nonpolar) solutes, in water, as indicated at the end of the previous section.

Concluding Remarks

The shell model,[36,37] with its recent improvements,[35] is a convenient one for including the effect of hydration in conformational energy calculations on proteins and in calculations of the interactions between macromolecules in aqueous solution. The parameters of this model can be improved by using the results of more accurate calculations on aqueous solutions of polar and nonpolar solutes. In the last analysis, the computations of the thermodynamic parameters for the interactions of such solutes in water must agree with experimental data. The theory can then be used to provide details of the interactions that are inaccessible to experiment; e.g., the free energy of hydration of a molecule can be partitioned into additive contributions from its component groups, for direct use in the shell model. The statistical mechanical methods described herein have the potential for yielding accurate theoretical data if a properly formulated potential function is used. The EPEN potential provides a framework, which, with improvements and modifications, can meet the requirements of a properly formulated potential function. Finally, the methods of umbrella sampling or integration with the use of a coupling parameter may help solve the problem of the computation of accurate free energies of hydration.

The work described here that was carried out in the author's laboratory was supported by research grants from the National Science Foundation (PCM75-08691) and the National Institute of General Medical Sciences of the National Institutes of Health, U.S. Public Health Service (GM-14312).

Origin of the Pigments of Life: The Type-III Problem in Porphyrin Biosynthesis

ALAN R. BATTERSBY* and EDWARD MCDONALD

University Chemical Laboratory, Cambridge CB2 1EW, United Kingdom

Received February 2, 1978

Life on this planet depends ultimately on the photosynthetic activities of the chlorophylls,[1a] which are macrocyclic complexes of Mg^{2+}. In addition, electron transport in living systems and other vital functions such as reduction of oxygen and hydroxylation reactions are based on the cytochromes,[1b] which carry one or more units of a number of different heme systems (cf. **5**). When one adds that protoheme (**5**) is the oxygen-carrying unit of hemoglobin and myoglobin, it becomes evident that this whole family of macrocyclic pigments is of vital importance.

All the natural tetrapyrrolic macrocycles mentioned so far are derived in living things from uroporphyrinogen III (**1**), abbreviated throughout to uro'gen-III. In animals, plants, and most bacteria, the major pathway[2a] involves decarboxylation of **1** to yield copro'gen-III[2b] (**2**), which by oxidative decarboxylation produces proto'gen-IX (**3**). Aromatization to **4** followed by metal insertion yields protoheme (**5**), and, with esterification, the magnesium complex (**6**) for photosynthetic organisms. The intermediates **5** and **6** are then used to build one set of life's pigments, as indicated in Scheme I.

A second recently discovered pathway operates in certain bacteria, yeasts, and spinach to convert uro'gen-III (**1**) into siroheme (**8**), the prosthetic group of several sulfite reductases and nitrite reductases. Presumably siroheme (**8**) is derived from sirohydrochlorin[3] (**7**), and this substance (or possibly a dihydro form of it) has been proved[3b] to be a precursor of cobyrinic acid (**9**) and so of coenzyme B_{12}. Scheme II shows the relationships of this second set of pigments.

The central role of uro'gen-III (**1**) as the parent of all these vitally important metalloporphyrins, -chlorins, -isobacteriochlorins, and -corrins highlights the problem of its biosynthesis, a problem which is fascinating in its own right.

Enzymes and Building Blocks for Uro'gen-III. The pioneering studies of Shemin, Granick, Bogorad, Neuberger, and Rimington[2] established that two enzymes are required to catalyze the conversion of 4 mol of the monopyrrole, porphobilinogen, PBG (**10**), into uro'gen-III (**1**) and ammonia (Scheme III). The names of these enzymes, PBG-deaminase and uro'gen-III

A. R. Battersby graduated at the University of Manchester and received the Ph.D. and D.Sc. degrees from the Universities of St. Andrews and Bristol. He spent a period in the United States at the Rockefeller Institute for Medical Research and at the University of Illinois. In 1962, he became Professor of Organic Chemistry at the University of Liverpool where he remained until 1969 when he moved to Cambridge. He is a Fellow of the Royal Society.

