison

In Figure 3, the absorption at 280 nm of the chloroform solution has been plotted against time (curve i); curve ii shows the absorption at 527 nm of the dithionite shows that products absorbing at 280 nm other than propentdyopents are formed during the photodecomposition of bilirubin. These increase in amount and

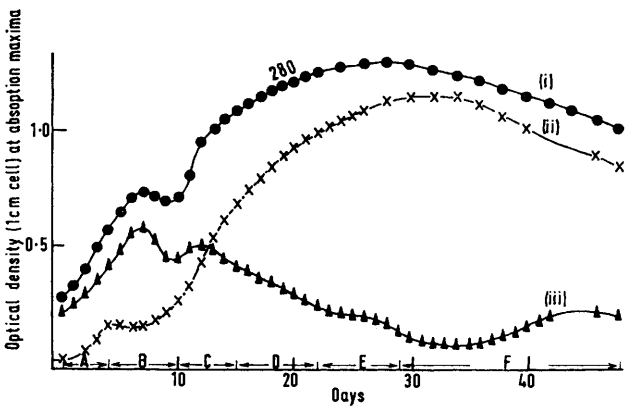


FIGURE 3 The change of maximal absorption at 280—290 nm of a chloroform solution of decomposing bilirubin (i) together with change of absorption at the 527 nm maximum (ii) due to total pentdyopents. The third curve (iii) shows the time of maximal formation of compounds other than propentdyopents and is derived from curves i and ii

reach a maximum in stages B and C, that is between 10 and 15 days, and then decline. At this stage little bilirubin and little biliverdin are present which possess significant absorption at 280 nm.

However, a quantitative consideration based upon the known extinction coefficients of bilirubin and biliverdin [4] and of variation of the 525 nm absorption of pentdyopent permits an assessment of the proportions of these compounds present at different stages

[4] C. H. Gray, A. Kulczycka, and D. C. Nicholson, *J. Chem. Soc.*, 1961, 2276.

of the decomposition. Figure 4 shows the percentage of remaining bilirubin (curve ii), that of biliverdin (curve iii), the relative concentration of propentdyopent (calculated from the pentdyopent absorption) (curve iv), and the differences (curve v) between the percentages of the two degradation products from the percentage of bilirubin which has disappeared. This confirms the qualitative finding that colourless compounds are formed during the photodecomposition of bilirubin.

The Table shows the results of paper chromatographic studies of the colourless products formed during the photodecomposition of bilirubin, and the Scheme

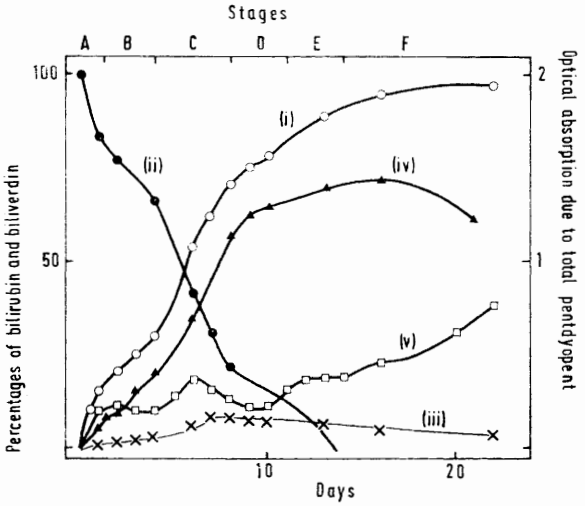


FIGURE 4 The total formation of non-rubinoid or non-verdinoid compounds (i) derived by difference from the rubin (ii) and verdin (iii) concentrations. Total propentdyopent variation throughout (iv) was determined from absorption at 527 nm after conversion to pentdyopent and the time of formation of other compounds (v) was derived by difference

illustrates the possible pathways of decomposition. At a very early stage of photodecomposition a propentdyopent with the probable structure (1b), 3-carboxyethyl-4-methylpyrrole-2,5-dicarboxylic acid (III), and the further degradation product, methylvinylmaleimide (VII), could be identified by paper chromatography. A few days later a second propentdyopent, probably (1a) and/or (1c), methylcarboxymaleimide (IV), and methylmaleimide (VIII) could be detected. At a later stage haematinimide (V) and methylethylmaleimide (VI) were present; throughout the early stages an unknown compound reacting with Bromocresol Green and 3,3'-dimethylbiphenyl-4,4'-diamine was also present.

