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SEPARATION AND IDENTIFICATION OF BILIVERDIN ISOMERS AND ISOMER ANALYSIS OF PHYCOBILINS AND BILIRUBIN

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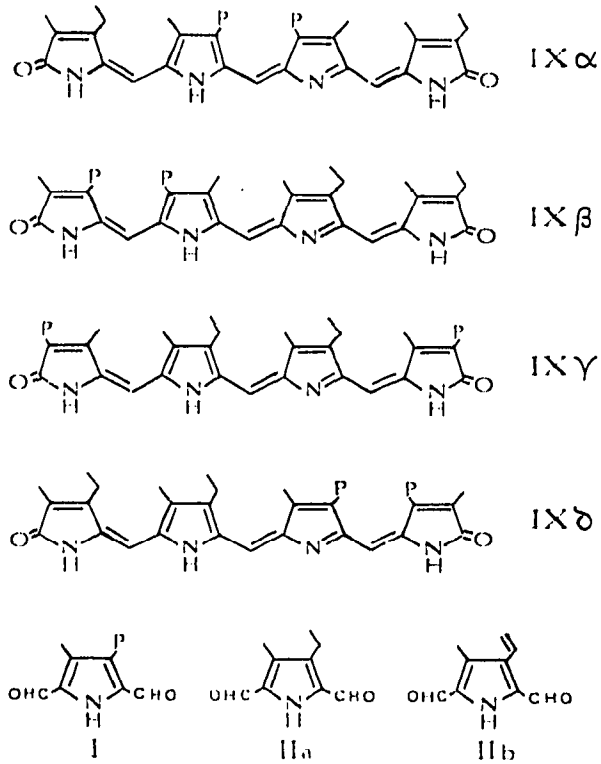
SUMMARY

One-dimensional thin-layer chromatographic systems are described for the resolution of the IXα, IXβ, IXγ and IXδ isomers of protobiliverdin and mesobiliverdin, and each isomer is unambiguously identified. The separations may be applied on a preparative scale or as a micro-scale method for isomer analysis of biliverdin preparations or of bilins which can be converted thereto. Besides being very sensitive and reliable, the analytical method also yields much more information than degradative methods. Applied to some naturally occurring bilins, it shows that bilirubin from pig bile is about 99.6% IXα with traces of IXδ and of IXβ and no IXγ. Phycoerythrobilin and phycocyanobilin seem to be entirely IXα. Mesobiliverdin IXα, prepared from bilirubin by the usual alkaline reduction followed by dehydrogenation, contains two extra minor, seemingly isomeric mesobiliverdins. A new method of preparation, avoiding alkaline reduction, yields only true mesobiliverdin IXα.

INTRODUCTION

Biliverdins are derived formally from haems by oxidative removal of a methine bridge and loss of the iron atom. The asymmetric nature of haem IX results in the possibility of four different biliverdin isomers, each corresponding to the removal of a different methine bridge and named accordingly. Thus the IXα, IXβ, IXγ and IXδ isomers illustrated in scheme 1 correspond respectively with removal of the α, β, γ and δ bridges of mesohaem. Such isomerism is of importance in studying haem and bilin metabolism (*cf.* refs. 1 and 2).

Until recently the only method available for investigating the isomeric nature of bilins was alkaline permanganate degradation[3,4], a destructive method requiring about 6–10 mg of material, an amount not readily available from most of the processes we have been studying. We have found that results obtained with smaller quantities of material are unreliable[5]. Moreover, only the pure IXα and IXγ isomers can be distinguished by this technique; the IXβ, IXδ, and all mixtures of isomers are indistinguishable. The recently introduced dichromate degradation technique of



Scheme 1. Structures of the mesobiliverdin isomers (IX α, β, γ, δ) and of pyrrole dialdehydes I, IIa, and IIb. In the protobiliverdins the ethyl groups are replaced by vinyl groups as in pyrrole dialdehyde IIb. P represents a methyl propionate side-chain in the esterified compounds (or propionic acid side-chain in the free pigments).