Edward McDonald was born in Ormskirk, Lancashire, in 1943, and is University Lecturer at the University of Cambridge. During 1967–1969, he worked as a postdoctoral fellow with E. J. Corey at Harvard, after graduating from Imperial College and obtaining his Ph.D. from the University of Liverpool. Besides his research interest in the chemistry of porphyrins and corrins, he works on phenol oxidation and the synthesis of natural products.

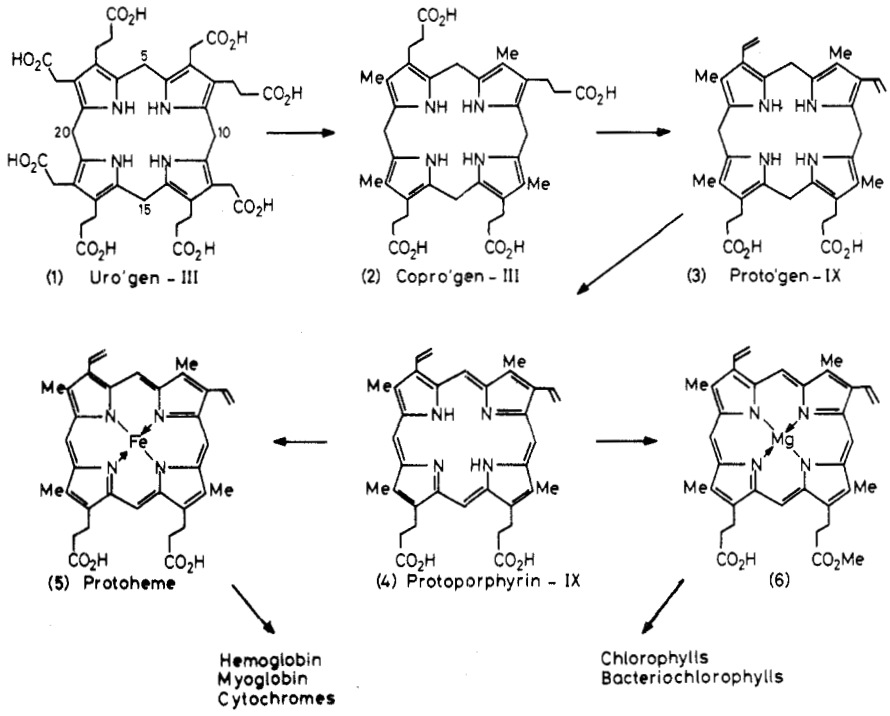
(1) Reviewed in (a) "The Chlorophylls", L. P. Vernon and G. R. Seely, Eds., Academic Press, New York, 1966; (b) R. Lemberg and J. Barrett, "Cytochromes", Academic Press, London, 1973.

(2) (a) Reviewed by A. R. Battersby and E. McDonald, "Porphyrins and Metalloporphyrins", K. M. Smith, Ed., Elsevier, Amsterdam, 1975, p 61. (b) Abbreviations used: PBG, porphobilinogen; ALA, δ-aminolevulinic acid; uro'gen-III, uroporphyrinogen III; copro'gen-III, coproporphyrinogen III; proto'gen IX, protoporphyrinogen IX.

