441. *The Chemistry of the Bile Pigments. Part III.*[1] *Prototropy of Bilirubin to a Verdinoid Pigment.*

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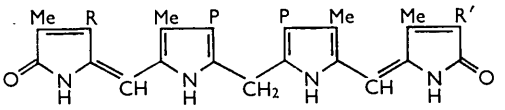
Treatment of bilirubin with alkoxides in the absence of oxygen affords a pigment spectroscopically similar to dihydrobiliverdin and containing a β-ethyl-β-methylpyrrole ring. Evidence is presented that the change is prototropic. Spectral absorption studies of verdins and their zinc complexes are reported.

Tautomerisation in bile pigments has been postulated to explain certain stages in the Gmelin reaction,[2] the weakly basic behaviour of bilirubin, which is unable to form complex salts,[3] the temperature-dependence of partition of purpurins between organic solvents and acids,[4] some properties of stercobilin,[5] formation of metal complexes by the dimethyl ether

[1] Part II, preceding paper.
[2] Fischer and Orth, "Die Chemie des Pyrrols," Akademische Verlagsges., Leipzig, 1937, p. 715.
[3] Lemberg and Legge, "Haematin Compounds and Bile Pigments," Interscience Publ., Inc., New York, 1949, pp. 108, 119.
[4] Ref. 3, p. 132.
[5] Gray and Nicholson, *Nature*, 1957, **179**, 264.

of mesobilirubin,[6] conversion of mesobiliviolin into mesobilirubin,[7] the anaerobic conversion of d-urobilin into violinoid pigments,[8] and differences in behaviour of d- and i-urobilin on treatment with ferric chloride.[9] Of these examples, only the last two are isomerisations

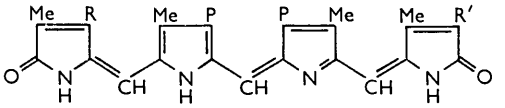


(Ia) R = R′ = CH:CH$_2$ (Ic) R = CH:CH$_2$; R′ = Et

(Ib) R = R′ = Et (Id) R = Et; R′ = CH:CH$_2$

involving the β-side chains of the pigments concerned. In this paper evidence is reported for the prototropic converson of a bisvinylrubin (bilirubin) into a monoethylmonovinylverdin (dihydrobiliverdin).

Rubins are bile pigments (I) containing two oxodipyrrylmethene units linked by a central methylene group. Bilirubin and mesobilirubin have structures (Ia and b respectively).[10] Dihydrobilirubin probably has structure (Ic) [11] but (Id) cannot be excluded.



(IIa) R = R′ = CH:CH$_2$ (IIc) R = CH:CH$_2$; R′ = Et

(IIb) R = R′ = Et (IId) R = Et; R′ = CH:CH$_2$

Biliverdin and mesobiliverdin (glaucobilin), with four conjugated pyrrole rings instead of two independent pairs, have the structures (IIa and b respectively).[12,13] Dihydrobiliverdin has not previously been described and may have either of the structures (IIc and d).

The bilirubin (10^{-3} ε = 56·6 at 450 mμ in chloroform) used in these experiments was free from pigments containing β-ethyl groups, because ethylmethylmaleinimide could not be detected after oxidation with nitric acid. Treatment of this bilirubin with boiling ethanolic sodium ethoxide in the absence of oxygen produced a verdinoid pigment which differed from the original bilirubin in containing a β-ethyl-β-methylpyrrole component, as shown by the formation of ethylmethylmaleinimide on oxidation. The isolation of this imide from the oxidation products of bilirubin treated with alkoxide has been reported previously.[14]

Since the verdinoid pigment was produced from bilirubin under conditions in which oxidation was excluded, its formation must be an isomerisation; mechanisms of prototropy of bilirubin consistent with these observations are shown in schemes A and B.