RÜDIGER[6-8] is far more sensitive and reliable, but it suffers from the same ambiguity with samples other than pure IXα or IXγ. Furthermore, degradative methods do not distinguish isomers of the IX series uniquely (from those of the III series for example).

NICOL AND MORELL[9] have recently described the combined use of mass spectrometry and NMR as a method of isomer analysis, but the technique is complex, requires relatively large quantities of material, and the results seem to be open to serious misinterpretation (see below).

RÜDIGER[6,7] has briefly described a method of isomer analysis applicable to deutero- and proto-biliverdins and based on thin-layer chromatographic separation of the isomers, but the IXβ and IXδ isomers were not distinguished and the system described for the protobiliverdins is a two-dimensional one, inconvenient for comparative or preparative work.

We describe here one-dimensional chromatographic methods which allow the complete resolution of the four isomers of both protobiliverdin IX and mesobiliverdin IX either on a preparative scale or as a microanalytical method, and we identify each isomer unequivocally. The method is applied here to the isomer analysis of biliary bilirubin and the phycobilin prosthetic groups of the algal biliproteins. We have also used the technique successfully in studying the specificity of coupled oxidation of haemproteins[1] and the specificity of biliverdin reductase[10].

MATERIALS AND METHODS

General

Crystalline protohaemin was obtained from Sigma. Mesohaemin was prepared by catalytic hydrogenation of protohaemin over palladium[11] followed by reinsertion of the iron atom into the mesoporphyrin formed[12,13]. Bilirubin IXα (twice crystallized) was obtained from Sigma. Its isomeric purity was checked by the pH 1.7 dichromate degradation technique of RÜDIGER[6,8]. Both the dichromate degradation technique and the chromic acid degradation (to imides) were performed by the "on the plate" technique and the pyrrole dialdehydes and imides were separated by TLC, detected and identified as described by RÜDIGER[6,8].

Handling, esterification and TLC of biliverdins

Biliverdins and other bilins are prone to oxidative and other modifications and throughout all procedures they were kept as far as possible under nitrogen and were stored at 2° in the dark. It is essential to ensure that all solvents used are completely free of peroxides.

Conversion to the dimethyl esters was achieved by dissolving the biliverdins in ice-cold 5% H_2SO_4 in methanol, the solutions being allowed to stand for 12–16 h at 0–4° under nitrogen. (Esterification of bilins at more elevated temperatures should be avoided as it may lead to formation of artifacts[7].) The mixtures were then diluted with four volumes of water and extracted with chloroform. The chloroform extracts were washed with distilled water until the washings were no longer acid (four washings with four volumes of water usually sufficed). The chloroform solutions were then evaporated to dryness under a stream of nitrogen and the pigment was dissolved in a drop of acetone for application to the thin-layer plates. (When the chloroform solutions were not washed sufficiently to remove all the acid, it was found that the pigment remained at the origin during subsequent chromatography. This could usually be overcome by drying the plate, spotting a little concentrated ammonia onto the biliverdin spot, drying off under a stream of nitrogen, and rechromatographing.)

Thin-layer chromatography was carried out on 20-cm plates coated with 0.25-mm layers of Silica Gel G (Merck) activated at 100° for at least 1 h. The chromatographic systems developed are sufficiently insensitive to deactivation of the silica gel to permit multiple development. It is necessary, however, during multiple development with those solvent systems containing acetic acid to ensure that the latter is well dried off in a stream of cool air between each development. The 13% gypsum present in the Silica Gel G (originally as a binder) appears to be essential for the separations reported here. Pre-coated silica gel plates containing other binders, or no binder, gave very poor results.

