Mechanism and Stereochemistry of the Porphobilinogen Deaminase and Protoporphyrinogen IX Oxidase Reactions: Stereospecific Manipulation of Hydrogen Atoms at the Four Methylene Bridges during the Biosynthesis of Haem

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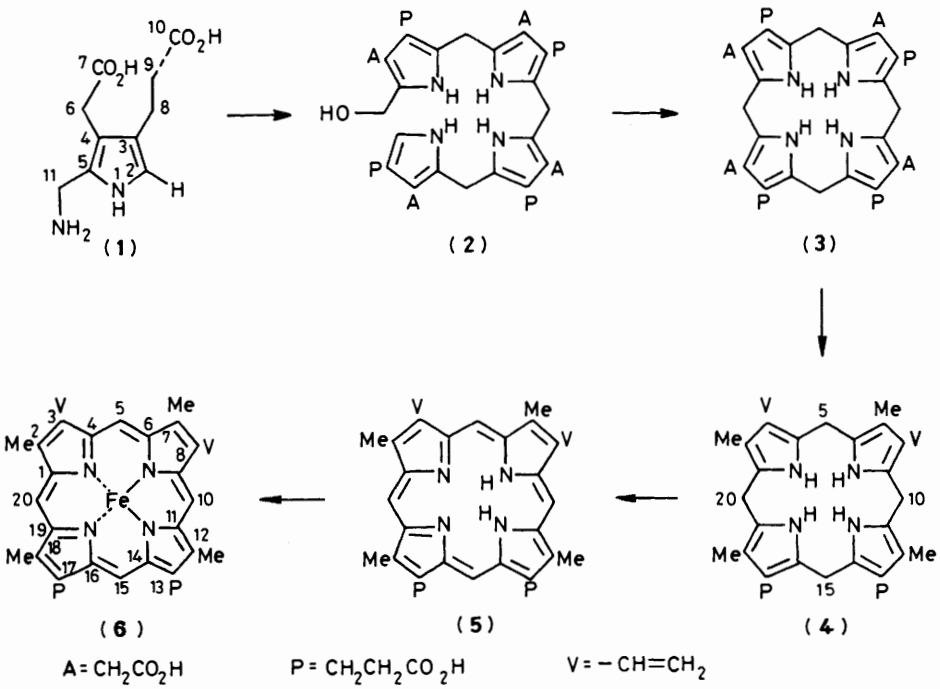
[2,11*S*-$^3H_2$;2,11-$^{14}C_2$]Porphobilinogen (PBG) with an enantiomeric excess greater than 81% was prepared in a coupled enzyme system from [2*RS*-$^3H_2$;2-$^{14}C_2$]glycine. Incorporation of this chiral PBG into haem using a broken cell enzyme system from chicken blood showed retention of only one tritium per haem molecule. Degradation of the haem showed that tritium had been incorporated specifically into the C-10 position of haem. Control experiments with [11*RS*-$^3H_2$;2,11-$^{14}C_2$]-PBG showed an equal distribution of tritium at all four *meso* positions of haem. The implications of this result are discussed in terms of the mechanism of two enzymes in haem biosynthesis: porphobilinogen deaminase (hydroxymethylbilane synthase) and protoporphyrinogen IX oxidase.

Uroporphyrinogen III (**3**), a key intermediate in the biosynthesis of all naturally occurring tetrapyrroles,[1,2] is formed from porphobilinogen (PBG) (**1**) through the sequential action of two enzymes.[3] The first enzyme, porphobilinogen deaminase (hydroxymethylbilane synthase), catalyses the polymerisation of four molecules of PBG to furnish a tetrapyrrole intermediate[4] originally termed preuroporphyrinogen and characterised as a hydroxymethylbilane of the type (**2**).[5] In this process, a number of enzyme-bound intermediates have been identified.[6] The next enzyme, uroporphyrinogen III cosynthetase (uroporphyrinogen III synthetase), then converts the intermediate (**2**) into uroporphyrinogen III (**3**). Notwithstanding an enormous body of important experimental work which is now available on these two enzymic reactions, the stereochemical events which attend the overall conversion of PBG into uroporphyrinogen III are not yet known. The paired hydrogen atoms at the C-11 of PBG provide a convenient mechanistic and stereochemical probe to follow the overall conversion since it is at this position that all the bond-forming and -breaking events occur.

Another problem in the biosynthesis of haem (**6**) relates to the enzymic oxidation of protoporphyrinogen IX (**4**) to protoporphyrin IX (**5**). The first indication that this stage is enzyme-catalysed came from the studies with *meso* tritiated protoporphyrinogen.[7,8] Protoporphyrinogen IX oxidase was subsequently demonstrated in yeast[9] and has now been isolated from a number of sources.[10] In aerobic systems the terminal electron acceptor for this enzyme is oxygen and although little is known about the mechanism of the oxidation reaction it has been assumed that hydrogen abstraction from each of the four



Scheme 1.

meso (bridge) positions (C-5, C-10, C-15, and C-20) occurs on the same face of the protoporphyrinogen IX macrocycle. Since the C-11 positions of the PBG molecules become the *meso* positions 5, 10, 15, 20 in protoporphyrinogen IX (**4**) and protoporphyrin IX (**5**), an evaluation of the status of hydrogen atoms at these positions would provide both mechanistic and *stereo*chemical information about the oxidase reaction.

Our previous work[11,12] had suggested a reliable synthetic route to the C-11 chirally labelled PBG [(**1a**) Scheme 2]. We anticipated that a study of the fate of the individual prochiral hydrogen atoms at C-11 when PBG is transformed into protoporphyrin IX would provide stereochemical information relevant to the mechanism of the formation of uroporphyrinogen III as well as the aromatisation catalysed by protoporphyrinogen IX oxidase. A preliminary account of a part of this work is available.[13]

Results and Discussion

Enzymic Synthesis of [11S-^{3}H;2,11-$^{14}C_2$]-*PBG* (**1c**).—The enzymic synthesis of stereospecifically tritiated porphobilinogen (at C-11) was accomplished in a two stage enzymic transformation using the enzymes, 5-aminolevulinate (ALA) synthetase and ALA dehydratase, similar to that reported by us previously.[12] Glycine (**7**) labelled randomly with tritium at C-2 (together with ^{14}C glycine) was initially converted into ALA (**8a**), a process known to result in ALA stereospecifically tritiated at C-5. The ALA was then transformed into the chiral PBG (**1a**) which was subsequently equilibrated with dilute acid to afford the required stereospecifically tritiated PBG (**1c**). This overall sequence is shown in Scheme 2. The principle underlying the synthesis and the knowledge of the stereochemistry at C-11 of the PBG's (**1a**) and (**1c**) follows from our earlier work,[12] but in the present study the requirement of larger amounts of PBG with higher stereochemical purity necessitated further methodological improvements.