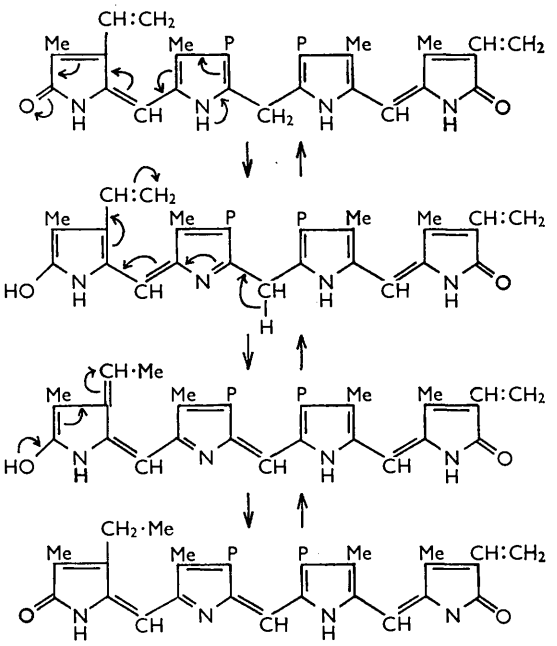
Mesobilirubin, which lacks the vinyl groups necessary for such isomerisation but like bilirubin is capable of oxidation, was almost unchanged by treatment with sodium ethoxide. Thus, little of the verdinoid pigment obtained by heating bilirubin with sodium ethoxide is likely to have arisen by oxidation.

Prototropic changes of the sort outlined in schemes A and B are usually catalysed in

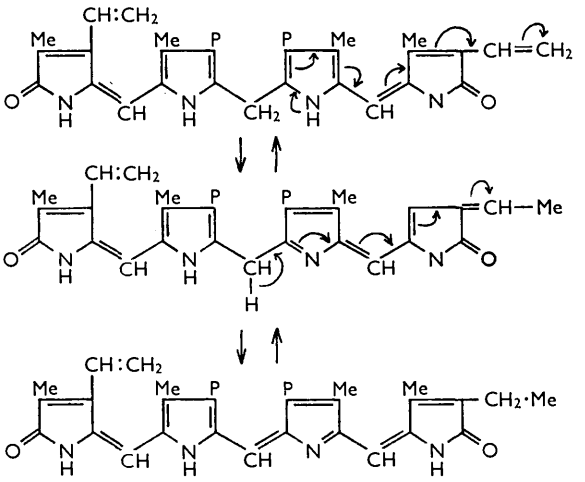
[6] Ref. 3, p. 123.
[7] Ref. 3, p. 126.
[8] Gray and Nicholson, *J.*, 1958, 3085.
[9] Gray and Nicholson, *Nature*, 1960, **185**, 380; Watson, Weimer, and Hawkinson, *J. Biol. Chem.*, 1960, **235**, 787.
[10] Gray, " The Bile Pigments," Methuen, London, 1953, pp. 1—10; Gray, Nicholson, and Nicolaus, *Nature*, 1958, **181**, 183.
[11] Ref. 2, p. 640.
[12] Fischer and Pleininger, *Z. physiol. Chem.*, 1942, **274**, 231.
[13] Siedel, *Z. physiol. Chem.*, 1935, **237**, 8.
[14] Fischer, *Z. Biol.*, 1915, **65**, 163.

increasing order of efficiency by methoxide, ethoxide, isopropoxide and t-butoxide ions.[15] This order was observed (Table 1) when bilirubin was converted into verdinoid pigment by treatment with these alkoxides at room temperature. The effect of using solvents of various polarities could not be investigated owing to low solubility of bilirubin.

Scheme A



Scheme B



The spectroscopic properties of verdins, together with recorded values for glaucobilin, are shown in Table 2. The recorded data for biliverdin are summarised in Part II of the series.[1] There was no significant difference between the spectra of the verdinoid isomerisation product and the other verdins.

[15] Baker, " Tautomerism," Routledge, London, 1935.

The zinc complexes of the verdins had two main absorption maxima, at 695 (685 mμ according to Lemberg [16]) and 375—385 mμ, and a third small maximum at 270 mμ (see Fig. 1); there were differences in the ratio of the intensities of absorption at the two main maxima (Table 3). The ratio for the isomerisation product of bilirubin was similar to that of dihydrobiliverdin.