Conversion of proto- to mesobiliverdins

It was found that reduction of the vinyl side chains of protobiliverdins could be carried out specifically on a micro-scale by hydrogenation over palladium in formic acid. (Other methods of reduction reduced the central methine bridge preferentially or concomitantly, yielding bilirubins.) The protobiliverdin (*ca.* 20–50 μg as the dimethyl ester or the free diacid) was dissolved in 99–100% formic acid (1 ml)

in a stoppered test-tube. Palladium black (4 mg) was added, hydrogen was bubbled through and the tube (filled with hydrogen) was stoppered and shaken at 40° for 10 min (until the color changed from green to blue). Water (6 ml) was added and the pigment extracted into chloroform which was then washed with 10% sodium acetate (10 ml twice) followed by water (10 ml twice) and evaporated to dryness.

Biliverdin preparations

Standard protobiliverdin IXα dimethyl ester was prepared by dehydrogenation of bilirubin IXα by the ferric chloride–glacial acetic acid method[14] followed by esterification. Standard mesobiliverdin IXα dimethyl ester was prepared by catalytic hydrogenation of protobiliverdin IXα dimethyl ester as described above. Mesobiliverdin preparations were also prepared from bilirubin IXα by catalytic hydrogenation to mesobilirubin or amalgam-reduction to mesobilirubinogen in aqueous alkali[15] followed by ferric chloride dehydrogenation and esterification. The latter type of preparation is termed mesobiliverdin IXα type II and was found to be inhomogeneous (see RESULTS AND DISCUSSION). Synthetic mesobiliverdin IXβ dimethyl ester[16] was kindly supplied by Professors G. W. KENNER and A. H. JACKSON.

Coupled oxidation of protohaemin with ascorbate was carried out as described by LEVIN[17], and of mesohaemin by mixing 30 mg mesohaemin in 50 ml pyridine with 100 mg sodium ascorbate in 150 ml water and incubating under an atmosphere of oxygen at 60°, with further additions of ascorbate if necessary, until the solution turned green (30–60 min). This procedure was necessary as mesohaemin was much more resistant to coupled oxidation than was protohaemin. The resulting verdohaemochromes were extracted as described by LEVIN[17] and were converted to biliverdins and at the same time esterified by following the standard esterification procedure.

Conversion of some naturally occurring bilins for isomer analysis

Phycocyanobilin, the blue chromophore of phycocyanins, was partly released from the apoprotein and simultaneously isomerized to mesobiliverdin[18,19] by heating 40 mg of C-phycocyanin (isolated from *Nostoc muscorum*[20]) in 1 *N* KOH (4 ml) at 100° for 5 min. 11 *N* HCl (8 ml) was then added and the mixture was allowed to stand at 40–50° for 2 h to complete the hydrolytic release of the pigment from the protein. The mixture was diluted with water (32 ml) and extracted with chloroform (10 ml three times), into which practically all the pigment went. The combined chloroform extracts were washed with water (20 ml) and evaporated to dryness, and the pigment was esterified for chromatography in 5% methanolic H_2SO_4 as usual.

Phycoerythrobilin, the red chromophore of phycoerythrins, was also isomerized to mesobiliverdin by similar KOH treatment of R-phycoerythrin (isolated from *Ceramium rubrum*[20]). The pigment was similarly released from the apoprotein, and esterified. Prior to analysis it was submitted to preliminary TLC in carbon tetrachloride–ethyl acetate (2 : 1) to remove traces of artifact pigments formed by modifications of the vinyl group initially present in phycoerythrobilin before its isomerization.

Biliary bilirubin for isomer analysis was prepared according to the method of OSTROW *et al.*[21] but the fractionation procedures were cut to a minimum to avoid possible loss of isomers present in small amounts. All washings were re-extracted

and any bilirubin recovered was returned to the bulk of the bilirubin. Crystallization steps were omitted. The total crude bilirubin was then converted to biliverdin by refluxing with ferric chloride in glacial acetic acid[14] and the biliverdin was esterified for TLC in the usual manner. Certain non-bilin impurities came through the limited fractionation procedures and were removed by preparative chromatography on Silica Gel G in carbon tetrachloride–ethyl acetate (2 : 1)[19]. (This solvent moved protobiliverdins as a homogenous band ahead of the impurities, with no fractionation of the isomers.) The biliverdin band was scraped off, eluted into acetone, concentrated and used for isomer analysis.