In our original work[12] both the enzymes, ALA-synthetase and ALA-dehydratase, required for the coupled system were obtained from *Rhodopseudomonas spheroides*. However, in the present study the pure ALA dehydratase was isolated from bovine liver. Details of the incubation conditions which were developed for the optimal functioning of the two enzymes in the coupled reaction are discussed in the Experimental section.

Accordingly, a protocol was designed which ensured that ALA produced in the first phase of the linked-process had a relatively short mean life of *ca.* 90 s and, hence, without suffering an appreciable racemisation at its sensitive C-5, was trapped by the ALA-dehydratase as PBG. The examination of a portion of the incubation by analytical t.l.c. revealed that the overall conversion of the labelled glycine into PBG had occurred in *ca.* 30% yield. For the preparative isolation, the biosynthetic PBG was purified initially from the incubation medium using Dowex-2 acetate. T.l.c. of this product showed it to be >95% radiochemically pure; the minor radiochemical impurities were ALA and glycine which were removed by further purification using t.l.c.

The $^3H/^{14}C$ ratio of the PBG was about half that of the precursor glycine (**7**) (Table 1). Since it has been shown previously that one of the two C-2 hydrogen atoms of glycine is removed in the ALA synthetase-catalysed formation of ALA,[14] the absence of a further loss of ^3H in the production of PBG confirms that the glycine-derived hydrogen atoms in both the molecules of ALA are conserved during their conversion into PBG. That the ^3H in the PBG was equally distributed between its C-2 and C-11 was shown by equilibrating the PBG with dilute acid, a process which catalyses the exchange of the ^3H label at C-2 (Table 2).

Analysis of the Chirality of [11S-^{3}H;2,11-$^{14}C_2$]-*PBG* (**1c**).—The principle underlying the determination of the steric distribution of ^3H at C-11 of the biosynthetic PBG involved an oxidative degradation to glycine. The latter was then subjected to chirality analysis using serine hydroxymethyltransferase (SHMT), an enzyme known to exchange the H_{Si} of glycine with the protons of the medium.[15] For this purpose the chiral PBG (**1c**) was first converted into its *N*-acetyl derivative with acetic anhydride in ammonium hydrogen carbonate buffer at 0 °C, followed by ozonolysis in formic acid–chloroform (3:1) at 0 °C. Oxidative work-up produced *N*-acetylglycine in 8% yield which had essentially the same $^3H/^{14}C$ ratio as the unequilibrated PBG. Hydrolysis and chirality assay of this glycine with serine hydroxymethyltransferase (SHMT) revealed 81.2—84.2% enantiomeric excess of the 11*S*-isomer.

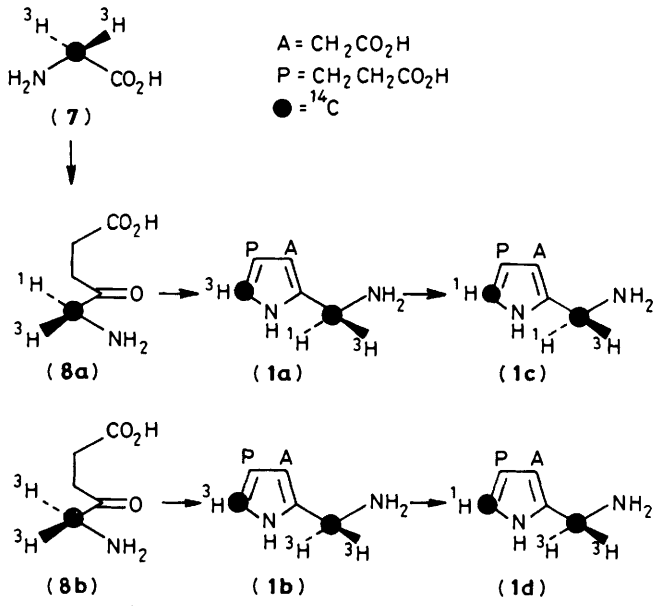
Table 1. Estimation of stereochemical purity of [2,11*S*-$^3H_2$;2,11-$^{14}C_2$]-PBG by oxidative degradative and enzymic analysis with serine hydroxymethyltransferase (SHMT)

[2,11*S*-$^3H_2$;2,11-$^{14}C_2$]-PBG was synthesised from [2*RS*-$^3H_2$;2-^{14}C]glycine in a coupled enzyme system consisted of ALA synthetase and ALA dehydratase. The PBG was *N*-acetylated and oxidatively degraded by (a) ozonolysis and (b) coupled permanganate–periodate oxidation to produce, after work-up, glycine from C-5 and C-11 of PBG. The glycine derived from the degradation was incubated with serine hydroxymethyltransferase (SHMT) to equilibrate specifically the H_{si} and the proportion of label lost was calculated. Glycine was always derivatised to the benzyloxycarbonyl derivative before radioactive counting.

Sample		$^3H/^{14}C$ Ratio	% Loss of ^3H	Enantiomeric excess of 11*S*-^3H isomer in PBG
1. [2*RS*-$^3H_2$:2-^{14}C]Glycine[a]		38.45		
2. [2,11*S*-$^3H_2$:2,11-$^{14}C_2$]-PBG[b]		18.76		
3. [2*S*-^3H:2-^{14}C]Glycine[c]	Before equilibration with SHMT	17.95	90.6	81.2
	After equilibration with SHMT	1.67		
4. [2*S*-^3H:2-^{14}C]Glycine[d]	Before equilibration with SHMT	30.15	92.1	84.2
	After equilibration with SHMT	2.38		

[a] Randomly tritiated glycine used for coupled enzyme synthesis of chiral PBG. [b] PBG enzymically synthesised from [2*RS*-$^3H_2$:2-^{14}C]glycine. [c] The ^3H:^{14}C ratio of glycine derived from PBG by ozonolysis. [d] The ^3H:^{14}C ratio of glycine derived from PBG by permanganate–periodate oxidation.

Coupled periodate–permanganate oxidation after Lemieux[16] was also attempted followed by acid hydrolysis to glycine. By this latter method the yield was very low (*ca.* 1%) and the $^3H/^{14}C$ ratio of the glycine was high, consistent with a side reaction involving attack on the acetamido methylene hydrogens of *N*-acetyl-PBG that showed a tritium isotope effect. However, enzymic assay of the glycine with SHMT showed a high degree of stereochemical purity had been maintained (Table 1).



Scheme 2.

