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The synthesis of stereoselectively labelled porphobilinogen and its incorporation into protoporphyrin-IX

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Summary. 11-(R)-^{2}H porphobilinogen, stereospecifically labelled with deuterium in the aminomethylene group has been incorporated into protoporphyrin-IX by haemolysates of chicken erythrocytes. High field NMR spectroscopy confirms that the overall biochemical process is stereospecific, deuterium being retained at the α-, γ- and δ-*meso* positions and lost from the β-*meso* position.
Key words. Porphobilinogen; stereoselectively deuterium-labelled protoporphyrin-IX; biosynthesis from porphobilinogen.

Some years ago we showed[1] that the dehydrogenation of protoporphyrinogen-IX **1** to protoporphyrin **2** in chicken red-cell haemolysates was a stereospecific process, but these experiments did not enable us to deduce the precise nature of the process, e.g. the stereochemistry of the hydrogen removal at each *meso*-bridge. Similar conclusions were reached in experiments in which doubly labelled (^{14}C, ^{3}H) porphobilinogen was incubated with a cell-free system from *Euglena gracilis*[2]. More recently Akhtar and Jordan have described[3] studies with tritium-labelled glycine which have effectively shown that both the enzymic formation of the macrocycle and the subsequent dehydrogenation process must be stereospecific.

We now describe experiments on the chemical synthesis of stereospecifically labelled porphobilinogen intended as a preliminary to further studies of the mechanism and stereochemistry of the in vivo formation[4] of the porphyrinogen macrocycle, and of related chemical processes. The route chosen to synthesise porphobilinogen was based on the original method worked out in MacDonald's laboratory from a suitably substituted pyrrole[5]. Following model experiments with simple alkyl pyrroles, the pyrrole aldehyde **3** related to porphobilinogen was converted into the imine **4** by condensation with R(+)-1-phenylethylamine. This was reduced with diborane and hydrolysed to give the corresponding amine **5a**, and with perdeuterodiborane followed by hydrolysis to give the mono-deutero analogue **5b**. Comparisons of the 360 MHz proton NMR spectra of the deuterated and undeuterated materials (fig. 1) showed that the latter was stereoselectively labelled (ca 40% e.e.). Attempts to improve the degree of specificity were of little avail. However, the stereoselectively deuterated phenylethylaminomethyl pyrrole **5b** was hydrogenolysed over palladium on charcoal to the corresponding aminomethyl pyrrole tri-acid **6** (with retention of stereochemical integrity). The latter was then converted[5] via the lactam **7**, decarboxylation and hydrolysis to deuterium-labelled porphobilinogen **8**.

The absolute stereochemistry of our deuterium-labelled porphobilinogen has been deduced as R by conformational analysis. There is a considerable body of evidence in the literature[6] that α-formylpyrroles have a preference for the *syn*-conformation as shown in structure **3**, and a priori it may be concluded that related imines also have a similar preference as shown in structure **4**. This was confirmed by the n.O.e. difference spectra of several imines of this type which

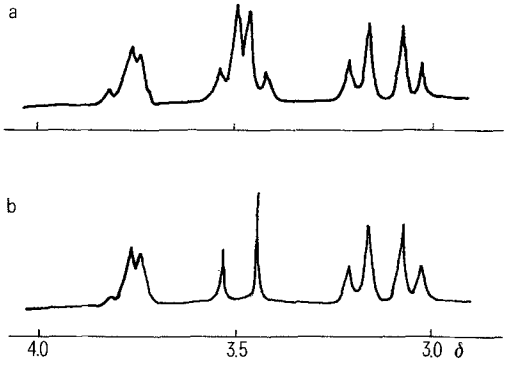


Figure 1. Partial 360 MHz proton N.M.R. spectra showing the resonances circa δ 3.5 of the α-aminomethylene groups in *a* the unlabelled pyrrole (**5a**) (an AB quartet), and *b* the stereoselectively deuterium-labelled pyrrole (**5b**) (two singlet resonances, ratio 7:3, corresponding to the two diastereoisomers).

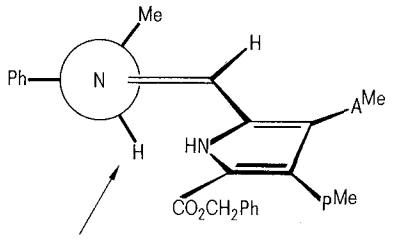


Figure 2. Newman projection of the preferred Conformation of the Imine (**4**) showing that reduction by Deuteriated Diborane (or a derivative) occurs preferentially from the side indicated by the arrow to afford the Deuteriated Amine (**5b**).

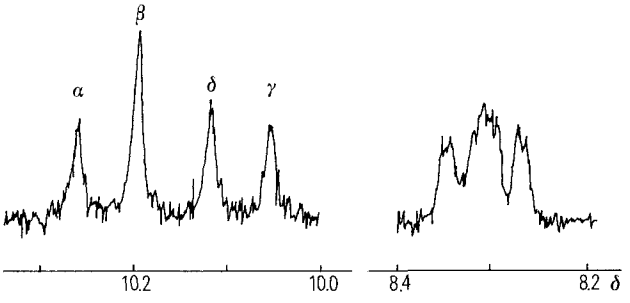


Figure 3. 360 MHz proton N.M.R. spectrum of the meso- and vinyl α-methine proton regions of the dimethyl ester of protoporphyrin-IX (**2**) obtained from incubation of the stereoselectively labelled porphobilinogen (**8**) with chicken red cell haemolysates.