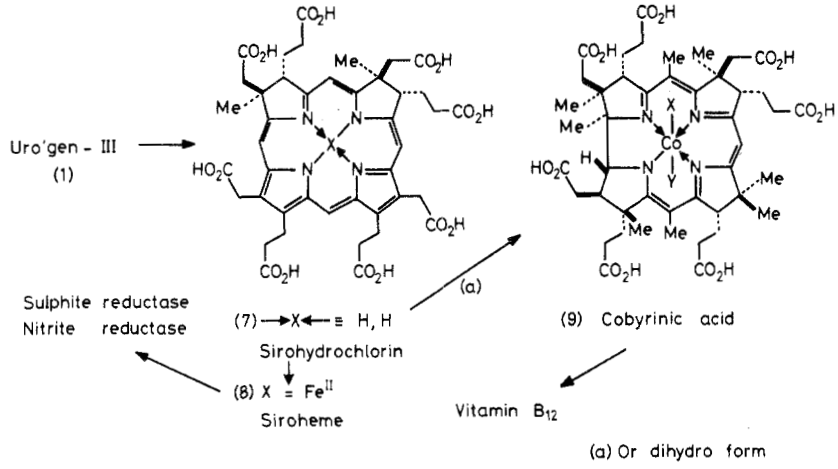
(3) (a) Isolation: M. J. Murphy, L. M. Siegel, H. Kamin, and D. Rosenthal, *J. Biol. Chem.*, **248**, 2801 (1973); M. J. Murphy and L. M. Siegel, *ibid.*, **248**, 6911 (1973); and references therein; see also H. E. Jones and G. W. Skyring, *Aust. J. Biol. Sci.*, **27**, 7 (1974). (b) Structure: A. R. Battersby, K. Jones, E. McDonald, J. A. Robinson, and H. R. Morris, *Tetrahedron Lett.*, 2213 (1977); A. R. Battersby, E. McDonald, H. R. Morris, M. Thompson, D. C. Williams, V. Ya. Bykhovsky, N. I. Zaitseva, and V. N. Bukin, *ibid.*, 2217 (1977); A. R. Battersby, E. McDonald, M. Thompson, and V. Ya. Bykhovsky, *J. Chem. Soc., Chem. Commun.*, 150 (1978); R. Deeg, H.-P. Kriemler, H.-H. Bergmann, and G. Müller, *Z. Physiol. Chem.*, **358**, 339 (1977); A. I. Scott, A. J. Irwin, L. M. Siegel, and J. N. Shoolery, *J. Am. Chem. Soc.*, **100**, 316 (1978); A. I. Scott, *Acc. Chem. Res.*, 11, 29 (1978).

0001-4842/79/0112-0014$01.00/0

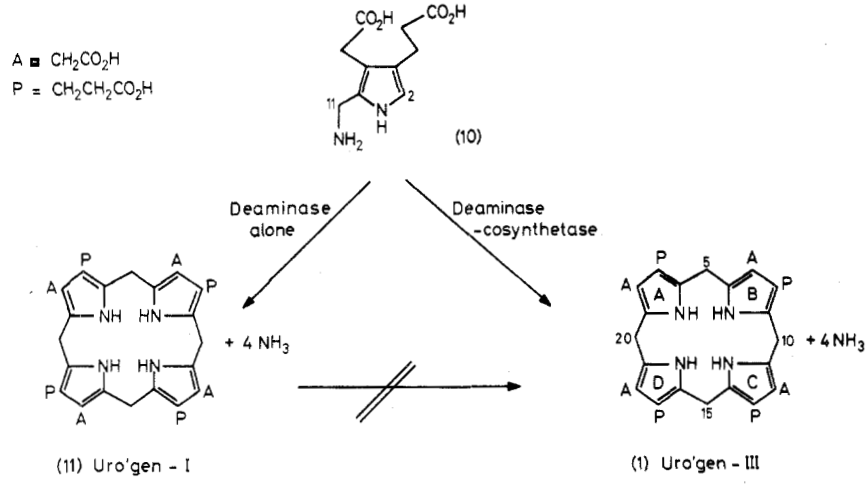
Scheme I



Scheme II

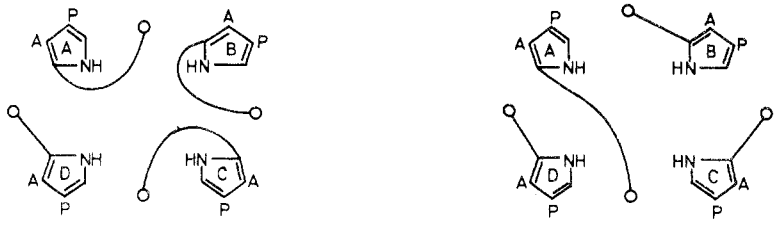


Scheme III



cosynthetase, will be abbreviated here to "deaminase" and "cosynthetase". In the absence of cosynthetase, deaminase catalyzes the conversion of PBG into uro'gen-I (11), an unnatural isomer but that expected from simple head-to-tail combination of four PBG units. However, uro'gen-I (11) is not transformed into