Table 2. Biosynthesis of haem from PBG

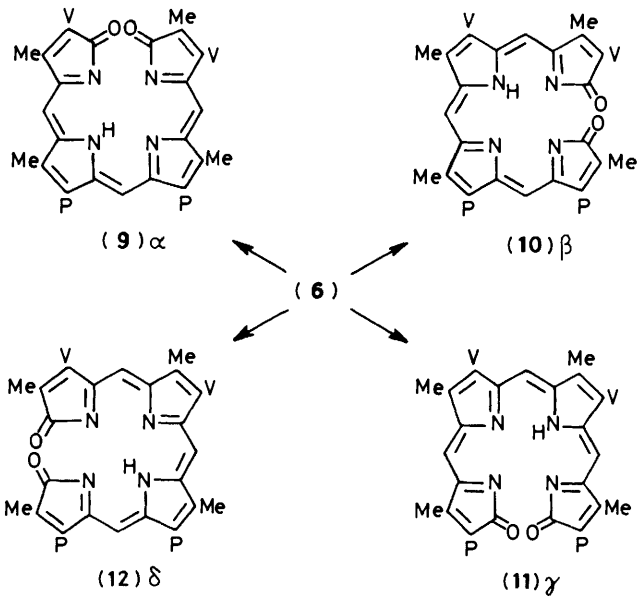
Broken red cell preparations from chicken blood were incubated with [2,11*S*-$^3H_2$;2,11-$^{14}C_2$]-PBG (sample 1, Table 1), [11*S*-^3H;2,11-$^{14}C_2$]-PBG (sample 2, Table 1 after the exchange of hydrogen atom at C-2) or [11*RS*-$^3H_2$;2,11-$^{14}C_2$]-PBG (C-2 hydrogen exchanged). Haem was extracted into organic solvents and purified as described. The haem was counted for radioactivity after decolourisation by a complex procedure (see methods). PBG and benzyloxycarbonylglycine were treated in identical regimes. The differences in $^3H/^{14}C$ ratios of the PBG samples below ($^3H/^{14}C$ = 15.58) with respect to those in Table 1 ($^3H/^{14}C$ = 18.76) are due to different sample processing methods and radioactive counting procedure (see Experimental section). The values in Table 2 have been used to calculate final results.

Substrate	$^3H/^{14}C$	$^3H/^{14}C$ of haem	% Retention ^3H (from C-11)
[2,11*S*-$^3H_2$;2,11-$^{14}C_2$]-PBG[a]	15.58	1.99	25.4
[11*S*-^3H;2,11-$^{14}C_2$]-PBG[b]	7.94	2.12	26.3
[11*RS*-$^3H_2$;2,11-$^{14}C_2$]-PBG	3.45	1.71	49.5

[a] The sample contained 50.3% and 49.7% ^3H at positions C-11 and C-2 respectively. The label at C-2 is lost on forming the macrocyclic ring. [b] The equilibration of ^3H at C-2 was carried out prior to incubation.

The Conversion of Variously Tritiated PBG into Haem.—The enzyme system used to convert PBG into haem was essentially that as described earlier,[17,18] except that neither succinate nor glycine were added. For good incorporation, a slow stream of air through the system and only slow shaking were required. Using 200—300 μg of the radioactive chiral PBG 25—30% of the ^{14}C label was incorporated into haem (**6**), which was isolated as haemin by butanone extraction and precipitation with light petroleum. These samples were then crystallised to constant specific activity and $^3H/^{14}C$ ratio. Results from feeding [2,11*S*-$^3H_2$;2,11-$^{14}C_2$]-PBG and [11*S*-^3H;2,11-$^{14}C_2$]-PBG indicated that the tritium from C-2 was lost as expected, but that, more interestingly, the tritium from only one of the four C-11 positions remained in haem giving rise to the retention of about a quarter of the ^3H label. These results are summarised in Table 2. Equivalent experiments using the racemic tritiated PBG (**1b**) ([11*RS*-$^3H_2$;2,11-$^{14}C_2$]-PBG) showed, on the other hand, that half the tritium present at C-11 of each of the four PBG units was incorporated into haem.*

The Location of the Position of ^3H in Haem Biosynthesised from Variously Tritiated PBG.—Cumulatively, these results imply that one of the four chirally labelled PBG molecules retains its tritium during the formation of the porphyrin nucleus of haem. To determine the precise position of the tritium label(s) a degradation method was chosen which involved the oxidation of haem (haemin) with molecular oxygen to yield (after removal of iron and esterification) a separable mixture of the four biliverdin isomer esters:[19] α-biliverdin ester (**9**; P = $CH_2CH_2CO_2Me$), β-biliverdin ester (**10**; P = $CH_2CH_2CO_2Me$), γ-biliverdin ester (**11**; P = $CH_2CH_2CO_2Me$), and δ-biliverdin ester (**12**; P = $CH_2CH_2CO_2Me$) (see Scheme 3) which were characterised by n.m.r. spectroscopy from their aromatic methyl resonances.[20]



Scheme 3.

* An experiment using the same enzyme system but using [2*RS*-$^3H_2$;$^{14}C_2$]glycine ($^3H/^{14}C$ = 21.59) as the precursor gave a low (*ca.* 0.1%) incorporation of glycine (**7**) into haem with 5.6% retention of ^3H ($^3H/^{14}C$ of haem = 1.20). This compares favourably to the ^3H retention of 5.9% obtained in haem in the two-stage process in which glycine is first converted into PBG and the latter then used to obtain haem. Although the haem from this experiment was not subjected to degradation to confirm that the ^3H was present at the β position (C-10), it may be assumed that the label had been stereospecifically manipulated throughout the overall transformations and that the chicken erythrocyte system had generated the same chiral substrate, namely [2,11*S*-$^3H_2$;2,11-$^{14}C_2$]-PBG. Apart from these conclusions the results may be extrapolated to suggest that the H_{Re} of glycine is lost serving to illustrate that the mechanistic and stereochemical course of the ALA synthetase reaction in avian systems is identical with that of the *Rhodopseudomonas spheroides* enzyme.[15]

Table 3. Degradation of haemin to α, β, γ, and δ biliverdins

Purified labelled haemin, synthesised in the chicken blood system (see Methods) was degraded to the methyl esters of α, β, γ, and δ biliverdin isomers which were purified by t.l.c. on silica. The radioactivity in the initial haemin and in the biliverdin isomers was determined after decolourisation as described in the methods.

		$^3H/^{14}C$ Ratios				
			Biliverdin methyl esters			
Haemin degraded	Structures	Haemin (**6**)	α (**9**)	β (**10**)	γ (**11**)	δ (**12**)
Haemin from [11*RS*-$^3H_2$;2,11-$^{14}C_2$]-PBG[a]		1.63	1.62	1.80	1.85	1.75
		1.63	1.50	1.59	1.63	1.53
Theoretical prediction		1.63	1.40	1.40	1.40	1.40
Haemin from [11*S*-^{3}H;2,11-$^{14}C_2$]-PBG[b]		2.42	2.19	0.86	2.46	2.48
		2.20	2.13	0.81	2.49	2.32
		1.99	2.15	0.56	1.91	1.78
Theoretical prediction[c]		2.00	2.11	0.52	2.11	2.11