TABLE 1. *Influence of alkoxides on the isomerisation of bilirubin.*

Medium used	NaOH	NaOMe	NaOEt	NaOPr^{i}	KOBu^{t}
Yield (%) of verdinoid isomerisation product	—	4·6	5·7	8·5	11·3
Recovered bilirubin (%):					
(*a*) as rubin	3·2	2·3	2·0	1·5	1·4
(*b*) as verdin after atmospheric oxidation	75·1	73·0	70·1	65·2	66·1
Total (%) accounted for	78·3	79·9	77·8	75·2	78·9

TABLE 2. *Absorption maxima* (mμ) *of verdins.*

	In $CHCl_3$			In HCl–MeOH (5% w/v)		
Pigment	λ_1	λ_2	E_2/E_1 *	λ_1	λ_2	E_2/E_1
Biliverdin (*a*)	640—645 (13·4)	377·5 (41·7)	3·10	670—680 (23·0)	377·5 (46·35)	2·02
Verdinoid isomerisation product (*a*)	640—645	387	3·20	685	378	2·14
Dihydrobiliverdin (*a*)	640—645	377	3·25	680	375	2·10
Glaucobilin (*a*)	635—640 (13·5)	367·5 (43·12)	3·20	680—685 (24·5)	365 (45·8)	1·87
Glaucobilin dimethyl ester (*b*)	Not given			670 (30·9)	Not given	
(*c*)	645 (17·0)					
(*d*)		363 (46·8) 309 (17·8)		Not given		
(*e*)		363 (46·7) 315 (24·6)		Not given		
(*f*)	636	367	1·79	647	367	1·54

The millimolar extinction coefficients, when available, are shown in parentheses.
* Ratio of absorption at the two maxima calculated from reported data.
References: (*a*) Present preparation. (*b*) Lemberg, *Annalen*, 1932, **499**, 25. (*c*) Siedel, *Z. physiol. Chem.*, 1935, **237**, 8 (in benzene). (*d*) Pruckner and Stern, *Z. phys. Chem.*, 1937, *A*, **180**, 25 (in dioxan). (*e*) *Idem*, *ibid.*, 1939, *A*, **182**, 117 (in dioxan). (*f*) Goodwin and Srisukh, *Biochem. J.*, 1951, **48**, 199.

TABLE 3. *Absorption maxima* (mμ) *of zinc complexes of verdins in ethanolic zinc acetate.*

Pigment	λ_1	λ_2	E_2/E_1	Inflections
Biliverdin	695	385	1·91	640, 320, 270
Verdinoid isomerisation product	695	385	1·65	640, 330, 270
Dihydrobiliverdin	695	385	1·71	640, 330, 270
Glaucobilin (*a*)	695	380	1·41	
Glaucobilin (*b*)	695	380	1·40	
Glaucobilin XIIIα	695	375—380	1·39	
Lemberg's value for glaucobilin (*c*)	685			

References: (*a*) Prepared by ferric chloride oxidation of mesobilirubin. (*b*) Prepared by ferric chloride oxidation of mesobilirubinogen (following paper). (*c*) Lemberg, *Biochem. J.*, 1934, **28**, 978.

The zinc complexes are readily oxidised in solution to violinoid pigments, and this results in progressive changes of absorption spectra. All solutions of the complexes of pure verdins are green and do not fluoresce in ultraviolet light. Curve 1 in Fig. 2 shows the spectrum of a freshly prepared solution of the zinc complex of pure biliverdin. When this solution was kept, there was no change in its appearance but the absorption spectrum changed. The small maximum at 270 mμ increased, a maximum appeared at 310—320 mμ, the absorption at 430—510, 580—600, and 630—650 mμ increased, and the initial maximum at 695 mμ shifted gradually to lower wavelength; often another maximum at 650 mμ appeared. The solutions then exhibited orange fluorescence in ultraviolet light. Sometimes the maximum at 650 mμ did not appear but that at 695 mμ gradually shifted

[16] Lemberg, *Biochem. J.*, 1934, **28**, 978.

towards 640 mμ. Orange fluorescence could not be detected while this maximum was above 660 mμ. Similar changes in the spectra and fluorescence were observed when the complexes were prepared from impure verdins containing traces of violinoid pigments.