Scheme IV



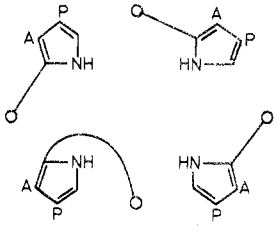
uro'gen-III (1) by cosynthetase alone nor by the complete deaminase–cosynthetase system. How then does the complete enzyme system bring about the molecular rearrangement required to produce the unexpected type-III isomer, and what are the intermediates in this intriguing transformation? At the outset of our studies in 1969–1970, there were no answers to these questions, but they had attracted so much attention that ca. 25 different hypothetical mechanisms had been proposed. A few of the compounds suggested as intermediates in these mechanistic schemes had been synthesized and tested as biosynthetic precursors, but none had been incorporated into a type-III porphyrin.

Nature of the Rearrangement. In order to produce uro'gen-III (1) from PBG (10), deaminase–cosynthetase must be capable at some stage of breaking the bond linking *one or more* of the C-11 methylene groups to their pyrrole nuclei of the four PBG units. On this basis, there are in principle 24 different ways in which four PBG molecules (10) may be assembled to produce uro'gen-III; two of these are illustrated in Scheme IV. Assembly W[4] would require the units corresponding to rings B, C, and D to be rearranged intramolecularly en route to uro'gen-III (1), whereas assembly X[5,6] corresponds to a different type of rearrangement involving only that PBG molecule which becomes ring A. Reasonable chemical mechanisms can be written for the pathways corresponding to many of the 24 hypothetical possibilities.

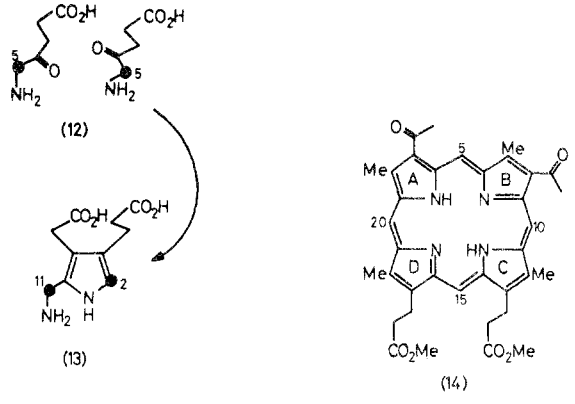
These various possible assemblies differ in the origin of the four bridge carbons of uro'gen-III (carbons 5, 10, 15, and 20) *relative to the rings to which they were originally attached*, and this analysis led us to studies with diluted doubly labeled PBG carrying ^{13}C at C-2 and C-11 (13). This approach proved conclusively,[7] as follows, that uro'gen-III (1) is biosynthesized from four PBG units assembled uniquely as indicated in Scheme V.

The doubly labeled sample of PBG (13) was prepared from 90 atom % carbon-13 [5-^{13}C]aminolevulinic acid[8] (12), ALA, using the enzyme ALA-dehydratase. No dilutions were made throughout this synthesis, so 81%

Scheme V



of the PBG molecules were doubly labeled as in structure 13. This PBG sample (one part) was diluted with normal unenriched PBG (four parts) in which only 0.01% of the molecules carry two ^{13}C atoms, as in 13.



The resultant PBG sample thus had a strongly nonstatistical distribution of ^{13}C atoms, and it (140 mg) was incubated with an enzyme system from the alga, *Euglena gracilis*. The product (35 mg) was protoporphyrin IX (labeled 4), formed because the biological system contained all the enzymes necessary to effect conversion of the uro'gen-III (labeled 1), as it was formed, through the steps in Scheme I as far as labeled 4. This product was then converted chemically into the diketone (labeled 14).

The low-field part of the ^1H-decoupled ^{13}C NMR spectrum of the labeled diketone 14 is shown in Figure 1. The signals from the bridge carbons C-5, C-10, and C-20 were 5.5 Hz doublets, and that for C-15 was a 72-Hz doublet.[9] This spectrum established that the

(4) R. Robinson, "The Structural Relations of Natural Products", Clarendon Press, Oxford, 1955, p 22.