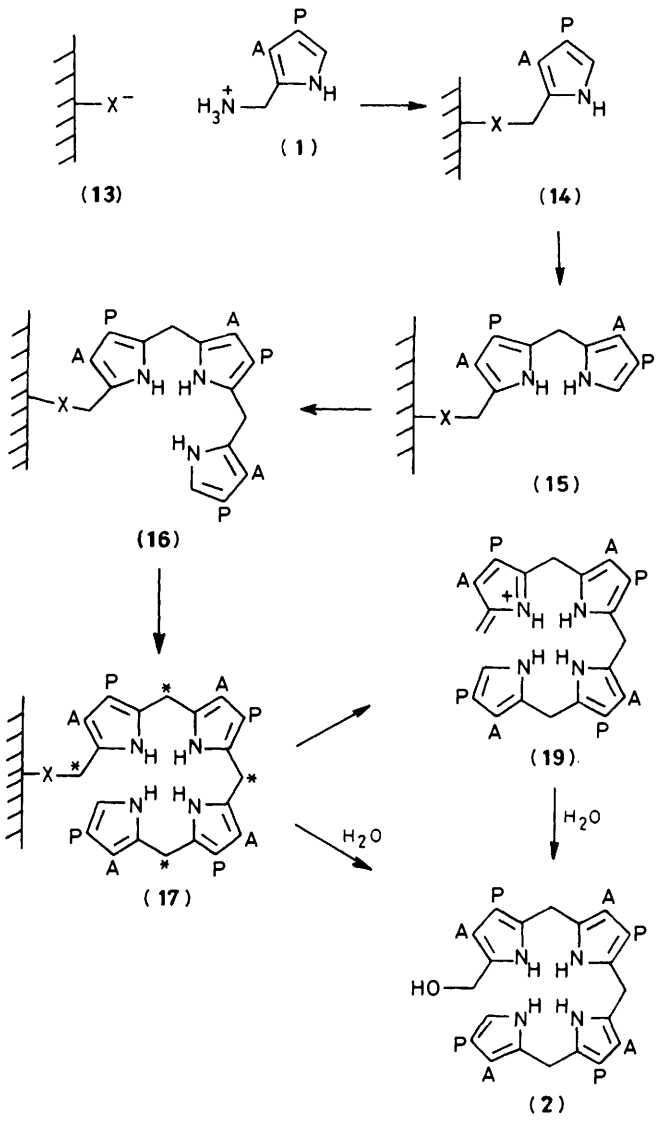
[a] Two experiments [b] Three independent experiments: the first experiment was performed using a batch of PBG different from that used for experiments 2 and 3. [c] Calculated (for the last two experiments) on the basis that the samples of PBG consisted of the 11*S* and 11*R* isomers in the ratio 91 : 9 and that the ^3H from the 11*S* enantiomer retained in haem is at C-10 (β).

In the degradative conversion, each biliverdin isomer is formed by the loss of a single *meso* hydrogen and that *meso* carbon. Assuming that the oxidative process producing the isomers occurs without the involvement of an isotope effect, two main possibilities exist. (1) That the tritium is randomly distributed between the four *meso* positions. In this case the four biliverdin isomers will each have a carbon specific activity 7/8 that of the haem, and a tritium specific activity 3/4 that of the haem. Hence the $^3H/^{14}C$ ratio of each isomer will be 6/7 that of the haem. This is what we expected, and indeed found, for the haem from racemically tritiated PBG (Table 3).

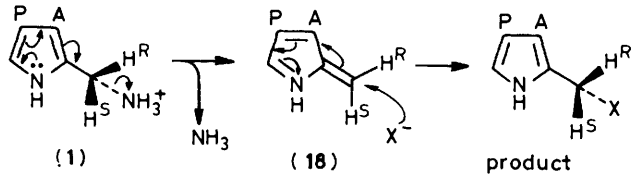
(2) That the tritium is localised on one *meso* position (*e.g.* the β). In this case the α, γ, and δ isomers will lose 1/8 of their ^{14}C, but retain their tritium, and the $^3H/^{14}C$ ratio will be 8/7 that of the haem. The β isomer will lose all its tritium and its $^3H/^{14}C$ ratio will be zero. This analysis, however, is very dependent on the stereochemical purity of the dual-labelled PBG, since the 'wrong' enantiomer will introduce ^3H into three positions while the 'right' isomer will introduce it into only one.

The results from five experiments are given in Table 3 along with 'theoretical' values calculated using the models above and taking account of the stereochemical purity of the PBG. These experimental results are in good agreement with the values calculated on the basis that incorporation of [11*S*-^{3}H;2,11-$^{14}C_2$]-PBG results in tritium retention only at C-10 in haem (the β *meso* position), and that incorporation of the racemic sample leads to an equal distribution at each *meso* position.

Mechanistic and Stereochemical Implications.—The interpretation of the above results requires the simultaneous consideration of the reaction mechanisms of porphobilinogen deaminase (hydroxymethylbilane synthase), uroporphyrinogen III cosynthase, and protoporphyrinogen IX oxidase. The first event in the transformation of PBG into uroporphyrinogen III involves the displacement of ammonia and the covalent attachment of the pyrrole moiety to the deaminase (**13**)→(**14**). Thereafter, the sequential addition of three further molecules of PBG *via* (**15**) and (**16**) furnishes the enzyme bound tetrapyrrole (**17**). Since all the stages in Scheme 4 involve the reaction of PBG, an elimination–addition process (**1**)→(**18**)→products, for what otherwise may appear to be simple displacements, would be the preferred course. The closest precedent for such a transformation from reactions in enzymology is a class of pyridoxal phosphate-dependent β-replacement reactions which are known to operate *via* an overall retention of stereochemistry.[21] If the same stereochemical preference was shown by the example under consideration, then all the methylene bridges of the enzyme bound tetrapyrrole (* **17**) would be formed by a retention mechanism and hence the steric



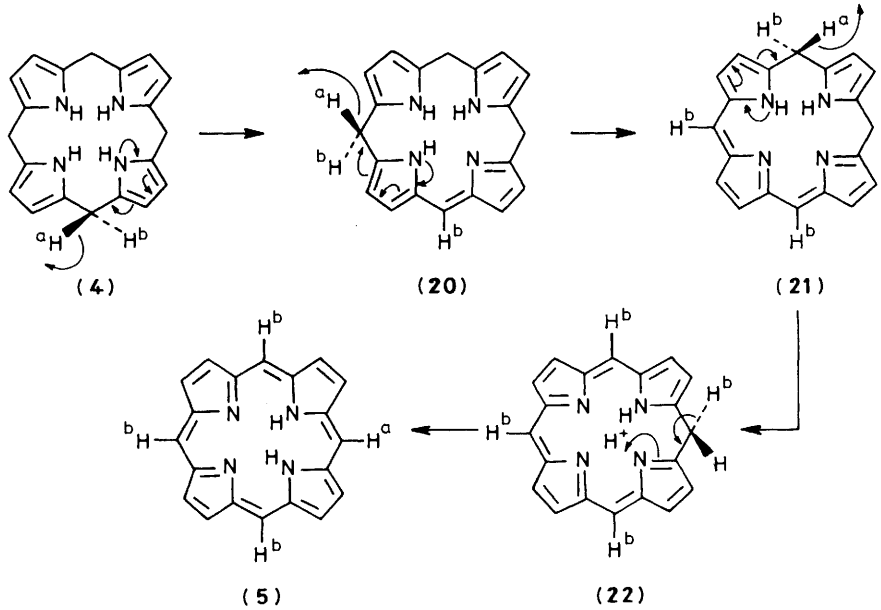
Scheme 4.