Since in the decomposition of bilirubin the maximal formation of propentdyopents occurred at stage D or E, or at the beginning of stage F when all bilirubin had disappeared, the formation of some intermediate precursor of the propentdyopent must be considered probable.

Pyruvic, succinic, and possibly oxalic and propionic acids were detected. The presence of oxalic and succinic acids and other unidentified acidic substances in oxidation products of bile pigments has been reported.[2,5]

In methanol–chloroform and in weakly alkaline methanol or aqueous solutions bilirubin decomposed, as in chloroform, with rapid degradation of the tetrapyrrolic structure. Decomposition was more rapid and the propentdyopents were formed earlier and in greater amount. In strongly alkaline solutions the vivid orange colour of bilirubin changed to brown and slowly diminished in intensity. On the other hand, in acidic media spectrophotometric measurements showed that the pigment decomposed with little destruction of the tetrapyrrole structure, propentdyopents, imides, and dipyrrylmethenes being detected only with weakly acidic solutions. In acid the overall rate of decomposition was less than in alkali. Formation of verdin occurred both in the dark and in light but the latter was essential for the formation of purpurins.

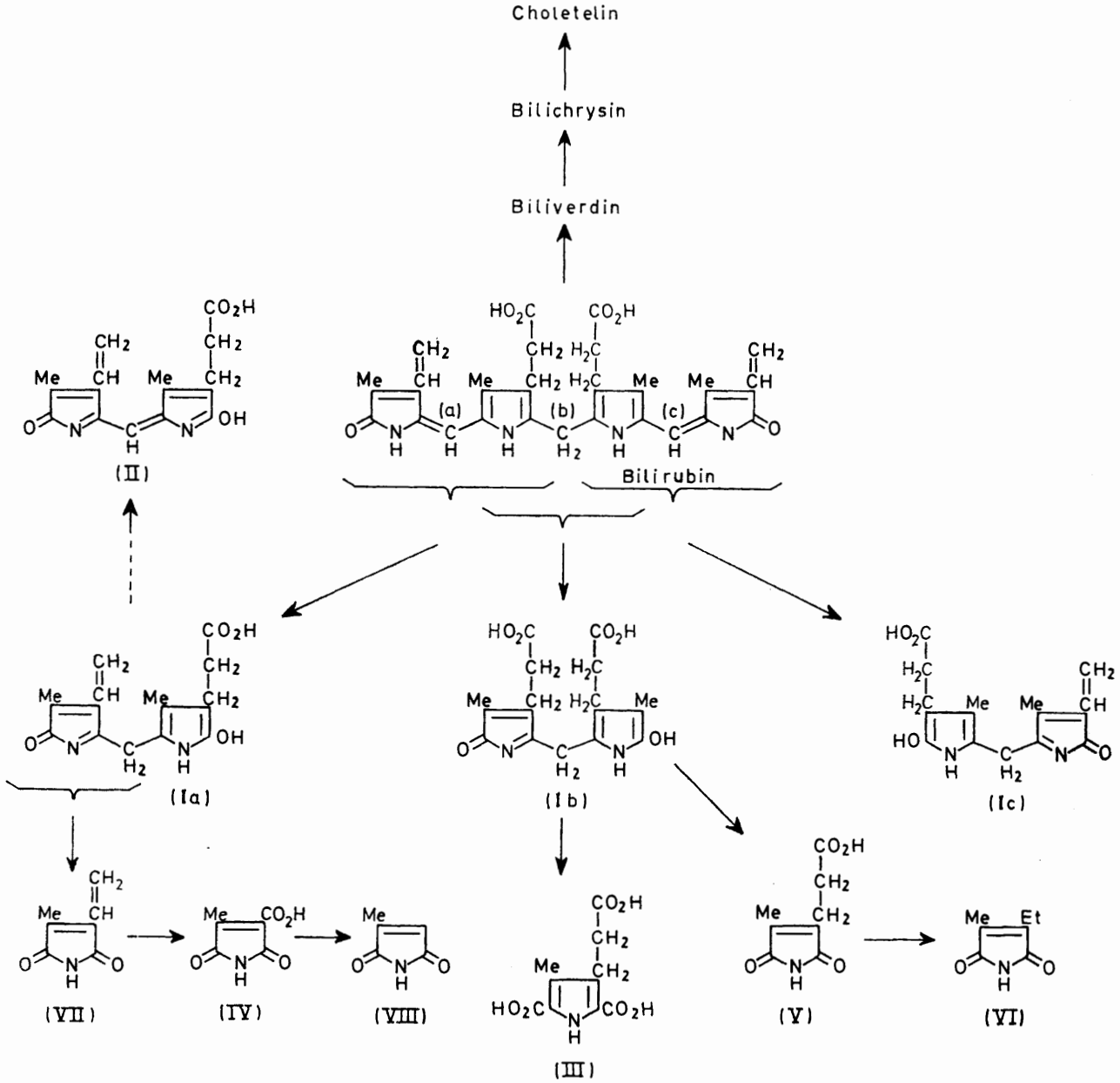
Photodecomposition of Other Bile Pigments.—Verdins, mesobiliviolins, and i-urobilin subjected to similar treatment differed from rubins in that they decomposed mainly through a Gmelin-type reaction, there being little indication of propentdyopent formation; only in the case of biliverdin were traces of propentdyopents detected.