RESULTS AND DISCUSSION

Separation of the isomers

Bilins are, in general, more easily chromatographed as the diesters than as the free diacids. The dimethyl esters of the biliverdins were therefore used for TLC throughout this investigation. Silica Gel G was found to be the most suitable adsorbent; as mentioned above, the calcium sulfate present as binder plays an important role in the separations reported here and cannot be omitted.

The esterified protobiliverdin and mesobiliverdin preparations, produced by coupled oxidation of pyridine haemochromes followed by treatment of the resulting verdohaemochromes with acid methanol, were used to search for suitable chromatographic systems, as it had been shown[6,22] that such a preparation of protobiliverdin is a mixture of isomers and it was anticipated that the same would be true of the mesobiliverdin preparation.

The mesobiliverdin preparation was indeed eventually separated into four blue components by multiple development in one dimension with either of the solvent systems A, B or C listed in Table I. Solvent A gave the most consistent results and was adopted for further studies although it requires about eight-fold development for full resolution (Fig. 1A, position 1). Sharper resolution was obtained by multiple development than by continuous (open ended) TLC. The two top spots were the most difficult to resolve with all solvent systems, and the composition given for solvent system A seems to be optimal, and rather critical, for this separation.

The corresponding protobiliverdin preparation was separated into four green components by triple development in one dimension in solvent system D (Fig. 1B, position 1) or by double development in solvent E (Table I). Solvent D was marginally

TABLE I

SOLVENT SYSTEMS DEVELOPED FOR TLC SEPARATION OF BILIVERDIN ISOMERS, AS THE DIMETHYL ESTERS, ON SILICA GEL G

For mesobiliverdins	
(A)	Carbon tetrachloride–ethyl acetate–cyclohexane (32:9:4)
(B)	Carbon tetrachloride–ethyl methyl ketone (4:1)
(C)	Toluene–carbon tetrachloride–ethyl methyl ketone (5:5:2)
For protobiliverdins	
(D)	*n*-Heptane–ethyl methyl ketone–glacial acetic acid (10:5:1)
(E)	Toluene–ethyl methyl ketone–glacial acetic acid (10:5:0.5)

more reproducible, and was used thereafter. The slight "bearding" effect observable in Fig. 1 becomes progressively worse when the plates are overloaded, but at concentrations of biliverdin sufficient to give good visible spots the effect is not troublesome, and when bands rather than spots are applied only the ends of the bands are affected.

Identification of the isomers

The results establishing the identities of the separated isomers are summarized in Fig. 1. The separated spots were subjected to RÜDIGER's pH 1.7 dichromate degradation technique[6,8] as illustrated in Fig. 2. The pyrrole dialdehydes produced from each spot are indicated on the left of Fig. 1. As reported by RÜDIGER[6,8], only the two internal pyrrole rings of bilins yield dialdehydes; the outer rings yield maleimides. Therefore, the production of I as the only dialdehyde from the front mesobiliverdin spot (see Fig. 1A) identifies this as the IXα isomer, since this is the only isomer both of whose internal rings carry a propionate side-chain (see scheme 1). This isomer is also identified by its chromatographic identity with the authentic IXα preparation (Fig. 1A, position 2). The production of only dialdehyde IIa from the second spot identifies this as the IXγ isomer (see scheme 1). The remaining two spots yield both pyrrole dialdehydes I and IIa indicating that they are the IXβ and IXδ