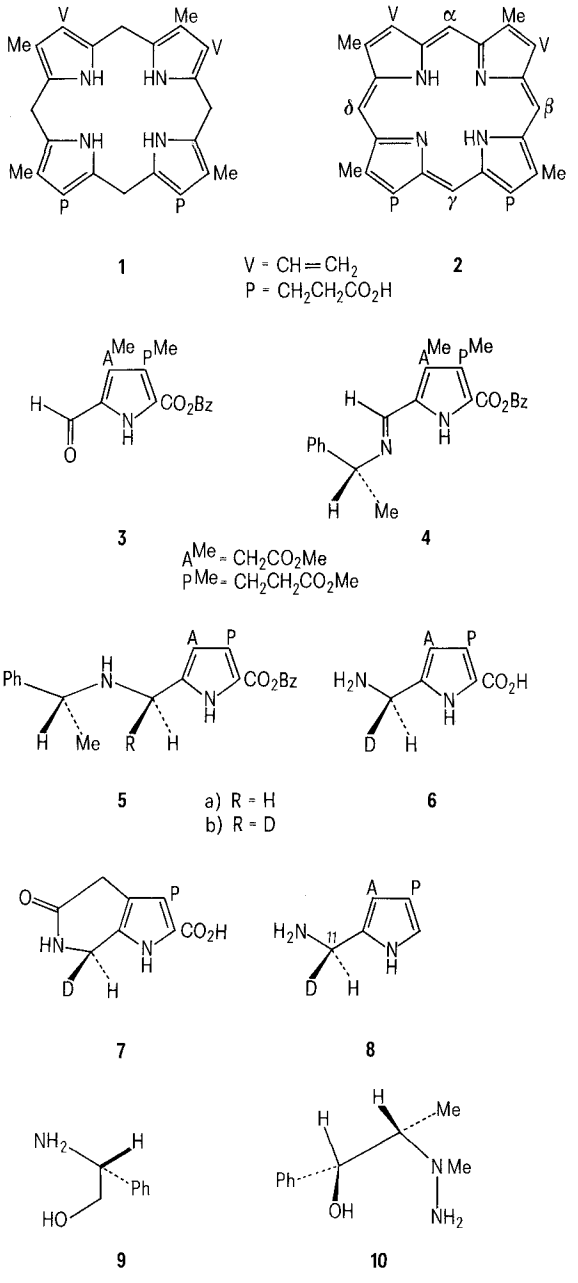
clearly show n.O.e.'s (5–6%) between the imine CH resonances and those of the CH_2 protons of the neighbouring β-substituent in the pyrrole ring; thus the preferred conformation of the imine **4** is that shown in figure 2. By applying the same conformational principles as in Cram's rule for the reduction of chiral ketones[7] we may deduce that deuterated diborane (and related reducing agents) will approach the imine as shown by the arrow (fig. 2) and lead predominantly to deuterated amine of structure (**5b**) in which the configuration of the deuterium bearing carbon is R.

Incorporation of labelled porphobilinogen **8** (3 mg) into protoporphyrin-IX **2** was studied with a chicken red-cell haemolysate preparation and the product (0.8 mg) was isolated as the dimethyl ester. Careful measurements of the integrals of the *meso*-proton resonances in the 360 MHz spectrum (fig. 3) of the protoporphyrin-IX dimethyl ester (using the vinyl proton resonances as internal standards) gave the labelling pattern shown in the table. The correspondence between the

Incorporation of labelled porphobilinogen into protoporphyrin-IX by chicken red cell haemolysates

Porphobilinogen			Percentage retention of hydrogen isotope at the *meso*-positions of protoporphyrin or haemin			
			α	β	γ	δ
a) 11R-^{2}H, 40% e.e.	^{1}H	obs.	32	70	34	36
		calc.	32	72	32	32
b) 11S-^{3}H, 2,11-$^{14}C_2$ (91% 11S-^{3}H)	^{3}H	obs.	9	73	9	9

a) The sample used for these experiments was estimated by NMR and mass spectrometry to contain 68% 11R-^{2}H, 28% 11S-^{2}H and 4% unlabelled PBG. The intensities of the four *meso*-proton signals in the protoporphyrin-IX dimethyl ester isolated were measured by careful comparisons with the intensity of the signal at δ 8.3 corresponding to the α-methine signals of the vinyl groups (see fig. 2). The accuracy of the integrations is estimated at ± 2%. b) Results reported by Akhtar and Jordan[3] for haemin obtained from tritium-labelled PBG.