Biliverdin. The initial biliverdin solution was green, $\lambda_{max.}$ 640 and 380 nm, $\lambda_{min.}$ 500 nm. In daylight, the maxima slowly decreased, owing to decomposition of verdin. Absorption between 550 and 650 nm and below 350 nm increased owing to production of violinoid compounds, and the minimum shifted to 450 nm. On the tenth day a well defined maximum at 580 nm had appeared and this later shifted to 540 nm. The well defined maximum at about 330 nm, characteristic of violinoids, was obscured by greatly increased absorption below 300 nm, which on the twentieth day had developed into a definite maximum at 270 nm. A violinoid pigment was isolated by chromatography on calcium carbonate, and appeared spectroscopically to be a purpurin in not yielding biliverdin on iron(III) chloride oxidation. On about the twenty-third day approximately half the biliverdin had disappeared, and increased absorption at 490 to 510 nm and at 415 and 315 nm suggested the formation of 'choletelins' (*i.e.* bilatrienes in which a- and c-methyne bridges are saturated by the addition of groups other than hydrogen atoms) and 'chrysins' (*i.e.* bilatrienes in which either an a- or a c-methyne bridge is saturated by groups other than hydrogen atoms), respectively. Later absorption at 540—560 and at 420—450 nm increased, and at this stage absorption at 500—580 nm found for the zinc complex of the mixture indicated the formation of rhodinoid purpurins.

A few days later, paper chromatography showed that imides had been formed. By about the forty-fifth day of the experiment the solution was violet-brown, virtually all verdin had disappeared, and the solution contained mainly violinoid purpurins with some rhodinoid purpurins and choletelins. Increasing amounts of

[5] C. H. Gray, D. C. Nicholson, and R. A. Nicolaus, *Nature*, 1958, **181**, 183.

colourless products had been formed, as shown by generally increased absorption below 300 nm and absorption maxima at 230 and 270 nm without associated increase in absorption in the visible region. No propentdyopents were detected. On the sixtieth day purpurins and choletelins were still present, and of the tetrapyrrolic structure was low and propentdyopents were formed; the corresponding pentdyopents having $\lambda_{max.}$ 529 nm. In contrast, in methanolic acid solutions a slow decomposition took place with the formation of purpurins, chrysins, and choletelins. There was progressive diminution of the 380 and 680 nm



SCHEME Possible routes of photodecomposition of bilirubin in chloroform

the colourless products included haematinimide and an unidentified acid imide of R_F 0·28—0·35. The solution became colourless about the eightieth day and contained a pyrrole of R_F 0·39, but the pentdyopent reaction was barely positive even after a ten-fold concentration of the solution.

In methanol and aqueous alkaline solutions the proportion of pigment decomposing without destruction verdin absorption bands, which gave place to the 340 and 600 nm region bands of violinoid pigments, indicating saturation of the a- or c-bridge methyne groups. Considerable verdin remained even when the solution was distinctly purple and when choletelin was forming by saturation of a second methyne group.