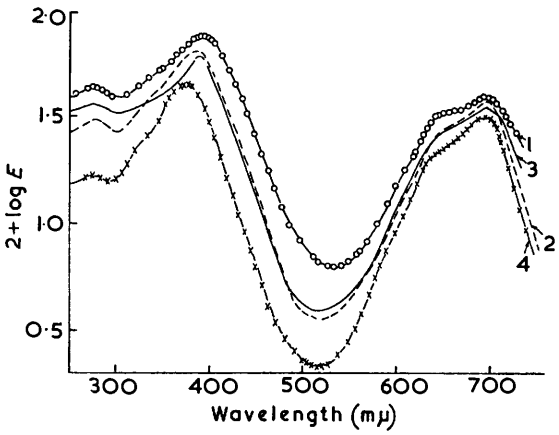


FIG. 1. *Absorption spectra of zinc complexes of verdins in ethanol*: 1, *Biliverdin*; 2, *dihydrobiliverdin*; 3, *verdinoid isomerisation product*; 4, *glaucobilin.*

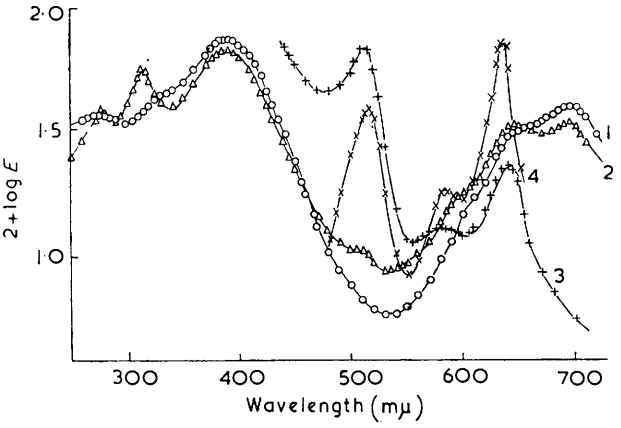


FIG. 2. *Absorption spectra of zinc complexes of pure and slightly oxidised biliverdin*: 1, *Pure biliverdin*; 2, *impure biliverdin*; 3, *as* 1, *after addition of iodine*; 4, *as* 2, *after addition of iodine.*

The zinc complexes of dihydrobiliverdin and of the verdin obtained by isomerisation were particularly susceptible towards oxidation in this way; that of glaucobilin was less readily oxidisable. The zinc complex of biliverdin itself was the most stable.

TABLE 4. *Absorption maxima* (mμ) *of zinc complexes of verdins after oxidation with iodine in ethanolic zinc acetate.*

Biliverdin	640—637·5	580	510—515
Verdinoid isomerisation product	632·5—635	580—585	515
Dihydrobiliverdin	632·5—635	580—585	515
Glaucobilin	630	585	515
Glaucobilin XIIIα	630—632·5	585	515

Optical densities of the maxima at 580—585 and 515 mμ increased, while that at 630—640 mμ decreased with time. The positions of the maxima at 580—585 and 510—515 mμ were not exactly reproducible and depended on concentration and time.

The zinc complexes of the different verdins could also be distinguished by determination of the red absorption maximum after oxidation of the complex with iodine (Table 4; Fig. 2, curve 3). Initially there was a red fluorescence in visible or ultraviolet light; this

rapidly changed through orange to lime-green, seen only in ultraviolet light. Such solutions had intense absorption peaks at about 635 mμ (stabilised by ammonia) and 515 mμ, with a subsidiary maximum at 580 mμ. The second of these maxima is due to choletelin formation; the others arise from violinoid pigments.

Similar maxima appeared on addition of iodine to aged solutions of zinc complexes of verdins (Fig. 2, curve 4), showing contamination by violinoid pigments.