(5) S. Granick, quoted by D. Shemin in "Ciba Foundation Symposium on Porphyrin Biosynthesis and Metabolism", G. E. W. Wolstenholme and E. C. P. Millar, Eds., Churchill, London, 1955, p 143; P. Cornford, *Biochem. J.*, **91**, 64 (1964); E. B. C. Llambias and A. M. del C. Batlle, *ibid.*, **121**, 327 (1971).

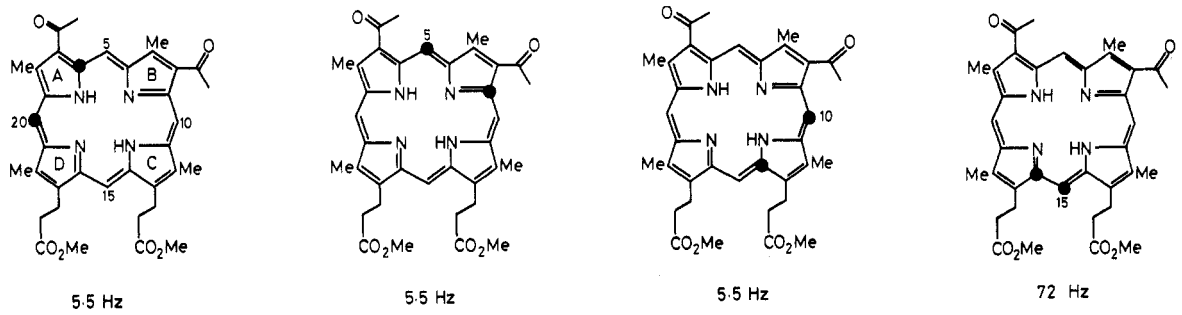
(6) R. Radmer and L. Bogorad, *Biochemistry*, 11, 904 (1972).

(7) A. R. Battersby, E. Hunt, and E. McDonald, *J. Chem. Soc., Chem. Commun.*, 442 (1973); A. R. Battersby, G. L. Hodgson, E. Hunt, E. McDonald, and J. Saunders, *J. Chem. Soc., Perkin Trans. 1*, 273 (1976).

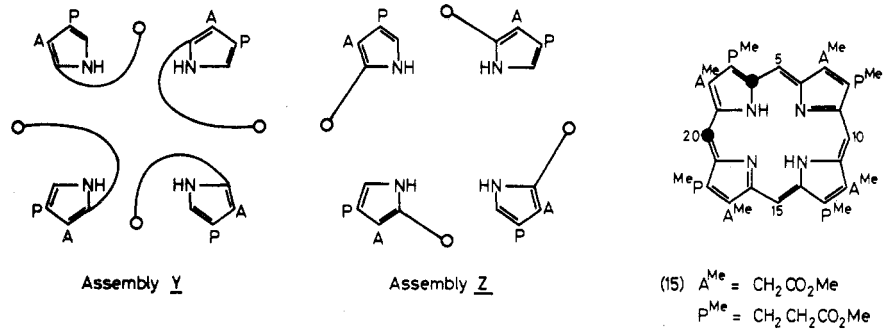
(8) A. R. Battersby, E. Hunt, E. McDonald, and J. Moron, *J. Chem. Soc., Perkin Trans. 1*, 2918 (1973).

(9) The chemical shifts for C-5, C-10, C-15, and C-20 were assigned by rational total synthesis of three samples of 14 specifically labeled at C-10, C-15, and C-20, respectively: A. R. Battersby, G. L. Hodgson, M. Ihara, E. McDonald, and J. Saunders, *J. Chem. Soc., Chem. Commun.*, 441 (1973); *J. Chem. Soc., Perkin Trans. 1*, 2923 (1973). Also, the couplings of 72 and 5.5 Hz were proved to correspond, respectively, to *direct bonding* of two ^{13}C atoms and to *through-three-bond coupling*, as in Scheme VI, by unambiguous preparation of the appropriate doubly labeled porphyrins: A. R. Battersby, M. Ihara, E. McDonald, J. Saunders, and R. J. Wells, *J. Chem. Soc., Perkin Trans. 1*, 283 (1976).