Mesobiliverdin (glaucobilin) and bihydrobiliverdin. In each case, the decomposition was similar to that of

biliverdin, except that the purpurins appeared at a later stage than with biliverdin. The proportion of rhodinoid purpurins was higher. Subsequent decomposition usually occurred faster than with biliverdin, decolourisation being complete after about 80 days. Crystalline methylethylmaleimide was isolated on the twentieth day and was characterised by m.p. and chromatography. Paper chromatography revealed also the presence of haematinimide (V), methylethylmaleimide (VI), and one other unidentified acid imide of R_F 0·28 and succinic acid. No propentdyopents or pyrrolic compounds were detected.

i-Urobilin. This was the least unstable pigment. In chloroform, there was a gradual decrease in the intensity of colour until the solution became colourless (*ca.* 150 days). The absorption maximum at 500 nm decreased at first rapidly and then more slowly. No other maxima in the visible region appeared, but in the u.v. region maxima at 264 and 270 nm appeared. In methanolic solution, the maxima were at 230 and 270 nm and gradually became more intense.

Methylethylmaleimide (VI) was detected on the fourth day and haematinimide (V) on the eighth. No propentdyopents were formed.

Mesobiliviolin. This was the least stable of the pigments studied, the solution becoming colourless in about 40 days. Initially the maximum at 580 nm shifted to 540 nm, indicating ready formation of purpurins which resisted iron(III) chloride oxidation. Methylethylmaleimide (VI) was detected on the fifth day and haematinimide (V) on the eighteenth day. At this time the absorption at 275 nm was intense, masking the violin absorption at 330 nm. Increased absorption at 500 nm indicated the presence also of choletelins. In methanol or acidic methanol, absorption between 380 and 420 nm increased, and formation of the zinc complex produced a maximum at about 460 nm.

In chloroform, methanol, and methanolic zinc acetate, the final colourless solution showed a large maximum at 230 nm and a smaller one at 275 nm, as did the final colourless solutions of bilirubin and glaucobilin. No propentdyopents were detected. Haematinimide (V) and methylethylmaleimide (VI) were also present.

Decomposition of mesobiliviolin thus resembled the later stages of decomposition of glaucobilin rather than of biliverdin.

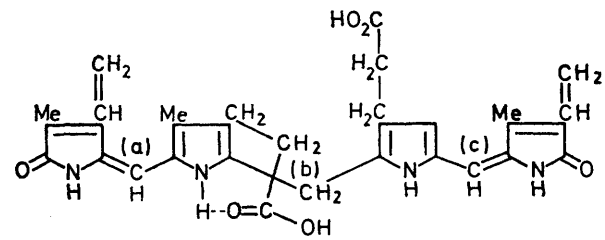
DISCUSSION

Solutions of bilirubin in chloroform were stable under nitrogen, showing that most of the bilirubin which disappeared in the described experiments decomposed by oxidation. The complexity of the products shows that several mechanisms operate simultaneously.

(1) *Gmelin oxidation.*—The Gmelin reaction displays a colour sequence caused by progressive conversion of the tetrapyrrole system through a number of different chromophoric structures followed by degradation to simpler compounds.[6] The occurrence of such a sequence in the present work is suggested by the isolation of biliverdin during the photodecomposition of bilirubin, and the detection of purpurins and choletelins. The small amounts of biliverdin detected (Figures 1 and 4) indicate that such decomposition proceeds gradually in stages A and B, continues more rapidly in stage C or early D, and thereafter proceeds more slowly. Biliverdin decomposed slowly in chloroform and, allowing for this, probably not more than about 20% of bilirubin appeared to be decomposed *via* this route.

(2) *Oxidation to Propentdyopents.*—Two products were isolated and characterised as propentdyopents (I), by conversion into the pink pentdyopent (II) absorbing at about 525 nm. The IXα-structure of bilirubin is formally capable of affording three different propentdyopents (Scheme). The propentdyopent extracted into water, having the lower R_F value and with absorption maximum of its pentdyopent at 523 nm, was assumed to have the structure (Ib) on account of the higher polarity expected from the presence of two propionic acid groups. The alkali-extractable compound (1a or c) might be assumed to be a less polar and less acidic monopropionic acid compound. Spectroscopically both compounds resembled the corresponding compounds synthesised by von Dobeneck.[7]

von Dobeneck [7,8] has reported the formation from bilirubin of propentdyopents (Ia) and (Ic) rather than (Ib). However, Heikel [9] has separated two propentdyopent fractions by electrophoresis and chromatography and showed that the less mobile compound (Ib) resulted from the partial oxidation of bilirubin, while both fractions (Ia) and (Ic) also appeared on longer oxidation. Possible reasons for the formation of the propentdyopent fraction (Ib) are not only that the electron-rich a- and c-methyne bridge groups would undergo ready oxidation compared with the saturated b-methylene group, but also that the propionic acid side chains are admirably situated for bonding of the pyrrole *N*-hydrogen atoms by the carbonyl groups as shown (IX). The central methylene group (b) in