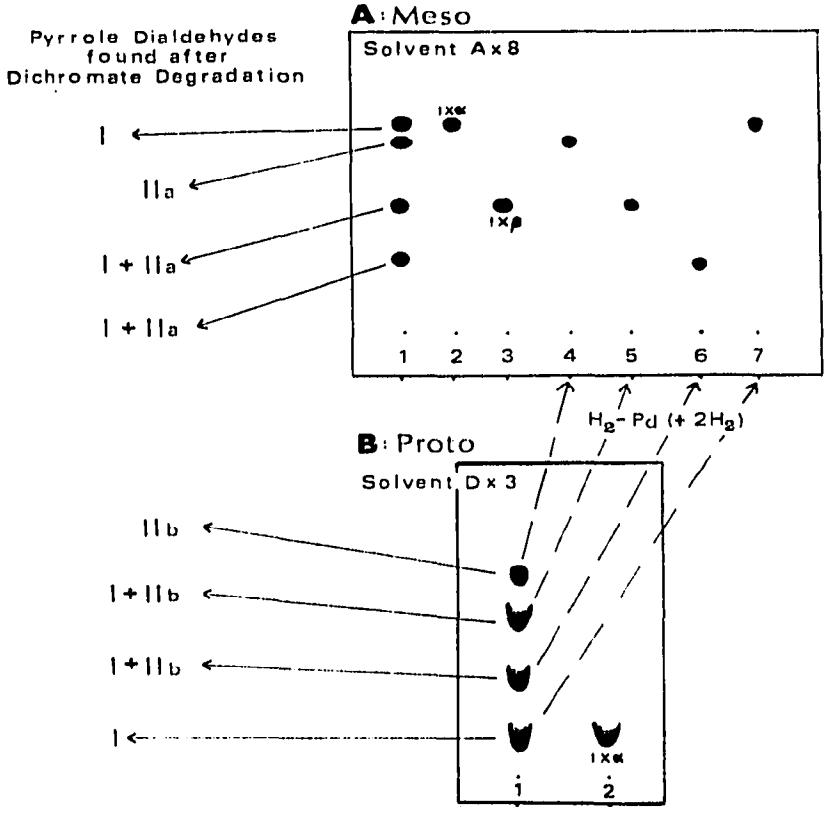


Fig. 1. Separation of (A) mesobiliverdins and (B) protobiliverdins as the dimethyl esters, and identification of the individual isomers (see text). For solvent systems see Table I; for structures of the pyrrole dialdehydes see Scheme 1. (1) Preparations obtained after coupled oxidation of haemins; (2) authentic biliverdin IXα dimethyl ester preparations (A,2 prepared by hydrogenation of B,2 in formic acid); (3) mesobiliverdin IXβ dimethyl ester; (4, 5, 6 and 7) separated isomers of B,1 hydrogenated to mesobiliverdin in formic acid.