orientations at these positions will be identical when this species is generated from [11*S*-^{3}H]-PBG (**1a**).*

Two broad possibilities now exist for the formation of the hydroxymethylbilane (**2**) from the enzyme-bound chiral tetrapyrrole (**17**). Firstly the free tetrapyrrole could be liberated by reaction of water with the enzyme-bound species (**17**) yielding (**2**) with chiral centres at C-5, C-10, C-15, and C-20 (Scheme 4). Alternatively, the release of the methylenepyrrole (**19**) from the enzyme with subsequent non-stereospecific hydration at C-20 (C-5, C-10, C-15 chiral) would yield the racemic mixture of the intermediate through the sequence (**17**)→(**19**)→(**2**) (Scheme 4). The comparison of the radiochemical data on 11*S*-PBG (**1a**) and the derived haem (Tables 2 and 3) clearly shows that all four *meso* hydrogen atoms which arose from the tritiated 11*S*-PBG are treated by stereospecific processes. This, therefore, eliminates the possibility in which methylenepyrrole is released as a 'free intermediate' and then is non-enzymically hydrated to (**2**). A key feature of the argument, its rearrangement into uroporphyrinogen III, the absolute stereochemistry of C-5 and C-10 should be identical as far as protoporphyrinogen IX (**4**). On the other hand, during the formation of the type III macrocycle, the positions 20 and 15 have been involved in several bond-forming and -breaking events, and we make no attempt to guess their absolute stereochemistry.†

In consideration of the second transformation, namely the formation of protoporphyrin IX (**5**) from protoporphyrinogen IX (**4**), it had been assumed that during the oxidation of protoporphyrinogen IX all four *meso* hydrogens are removed from the same face of the macrocyclic ring, but no mechanism was available in the literature. The results described above prompt us to suggest a hitherto unsuspected mechanism in which three of the *meso* hydrogens are abstracted as 'hydride' and one *meso* hydrogen is removed as a proton. Although it is speculative to attempt to define the precise order of events, several broad mechanisms exist. For instance, one mechanism would involve three oxidations using the same face of the macrocycle by the concerted loss of 'hydride' from each *meso* position, and a proton from the pyrrole NH through the sequence (**4**)→(**20**)→(**21**)→(**22**) (Scheme 5). Tautomerisation to the porphyrin would then occur by the loss of the fourth *meso* hydrogen atom as a proton (**22**)→(**5**). Another equally plausible mechanistic sequence involves two initial oxidations using the



Scheme 5. ^{a}H and H^b, hydrogen atoms above and below the plane of the ring

and one of paramount importance in the future considerations, is that the C-5 and C-10 of the growing polypyrrole chain are built into the tetrapyrrole essentially by identical reactions and since there is no mechanistic reason why these positions should be disturbed during the ensuing release of the intermediate and same face of the macrocycle by the abstraction of the *meso* hydrogens as hydride and the pyrrole NH as a proton. In this mechanism, however, the third oxidation occurs by the concerted removal of a proton from the third *meso* position and a third 'hydride' from the remaining *meso* position.

The results described above show that the chiral protoporphyrinogen IX biosynthesised from [11*S*-^{3}H;2,11-$^{14}C_2$]-PBG upon conversion into protoporphyrin IX stereospecifically loses the ^{3}H at C-5, C-15, and C-20 while retaining that at C-10. The possibility that the retention at C-10 may be due to the fact

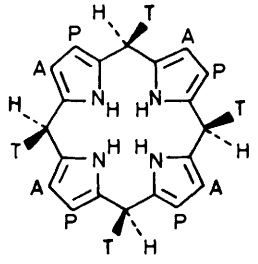
* We have deliberately not defined the absolute stereochemistry at positions 5, 10, 15, and 20 of any of the tetrapyrrole intermediates of haem biosynthesis since the stereochemistry has not yet been experimentally determined. Furthermore, the asymmetric disposition of ring substituents (at positions 2, 3, 7, 8, 12, 13, 17, and 18) and their changes in priority during the biosynthetic transformations leads to changes of notation which may be found confusing. A complete account of this latter point is available elsewhere (C. Jones, Ph.D. Thesis, University of Southampton, 1979).

† An important point to consider is that the subsequent elaboration of the side chains at positions 2, 3, 7, 8, 12, and 18, in the conversion of uroporphyrinogen III into protoporphyrinogen IX, need not affect the hydrogen atoms and their stereochemistry at C-5, C-10, C-15, and C-20.

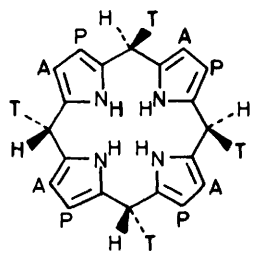
that the hydrogen from this particular position is removed by a non-enzymic process occurring through a high isotope effect was also considered. Indeed, a high isotopic effect has been observed for the non-enzymic [7] oxidation of [5,10,15,20-$^3H_8$]protoporphyrinogen IX. However, this possibility is eliminated by our experiment in which 50% of the original tritium was removed in the oxidase-catalysed aromatisation of [5,10,15,20-$^3H_8$]protoporphyrin IX produced from [11*RS*-^{3}H;2,11-$^{14}C_2$]-PBG (**1b**) (Table 2) (also see refs. 7 and 8).

The stereochemical findings, when taken in conjunction with the assertion that protoporphyrinogen IX biosynthesised from chiral PBG has the ^{3}H at C-5 and C-10 co-facial, make it mandatory that these two positions (C-5 and C-10) are treated differently in the overall reaction catalysed by protoporphyrinogen IX oxidase. We suggest that one of these two positions is a site for the oxidation reaction (loss of 'hydride') and the other for the tautomerisation process (loss of H^+), and that the two types of processes occur using hydrogen atoms from opposite faces of the macrocycle. These assumptions suggest two possibilities for the events occurring at the *meso* position during the oxidase-catalysed transformation. (1) That hydrogen atoms at C-5, C-15, and C-20 are lost through oxidation that at C-10 *via* tautomerisation. If this were the case the ^{3}H atoms at all the four *meso* positions of the chiral protoporphyrinogen IX, biosynthesised from [11*S*-^{3}H;2,11-$^{14}C_2$]-PBG would have been on the same face [*e.g.* structure (**23**)].