(IX)

such a structure would be screened from oxidation both sterically and electronically by the proximity of the nucleophilic carboxy-group. Fog [10] has suggested that, in contrast to bilirubin itself, its esters undergo a direct diazo-reaction, owing to the abolition of the hydrogen

[6] L. Gmelin and F. Tiedmann, 'Die Verdauung nach Versuchen,' Karl Groos, Heidelberg und Leipzig, 1826, p. 1.
[7] H. von Dobeneck, *Z. physiol. Chem.*, 1942, **274**, 1.
[8] H. von Dobeneck, *Z. klin. Chem.*, 1966, **4**, 137.
[9] T. Heikel, *Scand. J. clin. Lab. Invest.*, 1958, **10**, 191.
[10] J. Fog and E. Jellum, *Nature*, 1963, **198**, 88.

bonds in the free pigment. This theory is compatible with the work of Ostrow *et al.*[3] and with the fact that the proportion of propentdyopent (Ib) formed in pure chloroform is greater than that formed in the more polar methanol–chloroform mixture (1 : 1 v/v).

(3) *Oxidation to Monopyrroles and Imides.*—Degradation to monopyrroles with subsequent conversion into imides was indicated by detection of 3-carboxyethyl-4-methylpyrrole-2,5-dicarboxylic acid (III) and the imides (IV)—(VIII). Since the acid (III) was sometimes detected in stage A as well as in B and early C, the fission responsible for its production must have occurred very early. The imides (IV)—(VIII), which represent products of further oxidation, mostly appeared from stage B onwards. Preferential fission of the bilirubin molecule at the methyne bridges (a) and (c) producing propentdyopent (Ib) would be followed by end ring oxidation to methylvinylmaleimide (VII) as well as methylcarboxymaleimide (IV) and methylmaleimide (VIII), its products of oxidation and decarboxylation. All these imides were identified as products of bilirubin degradation, as well as haematinimide (V), which could arise from the decomposition of intact rubins or from decomposition of 3-carboxyethyl-4-methylpyrrole-2,5-dicarboxylic acid (III). This acid (III) might arise either from decomposition of bilirubin or of the propentdyopents (Ia—c). Decomposition of imides bearing β-methyl groups (IV)—(VIII) could formally give rise to pyruvic and propionic acids, and succinic acid could arise from compounds containing rings with β-carboxyethyl groups. The observation of oxalic acid and an unidentified acid presumably indicates further oxidation of these products.

EXPERIMENTAL

For spectrophotometry the Hilger H700 recording spectrophotometer and the Hilger Uvispek 705 spectrophotometer were used. Solvents were redistilled, the chloroform first being shaken with sodium hydrogen carbonate solution. For chromatography (descending) Whatman no. 4 paper was used.

Bile pigments were prepared as previously described.[1]

Decomposition of Bilirubin in Chloroform.—A standard solution of bilirubin (30 mg l^{-1}) was left unstoppered in diffuse daylight for several days; chloroform was added daily to replace losses due to evaporation. At first daily, and then at frequent intervals, the following were determined: (*a*) spectrophotometric absorption curves of samples in chloroform, methanol, methanolic zinc acetate, and methanolic hydrochloric acid; (*b*) spectrophotometric curves of propentdyopent solutions obtained by extraction of the chloroform solution with water and dilute alkali; and (*c*) curves of the pentdyopents formed from these. Concurrently four separate samples were measured out, the solvent was evaporated off and the residues were studied by paper chromatography. Four such experiments were conducted.