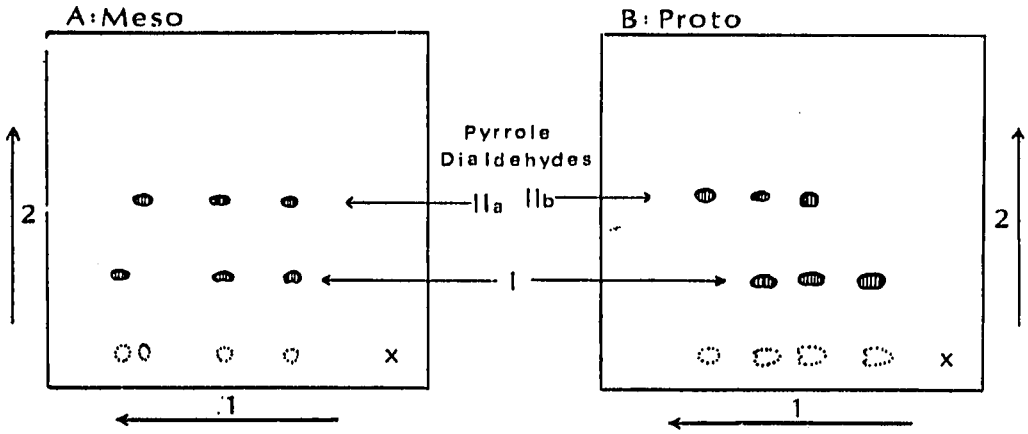


Fig. 2. Dichromate degradation of (A) separated meso- and (B) separated protobiliverdin isomers by the on-the-plate method of RÜDIGER[6,8]. The biliverdin ester mixtures were applied at ×, and, by chromatography in direction 1 as in Fig. 1, they were separated into the individual isomers (areas bounded by dotted lines). A 1% aqueous, pH 1.7 dichromate–acetic acid (2:1) mixture was carefully applied over each biliverdin spot and after 1 h the degradation products were separated by chromatography in direction 2 with carbon tetrachloride–ethyl acetate–cyclohexane (5:3:1). After evaporation of the solvent the pyrrole dialdehydes were visualized as red-brown spots (hatched areas) by spraying with dinitrophenylhydrazine[6,8]. The indicated identifications of the dialdehydes were confirmed by running standards (not shown) in direction 2.

isomers but without distinguishing between them. However, the synthetic mesobiliverdin IXβ dimethyl ester provided by Professors KENNER and JACKSON is chromatographically identical with the third mesobiliverdin spot (Fig. 1A, position 3; the chromatographic identity was confirmed by co-chromatography in solvents A, B and C). Thus the third spot is the IXβ isomer and the bottom one must then be the IXδ isomer.

Of the four protobiliverdin spots (Fig. 1B) the last spot corresponds with authentic protobiliverdin IXα dimethyl ester. Dichromate degradation (on the basis of the same reasoning as applied above to the mesobiliverdins) confirms the identity of the last spot as the IXα isomer and shows that the front spot is the IXγ and the middle two spots are the IXβ and IXδ isomers.

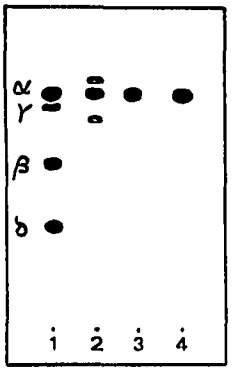
The catalytic hydrogenation procedure, described in MATERIALS AND METHODS, enabled the direct conversion of the separated protobiliverdin isomers (after elution from preparative-scale plates) to mesobiliverdins and thus allowed a correlation of the isomers of the two biliverdin series as illustrated in Fig. 1 (arrows from B to positions 4 to 7 of A). The second protobiliverdin spot (from the front) was thus identified unambiguously as the IXβ isomer and the third as the IXδ. The confirmatory cross-correlation of the IXα and IXγ isomers of the two series also adds further weight to the identifications.

The identities of the isomers are summarized in Figs. 3 and 4.

The separations may be carried out on a preparative scale on thick layers of Silica Gel G and, after elution from the silica gel with acetone, the isolated pigments may be converted to the free diacids by hydrolysis in 10 *N* HCl.

Isomer analysis

Micro-analysis of the isomer composition of a bilin preparation based on the



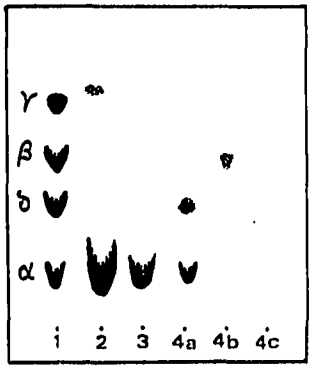


Fig. 3. Mesobiliverdin dimethyl ester preparations separated by eight-fold multiple development with solvent system A (carbon tetrachloride–ethyl acetate–cyclohexane, 32:9:4). (1) Standard preparation obtained after coupled oxidation of pyridine mesohaemochrome showing identities of isomers; (2) mesobiliverdin IXα, type II (see text); (3 and 4) mesobiliverdin prepared respectively from C-phycocyanin and from R-phycoerythrin.