Scheme VI



Scheme VII



major components[10] in the labeled sample of **14** were the four doubly labeled species in Scheme VI. It was thus rigorously proved[7] that the three PBG molecules that provide rings A, B, and C with their attached carbons at C-20, C-5, and C-10, respectively, are *incorporated intact*, whereas that providing ring D has undergone *intramolecular rearrangement*,[11] with the rearranging carbon appearing at C-15.

Exactly the same conclusion was derived from studies of the incorporation of the same diluted [2,11-$^{13}C_2$]PBG (**13**) into protoporphyrin IX (labeled **4**) by an enzyme preparation from chicken blood and beef mitochondria[7] and also into copro'gen-III (labeled **2**) by *Propionibacterium shermanii*.[12] Furthermore, it has been demonstrated[13] that during enzymic conversion of uro'gen-III (**1**) via copro'gen-III (**2**) into protoporphyrin IX (**4**) (see Scheme I) the macrocycle remains intact and unscrambled. All the foregoing results obtained for protoporphyrin IX (**4**) and copro'gen-III (**2**) therefore hold good for uro'gen-III (**1**). It follows that the deaminase–cosynthetase system assembles four PBG molecules to form uro'gen-III in exactly the same manner in a photosynthetic alga, in avian blood and in a bacterium, and the mode of assembly is that illustrated in Scheme V.

Is Uro'gen-I Formed Enzymically without Rearrangement? It may appear obvious that uro'gen-I (**11**) is formed from PBG by deaminase without rearrangement, but this is not necessarily so. For example, assembly Y in Scheme VII would require deaminase to bring about consistent head-to-head reaction of the PBG units and rearrangement at each stage of the pathway.

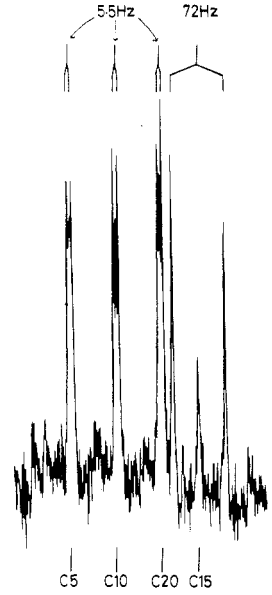


Figure 1. ^{13}C NMR signals from bridge carbon atoms of the diacetylporphyrin (Scheme VI).

To resolve this problem, [2,11-$^{13}C_2$]PBG (**13**), diluted with unlabeled material as earlier, was converted by deaminase from *E. gracilis* into uro'gen-I which was transformed chemically into uroporphyrin I ester (**15**) for isolation and ^{13}C NMR. The signals from the C-5, C-10, C-15, and C-20 bridges overlap and appeared as a 5-Hz doublet centered on a singlet. The ratio of the intensities of the split and unsplit signals was that expected for intact incorporation of four unrearranged PBG units, as indicated by assembly Z in Scheme VII.[12]

It is now appropriate to suggest a sequence[14] (Scheme VIII) for the formation of uro'gen-I (**11**) by deaminase,

(10) Minor amounts of species containing two or more labeled PBG units will also be present; these are unimportant for the argument.

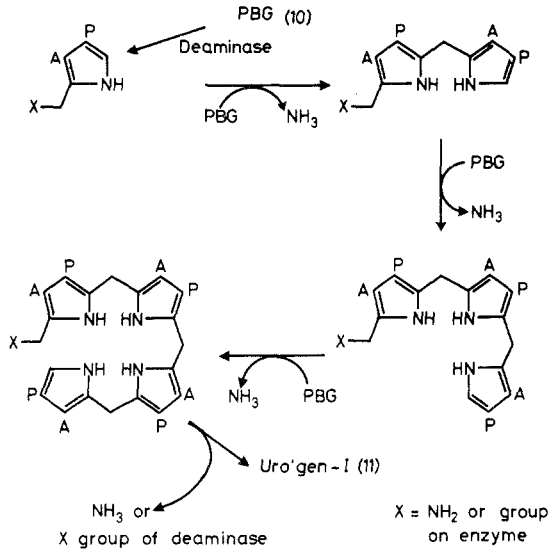
(11) Because of the heavy dilution with nonenriched PBG, an *intermolecular rearrangement* would have resulted in a statistical distribution of ^{13}C at C-15 and C-16. These ^{13}C atoms would therefore have been largely in *different molecules*, and the major signal from C-15 would have been a singlet.