Measurement of the Concentrations of Bilirubin, Biliverdin, and Other Bile Pigments.—The concentration of the residual bilirubin was calculated from the absorption in chloroform at 450 nm (ε 56,000).[11] In order to determine accurately the amount of biliverdin formed, some of the chloroform solution (50 ml) was evaporated to dryness under reduced pressure and the residue was treated with methanol (6 ml). After filtration and adjustment of volume to 6 ml the solution was divided into two; the spectrum of one part was measured and redetermined after addition of two drops of a saturated solution of zinc acetate in methanol. Almost all the methanol in the other half was evaporated off in a current of nitrogen and the residue was made up to 3 ml with methanolic hydrochloric acid (5% w/v). From the absorption in the latter solvent at 675 nm (ε 23,000),[11] the conversion of pigment was calculated. Overlapping of absorption bands of the mixture of bile pigments precluded quantitative estimation of violins, purpurpins, and choletelins. From a comparison of the absorption spectra in the four solvents the time of appearance of these pigments was derived.

Isolation and Properties of Propentdyopents and Pentdyopents.—The chloroform solution (5 ml) was extracted first with water (5 ml) and then with 0·5M-ammonium hydroxide (5 ml); a further 5 ml of the chloroform solution was extracted similarly with only the ammonium hydroxide solution. The spectra of the three aqueous solutions were recorded (' water-extractable ', ' alkali-extractable ', and ' total ' propentdyopents, respectively). To each of these solutions a freshly prepared saturated solution (3 ml) of sodium dithionite in 2M-sodium hydroxide was added, and exactly 5 min later the maximal absorptions of the resultant pentdyopent solutions were measured. The maxima for the three solutions were at 523—525, 527—529, and 526—529 nm, respectively. The wavelength maximum of the ' total ' was lower at the beginning of the experiment and higher towards the end. The pentdyopent extractable into alkali was considerably more stable than that extracted into water and was usually not formed at once, the red colour appearing about 2 min after addition of dithionite.

The propentdyopents could not be distinguished spectroscopically, both compounds absorbing maximally between 270 and 290 nm, and the wavelength being lower in acid, higher in alkali, and of intermediate value between pH 5 and 6. Previous reports [12,13] differ as to the wavelength of absorption for these compounds; and no molar extinction coefficients are recorded.

The two propentdyopents were unstable, being destroyed both by strong acid and by alkali. They were very soluble in water, being only partially extracted into ether and n-butanol. The propentdyopents were separable by paper chromatography, and located by spraying with a saturated solution of sodium dithionite in aqueous 2M-sodium hydroxide; the resulting red pentdyopent spots faded rapidly. The results of the chromatographic investigation are as follows:

	R_F Values	
Solvent system	Propentdyopent (Ib) (water-soluble)	Propentdyopent (Ia and c) (alkali-soluble)
EtOH–NH_3 (0·88)–H_2O (16 : 1 : 3)	0·58	0·75—0·71
PrOH–NH_3 (0·88)–H_2O (6 : 3 : 1)	0·74	0·87
Bu^nOH–AcOH–H_2O (4 : 1 : 5)	0·57	0·77

[11] C. H. Gray, A. Lichtarowicz-Kulczycka, D. C. Nicholson and Z. Petryka, *J. Chem. Soc.*, 1961, 2264.
[12] K. Kona, *Okayama Igakkai Zesshi*, 1959, **71**, 7203.
[13] R. Tachenchi, *Okayama Igakkai Zesshi*, 1959, **71**, 7653.

Electrophoresis in barbital buffer [14] of pH 8·6 for 4 h on Whatman 3 mm paper (3·2 V cm^{-1}; 1·5—2·0 mA) also separated the propentdyopents, both compounds moving towards the anode. Neither was made visible by u.v. light. Spraying the chromatograms with methanolic zinc acetate solution rendered propentdyopent (Ib) visible immediately. The 'alkali-extractable' compounds (Ia and c) appeared only after an interval. The absorption maxima for the corresponding pentdyopents were 523 and 527 nm, respectively.