The observations described are consistent with scheme A or B. The former would provide a verdin identical with that formally derived by ferric chloride oxidation of dihydrobilirubin. Dihydrobiliverdin thus obtained differs, however, from the verdinoid isomerisation product in the change of light absorption with pH.

Experimental

Ether was freed from peroxide by treatment with ferrous sulphate, and chloroform from acid by shaking it with potassium carbonate; the solvents were then distilled. The light petroleum used had b. p. 40—60°. Nitrogen was passed through alkaline sodium dithionite for the complete removal of oxygen.[17] Absorption spectra in the range 250—700 mμ were determined on the Hilger Uvispek Spectrophotometer of type H. 700, calibrated as recorded in Part II of this series.

Bilirubin.—The bilirubin used [ε = 55,600 at 450 mμ in chloroform; $\lambda_{max.}$ of iodine-oxidised zinc complex 639, 585, and 516 mμ (lit.,[18] 543 and 585 mμ] was purified as described in the preceding paper; special care was taken to ensure that the pigment was free from meso-impurities, which was demonstrated by oxidation as described below.

Dihydrobilirubin. Bilirubin (785 mg.; prepared from pig's bile) in 0·1N-sodium hydroxide (75 ml.) was treated with hydrogen in the presence of 10% palladium–charcoal (120 mg.) until absorption ceased. A further quantity of catalyst (120 mg.) was added and reduction was continued until 34·9 ml. of moist hydrogen at 21°/759 mm. had been absorbed. After acidification to Congo Red with 2·8N-hydrochloric acid, the product was separated by centrifugation and triturated with cold methanol. The catalyst was removed by dissolution of the pigment in boiling chloroform and filtration. Crystallisation from chloroform gave four crops. The two middle crops, united and recrystallised from chloroform (236 mg., 30%), had m. p. 310—315° (lit.,[19] m. p. 315°), ε 49,900 at 442 mμ in $CHCl_3$. The absorption maximum for the iodine-oxidised zinc complex in methanol was at 685, 586, and 515 mμ (lit.,[19] 639·5, 583·9 mμ).

Mesobilirubin. Bilirubin (0·5 g.) in 0·1N-sodium hydroxide (25 ml.) was shaken with hydrogen in the presence of 10% palladium–charcoal (1 g.) until the absorption ceased (3 hr.). After acidification with glacial acetic acid, the mixture was extracted with chloroform. The catalyst was separated and extracted with boiling chloroform. The united chloroform solution was green-yellow and gave no Ehrlich reaction; on evaporation it afforded a solid which crystallised poorly from chloroform. Sufficient chloroform (40 ml.) was added to redissolve the pigment which was then precipitated as a green-yellow mass by pouring the solution into stirred light petroleum. The crude substance was crystallised once from pyridine and then from glacial acetic acid, much verdinoid pigment remaining in the mother-liquors. The twice crystallised product was washed freely with alcohol for removal of further verdin and was recrystallised from chloroform, to give mesobilirubin (0·17 g., 34%), m. p. 315—320° (darkening from 250°) (lit.,[20] m. p. 315°), ε = 54,600 at 433—434 mμ in chloroform; $\lambda_{max.}$ of the iodine-oxidised zinc complex in methanol were at 626, 597, and 513 mμ (lit.,[19] 627·5, 576·2 mμ).

In a second preparation the yield was 58%.

Biliverdin, Dihydrobiliverdin, and Glaucobilin.—These were obtained by ferric chloride oxidation of the corresponding rubins in methanol. Because of the ease of further oxidation, conditions had to be strictly controlled. The method has been described in detail for biliverdin in Part II of the series.[1] All three pigments were obtained crystalline. Glaucobilin was also obtained by ferric chloride oxidation of mesobilirubinogen as described in the following paper.

[17] Linstead, Elvidge, and Whalley, "Modern Techniques of Organic Chemistry," Butterworths, London, 1955, p. 139.
[18] Ref. 2, Vol. II, p. 632.
[19] Ref. 2, Vol. II, p. 640.
[20] Ref. 3, p. 120, ref. 2, p. 650.