(12) R. Hollenstein, unpublished work, Cambridge, 1976.

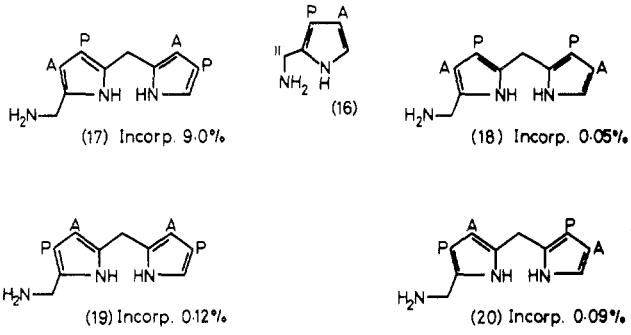
(13) B. Franck, D. Gantz, F.-P. Montforts, and F. Schmidtchen, *Angew. Chem., Int. Ed. Engl.*, **11**, 421 (1972); A. R. Battersby, J. Staunton, and R. H. Wightman, *J. Chem. Soc., Chem. Commun.*, 1118 (1972); A. R. Battersby, E. McDonald, J. R. Redfern, J. Staunton, and R. H. Wightman, *J. Chem. Soc., Perkin Trans. 1*, 266 (1976).

(14) Variants of this sequence are conceivable, but Scheme VIII provides a valuable framework for subsequent discussion.

Scheme VIII



Scheme IX



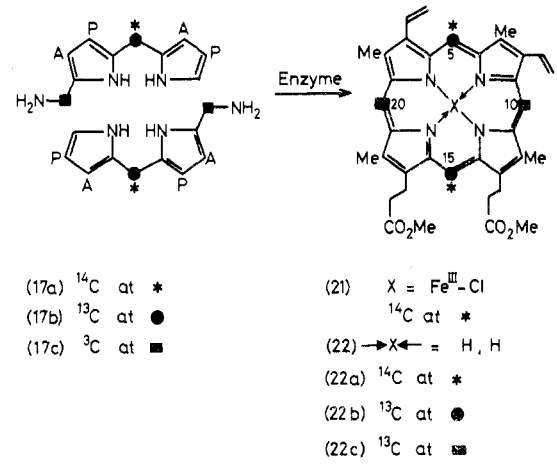
secure in the knowledge that this enzyme effects consistent head-to-tail reaction of the four PBG units. It is important to stress that some, or all, of the intermediates may be strongly bound to the enzyme, perhaps covalently (see Scheme VIII).

Role of Cosynthetase: Timing of Rearrangement. Previous sections have established that deaminase alone catalyzes head-to-tail coupling of four PBG units to produce uro'gen-I (**11**) and that deaminase–cosynthetase together catalyze the formation of uro'gen-III (**1**) from three intact PBG units and one intramolecularly rearranged unit (corresponding to ring D and C-15). Cosynthetase must bring about this rearrangement either by operating on an intermediate produced by deaminase or by modifying the way in which deaminase brings about one of the coupling steps. Cosynthetase might operate at any stage in the overall process, i.e., at the monopyrrole, dipyrrole, tripyrrole, or tetrapyrrole levels, and a distinction should be possible by testing the hypothetical intermediates of Scheme VIII, and rearranged isomers, as precursors of uro'gen-III (**1**).

(*a*) *Is the Rearrangement at the Monopyrrole Level?* If rearrangement is at the first step, then iso-PBG (**16**), Scheme IX, would be expected to be incorporated by deaminase–cosynthetase into uro'gen-III (**1**) in the presence of PBG (**10**). Earlier work[15] pointed against this possibility, but incorporation experiments were not studied. Accordingly, [11-^{14}C]iso-PBG (**16**) was synthesized[16] and with unlabeled PBG was incubated

(15) A. T. Carpenter and J. J. Scott, *Biochim. Biophys. Acta*, **52**, 195 (1961).