Identification of Products by Paper Chromatography.—At frequent intervals four separate samples (5 ml) were taken from each of the decomposing chloroform solutions. The solvent was evaporated off in a current of nitrogen at room temperature; each residue was dissolved in methanol (0·5 ml) and applied to Whatman no. 4 paper. Solid not dissolving in methanol was extracted with ethyl acetate and subsequently with chloroform, and from these solutions was subsequently transferred to the paper. Thus for each day four papers were prepared and these were developed simultaneously in ethanol–ammonia (0·88)–water (16 : 1 : 3 v/v/v) for 12—14 h. After air-drying the papers were inspected under u.v. light and later were examined separately for imides (3,3′-dimethylbiphenyl-4,4′-diamine after saturation with chlorine [15]), pyrrolic acids (diazotised sulphanilic acid [16]), pentdyopents (alkaline dithionite), and acids (Bromocresol Green).[2] Authentic specimens of these substances, except for pentdyopents, were used as markers. Sometimes saturated ethanolic zinc acetate was used for spraying instead of the Bromocresol Green, the chromatogram then being examined in u.v. light. Chromatograms were sometimes developed in n-propanol–ammonia (0·88)–water (6 : 3 : 1) or n-butanol–2N-ammonium hydroxide (1 : 1) (for better separation of propentdyopents), or in ethyl acetate saturated with water or n-butanol–acetic acid–water (12 : 3 : 5) (for better separation of aliphatic acids).

Decomposition of Other Bile Pigments in Chloroform.—Standard solutions of biliverdin ($1·62 \times 10^{-5}$M), glaucobilin ($1·62 \times 10^{-5}$M), mesobiliviolin ($3·9 \times 10^{-5}$M), and i-urobilin ($0·96 \times 10^{-5}$M) in chloroform were prepared and similarly left to decompose as described for bilirubin. The absorption spectra were frequently measured for solutions in chloroform, methanol, methanolic zinc acetate, and methanolic hydrochloric acid. Measured samples were examined by paper chromatography, by the methods already described, for imides, pyrrolic acids, propentdyopents, and carboxylic acids. Papers were also sprayed with methanolic zinc acetate and examined for fluorescence in u.v. light.

Column Chromatographic Separation of the Violinoid Pigment formed during the Photodecomposition of Biliverdin in Chloroform.—The chloroform solution (25 ml) was evaporated to dryness. The residue was dissolved in the minimum quantity of fresh chloroform and placed on a column of calcium carbonate (B.D.H. chromatographic grade) suspended in light petroleum (b.p. 60—80°)–chloroform (20 : 1 v/v). On elution with the same solvent much coloured material remained at the top of the column. The violinoid pigments formed the most mobile fraction, being followed by a series of green, verdinoid bands. The violinoid pigments were finally eluted from the column with light petroleum–chloroform (2 : 1 v/v). After removal of the chromatographic solvent the pigment in chloroform had $\lambda_{max.}$ 540—560 nm and the pigment was unchanged on treatment with methanolic iron(III) chloride.

Compounds formed throughout the photodecomposition of bilirubin in chloroform and identified by paper chromatography [ethanol–ammonia–water (16 : 3 : 1 v/v)]

Compound	R_F	Colour of spot developed by reagents (a)—(d) *		Occurrence in stage
Propentdyopent (Ib)	0·50—0·62	Pink	(a)	From A throughout
		Unstable		
Propentdyopents (Ia and c)	0·70—0·76	Pink	(a)	Throughout from C and sometimes from B
		More stable		
(III)	0·03—0·07	Red, turning blue	(b)	A, B, early C
		Brown	(c)	Not always detected
(IV)	0·38—0·55	Yellow	(b)	B, C, D, E, and when concentrated, F
		Yellow	(c)	
		Blue	(d)	
(V)	0·45—0·65	Yellow	(c)	From C onwards
	(usually 0·63)	Blue	(d)	
(VI)	0·69—0·89	Blue	(d)	From D onwards
(VII)	0·85—0·92	Red-blue	(d)	A, B
		Yellow	(c)	Sometimes throughout
(VIII)	0·80—0·93	Red, pink, or violet	(b)	B and C; later not always detectable
		Yellow	(c)	
		Red-blue	(d)	
Unknown	0·22—0·28	Blue	(c)	A, B, C
		Blue	(d)	
		Otherwise visible under u.v. light		

* (a) Freshly prepared saturated solution of sodium dithionite in 2N-sodium hydroxide solution. (b) Diazotised sulphanilic acid.[10] (c) Bromocresol Green.[2] (d) 3,3′-Dimethylbiphenyl-4,4′-diamine in an atmosphere of chlorine gas.[15]

One of us (A. K.) received a maintenance grant from the M.R.C.. We thank Professor R. Nicolaus for samples of methylvinylmaleimide, methlycarboxymaleimide, and methylmaleimide.

[1/1268 *Received, July 23rd,* 1971]

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