Fig. 4. Protobiliverdin dimethyl ester preparations separated by three-fold multiple development with solvent system D (*n*-heptane–ethyl methyl ketone–glacial acetic acid, 10:5:1). (1) Standard preparation obtained after coupled oxidation of pyridine protohaemochrome, showing identities of the isomers; (2) preparation obtained from one post-mortem human bile sample overloaded to show trace of artifact biliverdin; (3) preparation from crude bilirubin from fresh pig bile; (4a, b and c) rechromatography of concentrated eluates from: (a) the IXδ region, (b) the IXβ region, and (c) the IXγ region, scraped from a number of plates heavily overloaded with bands of the preparation from crude pig bilirubin.

above TLC systems may be achieved if the bilin can be converted to proto- or mesobiliverdin. The methylated biliverdin is chromatographed alongside a mixed-isomer standard (prepared by coupled oxidation and esterification of the appropriate haemin) and the isomer(s) are thus identified. A rough indication of the relative proportions of the isomers may be obtained by visual inspection of the chromatograms. Reflectance densitometry using a red filter and calibrating with different quantities of the appropriate biliverdin may be used to obtain accurate figures. Alternatively, the sample is applied as a band and, after separation, the isomer zones are scraped off and extracted twice with acetone, the silica gel being removed completely by centrifugation. The extracts are made up to a fixed volume (1–2 ml) with acetone, the absorbancies read at $\lambda_{max.}$ and the relative proportions of the isomers calculated (assuming equal extinction coefficients for the four isomers). The latter method yields results which are reproducible to within about 5% except where a particular isomer is present in large excess over the others. About 50 μg of the biliverdin preparation is required for this method, while less than 5 μg is sufficient if a suitable reflectance densitometer is available.

Besides the green or blue biliverdin spots, faint red-purple and yellow spots due to artifact oxidation products (not shown in the figures) can usually be seen. As these do not absorb in the same spectral region as the biliverdins they do not interfere significantly with the estimations even where they coincide with a biliverdin spot.

NICOL AND MORELL[9] interpreted results obtained by a combination of mass spectrometry, NMR and alkaline permanganate degradation to indicate that the protobiliverdin produced by coupled oxidation of pyridine haemochrome with

ascorbate consisted of a single isomer—either the IXβ or IXδ. Since our results and those of RÜDIGER[6] indicate unequivocally that the product is a mixture of all four isomers, the validity of isomer analyses obtained by the methods of NICOL AND MORELL must therefore be open to doubt. However their deductions concerning the isomeric composition of phycoerythrobilin[9] are consistent with ours (see below).

Other methods of isomer analysis are dealt with in INTRODUCTION.

The phycobilins and bilirubin

The esterified mesobiliverdin prepared from both C-phycocyanin and R-phycoerythrin was found to consist entirely of the IXα isomer (Fig. 3 and Table II),

TABLE II

ISOMER COMPOSITION OF NATURALLY OCCURRING BILINS

Original bilin	*Converted to*	*% isomer composition*			
		IXα	*IXβ*	*IXγ*	*IXδ*
Phycocyanobilin	mesobiliverdin	100	0	0	0
Phycoerythrobilin	mesobiliverdin	100	0	0	0
Biliary bilirubin	protobiliverdin	*ca.* 99.6	*ca.* 0.1	0	*ca.* 0.3

no trace of any other isomer being detectable even when the plates were overloaded. Dichromate degradation confirmed this—only pyrrole dialdehyde I was obtained and no trace of IIa. Dichromate degradation of phycocyanobilin and phycoerythrobilin themselves (isolated as described in ref. 19) also yielded only pyrrole dialdehyde I. These results confirm that both these phycobilin prosthetic groups are entirely of the IXα configuration.