(2) That hydrogen atoms at C-10, C-15, and C-20 are lost by oxidation and the tautomerisation then occurs involving a hydrogen at C-5. In this case the protoporphyrinogen IX would contain tritium at C-15 and C-20 on the same face while those at C-10 and C-5 would have ^{3}H on the other; for example, see structure (**24**).



(**23**)



(**24**)

The reader should note that a reaction course in which the partially oxidised intermediates of type (**20**) or (**21**) or (**22**) are released free into the medium, with these species then being handled by the enzyme using different faces of the macrocycle, is not susceptible to the mechanistic analysis by the approach used here. This route is, however, a possibility albeit rather remote.

Conclusions.—Cumulatively, the results obtained in the present paper using [11*S*-^{3}H;2-$^{14}C_2$]-PBG show that all the four methylene positions of porphyrinogens III and hence of the hydroxymethylbilane (**2**) are produced through stereospecific processes. Thus, if a methylenepyrrole of type (**19**) is an intermediate in the formation of the intermediate (**2**) then its formation as well as its hydration must occur at an enzyme active-site. Furthermore, the stereochemical results have been interpreted to suggest that the overall process leading to the formation of uroporphyrinogen III from [11*S*-^{3}H;2,11-$^{14}C_2$]-PBG results in the disposition of the labelled hydrogen atoms in one of two orientations [*e.g.* (**23**) or (**24**)]. The absolute stereochemistry of ^{3}H at the *meso*-position in the uroporphyrinogen III skeleton cannot be established directly, but may be inferred as shown in structures (**23**) or (**24**) by invoking that, as a minimum mechanistic requirement, the formation of the C–C bonds in the extension of the polypyrrole chain in Scheme 4 occur by an elimination–addition process operating through a retention mode. It is hypothesised that in the protoporphyrinogen IX oxidase-catalysed reaction, the three oxidative steps occur with the involvement of *one* particular face of the macrocycle, with the tautomerisation reaction using the *other* face.

Experimental

Materials.—All radiochemicals were obtained from the Radiochemical Centre, Amersham, except 5-amino[5-^{14}C]-levulinic acid (ALA), which was obtained from New England Nuclear. Tri-lithium coenzyme A was obtained from Boehringer Gmbh, Mannheim. Tris(hydroxymethyl)methylamine (Tris) and DEAE Sephadex were obtained from Sigma, London. Yeast extract was obtained from Oxoid, London, and agar from Difco, East Molesley, Surrey. NE250 Liquid scintillator was from Nuclear Enterprises, Edinburgh, Scotland. Solvents were obtained from Koch-Light, Colnbrook and all other chemicals from British Drug Houses, Poole, Dorset.

N.m.r. spectra were obtained on a Varian XL-100 spectrometer operating in the Fourier transform mode. Electron spectra were recorded on a Pye-Unicam SP1800 spectrophotometer. Liquid scintillation counting was performed on an Intertechnique SL20 or ABAC 30, using external standard channel ratio standardisation. Counting was performed in NE250 (10 ml) or 0.6% butyl PBD [5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1,3,4-oxadiazole] in toluene.

T.L.C. Systems.—System 1. Cellulose Sigmacell 20 (10 cm × 20 cm × 0.5 mm) developed in Bu^{n}OH–HOAc–H_2O (63 : 10 : 27) in equilibrated tanks at room temperature. System 2. Silica gel H (20 cm × 10 cm × 0.5 mm) irrigated overnight in dry methanol and reactivated at 120 °C. Samples were applied in chloroform and developed in acetone–chloroform (1 : 24) under an N_2 atmosphere in the dark. System 3. Silica gel H (20 cm × 10 cm × 0.5 mm), samples were applied in chloroform and developed in n-heptane–butanone–HOAc (10 : 5 : 1) under N_2 in the dark.

Growth and Harvesting of Rhodopseudomonas spheroides.—*Rhodopseudomonas spheroides* (NCIB 8253) was grown after the method of Lascelles.[22] To prepare large quantities of bacteria, 15 ml of medium MS also containing 0.2% w/v yeast extract was inoculated from the stale culture and the bacteria were grown semi-anaerobically for 3 days at 30 °C under tungsten light. These cultures were used to inoculate 150 ml of medium MS (no yeast extract), and again grown for 3 days at 30 °C under tungsten light. These cultures were used to inoculate 4 l of medium MS in a 5-l conical flask. Growth was maintained semi-anaerobically at 28 °C in water-baths under tungsten light and the bacteria were harvested after 5 days with a Sharples continuous-flow centrifuge. The bacteria were resuspended in 10% aqueous glycerol containing Tris-HCl pH 6.8 (100 mM), EDTA (1mM), and 2-mercaptoethanol (5 mM), to a final volume of 150 ml from 50 l of culture. The re-suspended bacteria were stored at −15 °C until required.

Enzymes.—ALA synthetase was purified by a modification of the method of Warnick,[23] and of Laghai,[24] except for the exclusion of EDTA from the buffers used in the final DEAE Sephadex column storage. One unit of enzyme is defined as the amount of enzyme required to catalyse the formation of 1 μmol of ALA per hour from succinyl-CoA and glycine. The enzymic production of ALA was measured[25] in a final volume of 375 μl containing potassium phosphate pH 6.8 (10 μmol), pyridoxal

phosphate (10 nmol), glycine (50 μmol), 2-mercaptoethanol (100 μmol), and 30 μl of ALA synthetase. Incubation was for 15 min at room temperature. The reaction was started by addition of succinyl-CoA (250 nmol) in 25 μl of water, and the mixture was incubated at 37 °C for 10 min. The reaction was terminated by the addition of 10% trichloroacetic acid (250 μl), 1M-sodium acetate buffer pH 4.6 (9.3 ml), and pentane-2,4-dione (0.2 ml). This mixture was heated at 100 °C for 10 min and cooled. An aliquot of this solution (2 ml) was added to 2 ml of modified Ehrlich's reagent.[21] After 15 min at room temperature, the optical density at 553 nm was read against a reagent blank. A standard curve was prepared with known quantities of ALA. Modified Ehrlich's reagent was prepared by dissolving *p*-aminobenzaldehyde (1 g) in glacial acetic acid (40 ml) and adding perchloric acid (60%; 10 ml).

ALA dehydratase, purified from bovine liver by Dr. A. G. Chaudhry,[26] was stored as an ammonium sulphate precipitate at 0 °C and was homogeneous as judged by SDS polyacrylamide gel electrophoresis. The enzyme, when activated, had a specific activity of 20 units/mg when assayed under the conditions of Gibson.[27] Quantities of the enzyme were activated as required by dissolving a portion of the precipitate in a small volume of 10 mM-potassium phosphate pH 6.8 containing 20 mM-2-mercaptoethanol and dialysing against this buffer for at least 3 h to remove ammonia. In later experiments 2-mercaptoethanol was replaced by 10mM-dithioerythritol.