Zinc Complexes of the Verdins.—These were prepared by dissolving the pure pigment in a saturated ethanolic solution of zinc acetate at room temperature. Light absorption was measured within 10 min. in a stoppered quartz cell (1 cm.). Two drops of 1% ethanolic iodine and one drop of concentrated aqueous ammonia were added to the solution in the cell; absorption was determined exactly 10 min. after addition of iodine (Fig. 2, curve 3). A second portion of the solution of zinc complex was left for 2 hr. and its absorption was again determined (Fig. 2, curve 2). Absorption of this solution was redetermined 10 min. after addition of the iodine solution and ammonia as before (Fig. 2, curve 4). Similar results were obtained with zinc complex solutions prepared from crude verdins known to be contaminated with violinoid pigments.

Oxidation of bile pigments by nitric acid. A suspension of the pigments (20 mg.) in water (1 ml.) was treated with concentrated nitric acid (2 ml.) and left for 48 hr. at 23° in the dark. Water (5 ml.) was added and the solution extracted with ethyl acetate (4 × 8 ml.). The extract was washed with water (3 × 5 ml.), and evaporated in a stream of air at 23°. The residue was dissolved in water (3 ml.), made alkaline (pH 8·5) by addition of sodium hydrogen carbonate, and extracted with chloroform (4 × 8 ml.). These extracts were washed as before, dried by filtration, and evaporated at room temperature, affording a neutral fraction (A). The alkaline aqueous residue was acidified with 11N-hydrochloric acid and again extracted with chloroform, and the extract was washed and dried. Evaporation afforded an acid fraction (B).

For identification of the products paper chromatography with descending development on Whatman No. 2 paper with 16 : 3 : 1 ethanol–water–ammonia (*d* 0·88) was used. The products (0·01 mg.) were applied to the paper in methanol. The developed chromatogram was momentarily immersed in 1 : 1 acetone–ethanol, blotted, and immersed in a tank containing chlorine. After removal of chlorine by aeration and immersion of the paper in a saturated solution of *o*-tolidine in 2N-acetic acid to which an equal volume of 0·05N-potassium iodide had been added, the imides appeared as blue spots.[21] Authentic hæmatinimide and ethylmethylmaleinimide were used as markers; they had R_F 0·56 and 0·83 respectively.

Hæmatinimide was found in all the acid fractions (B). The neutral fractions (A) from the verdinoid isomerisation product, dihydrobiliverdin, mesobiliverdin, crude commercial bilirubin, dihydrobilirubin, and d-urobilin contained ethylmethylmaleinimide; fractions (A) from biliverdin, bilirubin from pig's bile, and purified commercial bilirubin did not contain this imide.

Oxidation of the verdinoid isomerisation product on a larger scale (50 mg.) gave crystalline ethylmethylmaleinimide, m. p. 68° (lit.,[22] m. p. 68°), from the neutral fraction.

Isomerisation of Bilirubin to Verdin by Treatment with Sodium Ethoxide in Absence of Air.—Through a solution of pure bilirubin (50 mg.) in ethanol (10 ml.) nitrogen was passed for 10 min. to expel air. 1·22N-Sodium ethoxide (10 ml.) was then introduced and the mixture was boiled under reflux in the oxygen-free atmosphere. After about 3 hr. the orange-brown colour of bilirubin had changed to olive-green and later to deep green. The cooled mixture was diluted with water (75 ml.), acidified with glacial acetic acid (10 ml.), and shaken with ether (250 ml.). The ethereal layer was exhaustively extracted with N- and then 2·8N-hydrochloric acid; the two acid fractions had absorption maxima at 340—360, 650—680 mμ (the exact position depended on the concentration of the pigment), and 385, 660 mμ, respectively. The acid fractions were separately extracted with chloroform. All these operations were performed under nitrogen. The extracts were washed with water (4 × 20 ml.), dried by filtration, and evaporated to dryness *in vacuo*. The two fractions were chromatographed separately on magnesia. Biliverdin was first eluted with 1 : 1 light petroleum–chloroform, leaving a small amount of residual bilirubin on the column which was later eluted with chloroform. The yields of verdin were determined spectroscopically both in chloroform and in 5% w/v methanolic hydrogen chloride, molecular extinction coefficients being assumed as for biliverdin (Table 2). The results calculated from the two maxima in the two solvents were almost identical and an average value of these was taken. The ethereal layer was washed with water, dried by filtration, and evaporated *in vacuo*. The residue, dissolved in chloroform, was united with the bilirubin fractions eluted from the columns and was immediately analysed for residual bilirubin. The results of a series of these experiments in which bilirubin was refluxed for variable times are tabulated.