Protobiliverdin obtained by dehydrogenation of bilirubin from fresh pig bile at first seemed to consist entirely of the IXα isomer (Fig. 4, position 3), but when crude non-crystalline bilirubin (which had been submitted to a minimum of fractionation during its isolation) was used, and when the TLC plates were heavily overloaded with bands of the esterified biliverdin, a faint trace of some biliverdin in the IXδ position was detectable. The regions corresponding to the IXδ, IXβ and IXγ isomers were scraped off, eluted, concentrated and reapplied as spots on a new TLC plate as illustrated in Fig. 4 (positions 4a, b and c). In this way the occurrence of trace quantities showed up much better. The presence of a trace of the IXδ isomer was verified (still contaminated with some IXα owing to spreading on the overloaded preparative plates) and also an even smaller trace of the IXβ isomer. No biliverdin IXγ whatever could be detected. The proportion of the IXδ to total IXα was roughly estimated to be about 1 : 300 or 1 : 400, and of IXβ to IXδ about 1 : 3, giving the approximate figures listed in Table II. At this trace level it is not possible to give accurate figures and the risk of interference by trace quantities of artifacts is considerably increased. However it seems certain that bilirubin from pig bile consists of over 99.5% of the IXα isomer. PETRYKA[23], using the alkaline permanganate technique applied to large quantities of bilirubin (200 mg), previously obtained evidence that bilirubin from dog bile was not entirely the IXα isomer and that the

early labeled bilirubin fraction contained a higher proportion of other isomer(s). No indication of the nature or proportions of the other isomers could be obtained with that technique, however. Since early labeled bilirubin represents about 10% of the total bilirubin[24,25], the low level of non-IXα isomers which we find in the total bilirubin indicates that even the early labeled fraction must be at least 95% IXα.

Some unidentified biliverdin components

Some interesting biliverdin artifacts which we have encountered could cause confusion. Fig. 3 shows the pattern obtained with "type II" preparations of mesobiliverdin IXα dimethyl ester (see MATERIALS AND METHODS) prepared by the usual methods of preparing this substance—dehydrogenation of mesobilirubin or mesobilirubinogen obtained by alkaline reduction of bilirubin IXα. As illustrated in Fig. 3 (position 2), such preparations are resolved into two additional minor mesobiliverdin components which do not correspond with any of the isomers discussed above, besides a major spot corresponding with the single mesobiliverdin IXα spot observed in the other preparations (Figs. 1 and 3).

The three components were isolated on a preparative scale. Chromic acid degradation showed that all three had identical side-chain complements and they were also similar spectrally. The structural basis of the two minor components is therefore unclear and may possibly be due to some previously undetected type of isomerization, which presumably takes place during the alkaline reduction step, since the mesobiliverdin prepared from the same bilirubin, but avoiding such a step, does not contain the minor components (Fig. 1A, position 2). RÜDIGER and PAULMANN (unpublished results, quoted in ref. 7) have independently observed inhomogeneity in mesobiliverdin IXα prepared from mesobilirubinogen and have also suggested that an unknown type of isomerization may be responsible.

In the protobiliverdin series artifacts may arise readily by modifications of the labile vinyl side-chains, *e.g.* by methoxylation during esterification[7] or by hydroxylation or oxidation, but if the precautions outlined in MATERIALS AND METHODS are observed, such modifications are avoided. However, in one post-mortem sample of human bile, we observed a trace of a modified IXα derivative despite maximum precautions. This trace material could only be detected when the plates were overloaded (Fig. 4, position 2) and it ran just in front of the IXγ isomer, with which it might easily be confused. Sufficient material was available only for limited degradative studies and these indicated that it was a derivative of biliverdin IXα apparently with a modified (but not methoxylated) vinyl side-chain. It was not found in preparations from fresh biliary bilirubin and it may have been due to bacterial action.

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