The assay contained potassium phosphate buffer pH 6.8 (50 μmol), 2-mercaptoethanol (10 μmol) and activated ALA dehydratase in a final volume of 495 μl. After 15 min at room temperature the reaction was started by the addition of 5 μl of ALA·HCl solution (0.5 μmol) and the mixture was incubated at 37 °C for 10 min. The reaction was terminated by the addition of 500 μl of 10% trichloroacetic acid containing 0.1M-mercuric chloride. After bench centrifugation, 500 μl of this solution was added to 500 μl of Ehrlich's reagent[21] and the PBG production was estimated from the optical density at 555 nm after 15 min. The activity was calculated from a value of 62 000 mol^{-1} l^{-1} cm^{-1} for the PBG-Ehrlich's reagent complex at 555 nm.

Preparation of Carrier PBG.—To aqueous potassium phosphate (300 ml; pH 6.8; 50 mM) containing 2-mercaptoethanol (10 mmol), ALA dehydratase (90 mg) and ALA·HCl (300 mg, 1.79 mmol) were added. The reaction vessel was flushed with nitrogen and sealed. Incubation was at 37 °C with shaking. At hourly intervals, aliquots were taken to assay for the production of PBG. After 6 h the pH was adjusted to 8.0 and the flask was left at 0—4 °C overnight. The isolation of the PBG was performed at 0—4 °C. The incubation mixture was poured onto a column of Bio-Rad AG-1 × 8 acetate 100—200 mesh (2.5 cm × 10 cm high) and washed with distilled water (300 ml) to remove ALA. The washings were checked for the absence of PBG, and PBG was eluted with acetic acid (1M) in 10 ml fractions. The fractions containing most of the PBG were rotary evaporated in 30 ml batches under high vacuum at 20 °C. PBG was recrystallised from 0.5M-ammonia adjusted to pH 5.5 by addition of 0.5M-acetic acid. Recrystallised yield was 110 mg (56%).

Preparation of N-*Benzyloxycarbonylglycine for Counting.*—To an aqueous solution containing radioactive glycine (4 ml) was added unlabelled glycine (100 mg, 1.3 mmol), sodium hydroxide (14%; 1 ml) and benzyl chloroformate (300 μl, 2.26 mmol). The solution was stirred on ice for 30 min and excess of benzyl chloroformate extracted with diethyl ether (3 × 5 ml). After acidification with concentrated hydrochloric acid, the solid was filtered off and the benzyloxycarbonylglycine recrystallised twice from chloroform–light petroleum (b.p. 60—80 °C).

Preparation of [2,11S-$^3H_2$;2,11-$^{14}C_2$]*Porphobilinogen* (**1a**).—Before use, ALA synthetase was dialysed for 3 h against aqueous potassium phosphate (100 ml; pH 6.8; 10 mM), containing glycerol (20%), 2-mercaptoethanol (2 mM), and ALA dehydratase activated as described above.

The use of these two enzymes together in a coupled reaction system necessitated the utilisation of a compromise thiol concentration which was sufficient to maintain the activity of the oxygen sensitive dehydratase but which was not so high as to inhibit the synthetase. The omission of ethylenediaminetetraacetic acid which inhibits the bovine ALA-dehydratase and the stepwise addition of the highly unstable succinyl CoA ($t_{\frac{1}{2}}$ 15 min) also served to maximise yields. The undesirable pyridoxal phosphate-catalysed racemisation at the sensitive C-5 position of ALA was minimised by using a concentration of the cofactor (7 μM) lower than that normally used for optimal enzyme activity, and the radiochemical yield was maximised by employing a lower glycine concentration (3 mM) than that used under assay conditions.

In a final volume of 1 ml [2*RS*-$^3H_2$;2-^{14}C]glycine (1 mCi ^3H; 25 μCi ^{14}C, 3 μmol), pyridoxal phosphate (7 nmol), Tris-HCl pH 6.8 (1 μmol), ALA synthetase (20 units; 0.5 ml), and ALA dehydratase (20 units; 0.3 ml) were incubated. The final phosphate concentration was 8 mM and the final thiol concentration was 7 mM. The reaction was started by the addition of neutralised CoA solution (1 mg; 50 μl) and the incubation was carried out at 37 °C with shaking for 30 min. Two further aliquots (50 μl each) of succinyl CoA were added after 10 and 20 min. After addition of 2 mg of carrier PBG (**1**), the incubation mixture was applied to a column of Dowex 2 × 8 acetate (200—400 mesh) 0.5 cm × 6 cm high. ALA and glycine were eluted with 30 ml of water. PBG was eluted with ice-cold acetic acid (10 ml, 1M). The solution was rotary evaporated to dryness under high vacuum at 20 °C and the residue resuspended in 1 ml of 100mM-ammonia solution and freeze dried. The product was stored desiccated at −15 °C.

Thin layer chromatography of the product in System 1 showed that it contained *ca.* 1—2% each of ALA and glycine. The radiochemical yield of PBG (**1a**) was 30% over 30 min.

Preparation of Racemic [2,11RS-$^3H_3$;2,11-$^{14}C_2$]-PBG (**1a**).—This was prepared enzymically from [5*RS*-$^3H_2$;5-^{14}C]-ALA as described above for the unlabelled material.

Acid Labilisation of the α-Hydrogen of Tritiated PBG.—Tritiated PBG (1 mg, 4.47 μmol; 10^6 d.p.m. ^{14}C) (**1a**) or (**1b**) was dissolved in hydrochloric acid (100 μl; 0.15M) and allowed to stand at room temperature. After 30 min, 50 μl was taken for direct freeze drying. After 1 h the remaining 50 μl was also freeze dried. The lyophilised samples were purified by t.l.c. (System 1). After scanning, the bands corresponding to PBG were eluted with aqueous ammonia (100 mM) and recrystallised in the presence of 2 mg of carrier. The loss of ^3H was confirmed by radioactive counting.

Estimation of Stereochemical Purity of [11S-^3H;2,11-$^{14}C_2$]-*PBG* (**1c**).—[11*S*-^3H;2,11-$^{14}C_2$]-PBG (5 mg, 22.2 μmol; 150 000 d.p.m. ^{14}C) (**1c**) was dissolved in water (0.3 ml) containing ammonium hydrogen carbonate (30 mg, 380 μmol) at 0 °C. Acetic anhydride (10 mg, 127 μmol) was added and the solution was stirred at 0 °C for 30 min. The solution of *N*-acetyl-PBG was used for subsequent oxidation. Two methods of oxidation were employed.

(1) *Ozonolysis.*[12] To the solution of *N*-acetyl-PBG was added formic acid (98%; 3 ml) and chloroform (1 ml). Ozone was passed through this solution for 6—7 h at 0 °C at an oxygen flow rate of 50 ml/min. Hydrogen peroxide (30%; 1 ml) was added and reaction was left overnight. The solution was

adjusted to pH 7 with dilute aqueous ammonia and residual peroxide was destroyed by addition of a catalase suspension (1 μl). The solution was acidified with hydrochloric acid, evaporated to dryness, and *N*-acetylglycine was extracted from the residue with dry tetrahydrofuran (4 × 2 ml); the extracts were pooled. The THF was removed under reduced pressure and the residue was hydrolysed in 6M-HCl (1 ml) in a sealed tube overnight at 110 °C. After freeze drying, the glycine was purified by t.l.c. (System 1) (16%, 8% radiochemical yield).