[21] Ref. 17, p. 11.
[22] Muir and Neuberger, *Biochem. J.*, 1949, **45**, 164.

Refluxing (hr.)	Verdinoid isomerisation product (%)			Residual bilirubin (%)	Pigment accounted for (%)
	in N-acid	in 2·8N-acid	Total		
0·0	0	0	0	96	96
0·5	—	14	14	86	100
1·0	26	4	30	64	94
6·0	53	—	—	29	
8·0	67	8·6	76	16	92
8·0	69	4	73	14	87

If contact with air is not restricted to a minimum and the extraction is not performed rapidly, then the amount of pigment which requires extraction with 2·8N-acid increases and the total amount of verdin is considerably reduced.

Comparative Behaviour of Bilirubin and Mesobilirubin towards Sodium Ethoxide.—Bilirubin (58 mg.) and mesobilirubin (60 mg.) were separately treated, as described above, for 8 hr. with 1·22N-sodium ethoxide, the yields of verdinoid products being 75% and 7% respectively. No visible verdin was formed from bilirubin or mesobilirubin on similar treatment with methanol alone; however, extraction of these solutions by the procedure outlined above resulted in isolation of some verdin, undoubtedly arising by oxidation; the yield was greater in the case of mesobilirubin than of bilirubin.

Effect of Alkoxides on the Isomerisation of Bilirbin.—A standard solution of bilirubin in chloroform (0·0698 g./100 ml.) was prepared; 3 ml. portions were measured into five similar tubes and evaporated almost to dryness *in vacuo*. Approximately 3% solutions of sodium methoxide, sodium ethoxide, sodium isopropoxide, and potassium t-butoxide were prepared in corresponding alcohols and, together with aqueous 3% sodium hydroxide, were titrated against standard hydrochloric acid. An amount of each, equivalent to 2 ml. of 1·22N-solution/10 mg. of bilirubin, was added to each tube and the solutions were made up to 15 ml. with the appropriate alcohol. The solutions were stored under nitrogen in the dark at room temperature. After 6 days the solutions were diluted with water (10 ml.), the excesses of alkoxide were neutralised by glacial acetic acid (2 ml.), and the pigments were extracted into peroxide-free ether (40 ml.). The ether solutions were washed with water (10 ml.) and extracted with 2·8N-hydrochloric acid (14 ml.). Pigment in the acid extract was taken into chloroform, washed with water, dried by filtration, and diluted to 20 ml., and its verdinoid content was determined spectroscopically, ε values being assumed to be those of biliverdin. The acid-extracted ether containing residual bilirubin was washed with water, dried by filtration, and evaporated *in vacuo*; the residue was dissolved in chloroform (20 ml.). This residual bilirubin was estimated spectrophotometrically 1 hr. after dissolution of the ether residue and when most of the bilirubin had been oxidised by atmospheric air to biliverdin. Remaining bilirubin was estimated by spectrophotometry at 450 mμ, and the resulting biliverdin at 375 mμ. The results are recorded in Table 1.

We gratefully acknowledge grants for expenses and scientific assistance from the Medical Research Council.

THE DEPARTMENT OF CHEMICAL PATHOLOGY, KING'S COLLEGE HOSPITAL MEDICAL SCHOOL, LONDON, S.E.5. [*Received, October 20th*, 1960.]