Formulae (**5b**), (**6**), (7) and (**8**) show the configurations of the major deuterium-labelled isomers.

observed and calculated intensities is consistent with an overall stereospecific process in which the 11R-^{2}H of porphobilinogen **8** is lost from the β-*meso*-position, and retained at the other three *meso*-positions of protoporphyrin-IX. The results are also in clear accord with Akhtar and Jordan's findings[3] with tritium-labelled porphobilinogen, prepared enzymically from tritium-labelled glycine (table).

Experiments are in progress to attain a higher degree of selectivity in the synthesis of the labelled porphobilinogen including the use of R-2-amino-2-phenyl ethanol (**9**) and N-aminoephedrine[8] (**10**) as chiral inducing agents instead of R-1-phenylethylamine. In our hands the former reagent (**9**) afforded a cleaner product in higher yield than the N-aminoephedrine[9] (**10**), and material of 88% e.e. was obtained as will be described in detail in a full paper. However, the Cambridge group recently reported[10] that the use of 1R,2S-ami-

noephedrine, and its enantiomer afforded 11S and 11R-[^{2}H]-porphobilinogen (66% e.e.) respectively; our findings are in accord with these results as the use of R-1-phenylethylamine and R-2-amino-2-phenylethanol both afforded 11R-[^{2}H]-porphobilinogen (40 and 88% e.e. respectively).

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Reversible reduction in bone blood flow in streptozotocin-diabetic rats

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Summary. Tibial growth and blood flow were both found to be markedly reduced in anaesthetised streptozotocin-diabetic rats compared to controls. Insulin treatment restored tibial growth to approximately control values and increased tibial blood flow to above control values. The observations are likely to be related to reduced bone turnover in uncontrolled diabetes.
Key words. Streptozotocin; diabetes; rat; insulin; bone; blood flow.

The normal development of bone is impaired in human diabetes. Deficits in bone mass[1] and height[2] may be demonstrated in insulin dependent diabetes after several years of the disease. The incidence of osteoporosis is also higher in sex and age matched diabetics[3,4]. Decreased bone formation has been demonstrated in animal models of diabetes[5,6]. Reduced bone turnover has been reported to occur in streptozotocin-diabetic rats with calcium deposition being more severely depressed than calcium loss so that the balance may approach zero[7,8]. A study of regional blood flow in pithed rats has reported a decrease in bone flow in streptozotocin treated animals compared to controls[9]. Autonomic control of the cardiovascular system has been reported to be abnormal by numerous studies in human and experimental animal diabetes[10]. Since pithing suppresses autonomic control of the cardiovascular system, it cannot be assumed that changes in regional blood flow observed in pithed rats will also occur in intact animals. We, therefore, considered it of interest to measure regional blood flow, including bone blood flow, in intact anaesthetised streptozotocin-diabetic rats and to see if any changes observed were modified by insulin treatment.

Materials and methods. Male Wistar rats (200–250 g) received 1 ml · kg^{-1} of freshly prepared solution of streptozotocin (55 mg × ml^{-1}, pH 4.5 citrate buffer) or buffer alone via a tail vein. The animals receiving buffer only served as controls. 14, 28 and 56 days later streptozotocin-treated and control rats were anaesthetised with pentobarbitone (60 mg · kg^{-1}). Cardiac output and its distribution to a number of tissues, including the left tibia was then estimated using ^{46}Sc labelled 15-μm diameter polystyrene microspheres (New England Nuclear Chemicals, Drieich, FRG). The method has been described in detail elsewhere[9]. Briefly, cannulae were placed in the left ventrical of the heart for microsphere injection, in the right femoral artery for blood withdrawal. Approximately 150,000 microspheres suspended in 0.15 ml were injected slowly over about 10 s. Blood withdrawal (0.43 ml · min^{-1}) was started 5 s before the injection of microspheres and continued for 20 s after its completion. Cardiac output (ml · 100 g^{-1} min^{-1}) was estimated as withdrawal rate (0.43 ml · min^{-1}) × dpm injected × 100/b. wt (g) × dpm with withdrawn arterial blood sample. Tissues were removed within 20 min of the microsphere injection and their blood flows (ml · 100 g · min^{-1}) estimated as cardiac output × b.wt (g) × dmp of tissue × 100/wet wt of tissue (g) × dpm, injected.

The above procedure was also carried out using a diabetic group which had received 4U protamine Zn insulin. Kg^{-1} s.c. at 9.00 and 17.00 h each day from 3 days after the streptozotocin dose until use on day 14 (they received the morning insulin dose on this day). Blood flow estimations were all carried out at approximately 13.00 h. The length as well as the weight of each tibia was noted. Blood glucose was determined in 0.1-ml samples of whole blood taken approximately 30 s after the microsphere injection by a micro-colorimetric copper reduction method[11]. The rats were each weighed at the start of the experiment and again just before the blood flow measurements. At no time were they denied access to food or water.

Results. Diabetes was confirmed in the streptozotocin treated animals by their raised blood glucose concentrations and negative growth rates. Insulin treatment returned both variables in approximately control values.

Overall tissue blood flow (cardiac output) was not significantly different in 14- or 28-day untreated diabetic groups compared to their controls, while in 56-day untreated diabetic animals it was increased by approximately 50%. Tibial blood flow was markedly reduced in all three groups compared to controls. The effect appeared to be maximal by 14