(2) *Coupled permanganate–periodate oxidation.*[16] To the above solution of *N*-acetyl-PBG was added water (0.7 ml), sodium periodate (80 mg, 369 μmol), potassium permanganate (10 mg, 63 μmol) and potassium carbonate (10 mg). The reaction was for 24 h at room temperature. Solid was removed by bench centrifugation and the supernatant liquid was treated with hydrogen peroxide (30%; 100 μl). After 15 min, excess of peroxide was destroyed with catalase (1 μl). Concentrated hydrochloric acid (12M; 100 μl) was added and the solution freeze dried. The *N*-acetylglycine was extracted with tetrahydrofuran, hydrolysed and purified as above; yield 1% (radiochemical).

Equilibration of the H_{Si} of Glycine with Serine Hydroxymethyltransferase.—This was carried out by a modification of the method of Jordan and Akhtar.[14] In a final volume of 1 ml, the incubation mixture contained potassium phosphate pH 7.1 (60 μmol), pyridoxal phosphate (30 nmol), tetrahydrofolic acid (4 μmol), serine hydroxymethyltransferase (5 units, 0.4 ml), and the sample of radioactive glycine (2 μmol). An aliquot of 0.5 ml was taken immediately for re-isolation of glycine. The flask was then flushed with nitrogen and incubated at 37 °C for 3 h.

To each of the two samples of glycine was added potassium hydroxide (2M; 1 ml), glycine (50 mg, 0.67 mmol), and benzyl chloroformate (128 mg, 0.75 mmol) and the mixture processed for the preparation of the benzyloxycarbonyl derivative as described above.

Incorporation of Radioactive PBG into Haem.—To haemolysed red blood cells from anaemic chickens[17] (25 ml) a buffer (pH 7.4) was added (5.5 ml) containing 0.15M-sodium hydrogen orthophosphate and 0.15M-potassium dihydrogen orthophosphate in 0.483M-potassium chloride, and 5 ml of aqueous L-malic acid (7.5 g, 33.5 μmol), disodium malonate (75 mg, 507 μmol), magnesium chloride hexahydrate (10 mg, 49 μmol), calcium chloride dehydrate (10 mg, 68 μmol), ferrous sulphate heptahydrate (5 mg, 18 μmol), benzylpenicillin (1 mg), coenzyme A (0.5 mg), thiamine pyrophosphate (0.5 mg), and the sample of radioactive PBG (**1a**), (**1b**), (**1c**), or (**1d**) (0.25 mg, 2 × 10^6 d.p.m. ^{14}C).

The incubation was for 6 h at 37 °C with slow shaking. Air was passed through the incubation mixture at a rate of 60 ml/h. The reaction was stopped by freezing. Incorporation of PBG into haem in the experiments was 25—30%. Control experiments were carried out with a boiled enzyme system.

Incorporation of Glycine into Haem.—This was as for the incubations with PBG, except that the reaction was carried out on a 7 ml scale containing glycine (21 μmol; 10^7 d.p.m. ^{14}C); pyridoxal phosphate (1.3 μg, 50 nmol), and dithiothreitol (5 mg, 32 μmol). Succinyl CoA (0.8 mg) replaced Coenzyme A. The air flow was 20 ml/h (see footnote * on p. 2627 for results).

Isolation and Recrystallisation of Haemin Chloride.—The incubation mixture (35—40 ml) was adjusted to pH 2.5 (concentrated HCl) and haem was extracted into butanone (1 × 80 ml, 2 × 40 ml). The combined butanone extracts were backwashed with dilute HCl, pH 2.5. Haemin chloride was precipitated by the addition of light petroleum (b.p. 60—80 °C). The amorphous powder was collected by bench centrifugation. The haemin chloride was redissolved in butanone and reprecipitated with light petroleum. This process was repeated after which the solid was washed with a small volume of ethanol and dried under high vacuum.

The haemin chloride was crystallised from aqueous acetic acid–methylene chloride. Repeated crystallisation led to only small changes in specific activity at $^3H/^{14}C$ ratio from the amorphous material.

Oxidation of Haemin and Isolation of the Biliverdin Isomers.—The method used was essentially that of Bonnett *et al.*,[19] except that the purification of the crude biliverdin isomers by precipitation from chloroform–light petroleum was omitted. After chromatography of the biliverdin methyl esters in t.l.c. system 2, bands corresponding to the α + β, γ and δ isomers were separated. The α and β isomers were separated on the t.l.c. system 3, and the γ and δ isomers were further purified in this system. Bands were eluted from the silica with 10% acetone–chloroform (1:9). The biliverdin esters were estimated spectrophotometrically from the peak near 650 nm using values quoted[20] for the γ and δ bands, and the mean of these values for the α and β bands.

The assignment of isomers is that of Bonnett[19] and of Stoll,[20] and was confirmed by ^1H n.m.r. of biliverdin isomers similarly isolated. The n.m.r. (in $CDCl_3$) of ArMe signals were:

α Isomer	2.20 : 2.12 : 2.09 : 1.90
β Isomer	2.31 : 2.23 : 2.18 : 1.80
γ Isomer	2.19 : 2.12 : 2.11 : 2.09
δ Isomer	2.18 : 2.16 : 1.90 : 1.83

Radioactive Counting.—Samples of haem or pre-dried biliverdin esters were dissolved in NE 250 scintillant (1.2 ml) at 60 °C and hyamine hydroxide (0.1M in MeOH; 0.1 ml) was then added. After 5 min at 60 °C, hydrogen peroxide (30%; 0.15 ml) was added with swirling, and the capped samples were maintained at 60 °C for a further 12 min. NE 250 (12 ml) was added, and the sample stored overnight at 4 °C before counting.

All samples of haem and biliverdins were corrected for quenching after decolourisation using pre-determined parameters which were programmed into an Intertechnique SL40 Scintillation Spectrophotometer. These parameters were generated by using doubly labelled samples of different $^3H:^{14}C$ ratios and subjecting them to the decolourisation regime with hydrogen peroxide as described above. $^3H:^{14}C$ Ratios thus obtained were accurate to within 2%. When comparing $^3H:^{14}C$ ratios of coloured products with non-coloured starting material both were subjected to the same quench correction routine. Thus, in Tables 2 and 3, benzyloxycarbonylglycine or PBG was mixed with cold haem and processed by this regime.

In Table 1, benzyloxycarbonylglycine and PBG were counted by a different regime, in butyl PBD (10 ml) solubilised with aqueous methanol (2 ml).

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