Scheme X



with the enzyme preparation from *E. gracilis* rich in deaminase–cosynthetase. The incorporation of radioactivity into protoporphyrin IX (**4**) was <0.01% when parallel runs with [11-^{14}C]PBG (as **10**) gave incorporations up to 40%.

This negative result[16] indicated strongly that iso-PBG (**16**) is *not* involved in the formation of uro'gen-III (**1**) and that the rearrangement occurs at a later stage in the biosynthesis; interlocking positive results (see following sections) confirmed this view.

(*b*) *Is the Rearrangement at the Dipyrrole Level?* Pyrromethanes (e.g., **17**) have been prepared in several laboratories and tested for incorporation into type-III porphyrins in the presence of deaminase–cosynthetase. This part of the story of type-III porphyrins has been very confusing, and the interested nonexpert must be constantly assessing what has been unambiguously proved and what has not. In particular, he must remember that a ^{14}C-labeled precursor has not been proved to be incorporated into a product until the latter has (a) been shown to be radiochemically pure and (b) has been degraded to demonstrate a specific labeling pattern as distinct from scattered activity. Fortunately, the picture has clicked into sharp focus during the past year, which makes this Account a timely one. Discussion of the related studies of other workers will follow a survey of our results.

Two PBG units can in principle be joined in four different ways to produce the aminomethylpyrromethanes **17**, **18**, **19**, and **20**. All four have been synthesized[17,18] in methylene-^{14}C-labeled form and tested as precursors of uro'gen-III (**1**) in the presence of PBG and in its absence; at least two preparations of deaminase–cosynthetase from different biological

(16) R. E. Markwell, unpublished work, Cambridge, 1974.

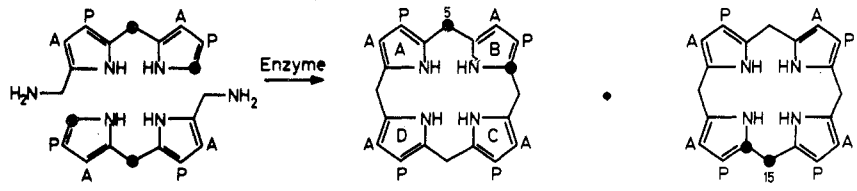
(17) A. R. Battersby, D. A. Evans, K. H. Gibson, E. McDonald, and L. N. Nixon, *J. Chem. Soc., Perkin Trans. 1*, 1546 (1973); A. R. Battersby, J. F. Beck, and E. McDonald, *ibid.*, 160 (1974).

(18) Our synthetic routes[17] and those from other laboratories[19,20] afford the crystalline lactam trimethyl esters corresponding to **17**, **18**, **19**, and **20**. Dr. R. E. Markwell (Cambridge) showed that a mixture of the four lactams can be separated by liquid chromatography and in this way it was demonstrated that each of our crystalline lactams was free of the other isomers. The aminomethylpyrromethane obtained by alkaline hydrolysis of each lactam was examined by ^{1}H NMR which established that no significant isomerization had occurred during the hydrolytic step.

(19) B. Frydman and R. B. Frydman, *Acc. Chem. Res.*, **8**, 201 (1975), and references therein.

(20) Cf. J. Bausch and G. Müller, *Enzyme*, **17**, 47 (1974); J. M. Osgerby, J. Pluscec, Y. C. Kim, F. Boyer, N. Stojanac, H. D. Mah, and S. F. MacDonald, *Can. J. Chem.*, **50**, 2652 (1972).

Scheme XI



sources were used in each case. The percentages in Scheme IX show our *highest* incorporations of radioactivity into type-III porphyrins found after a variety of different incubations.[21,22] It can be seen that the values for the rearranged compounds **18**, **19**, and **20** were so low[23] that the labeling pattern could not be checked by degradation.

In contrast (Scheme X), the relatively high level of incorporation of radioactivity from the unrearranged [^{14}C]pyrromethane (**17a**) (designated AP·AP in the sequel) into protoporphyrin IX ester (**22a**) and hemin ester (**21**) using hemolyzed duck blood allowed oxidative degradation[24] of **21**, which showed that its ^{14}C activity was essentially equally distributed between C-5 and C-15. It followed that [^{14}C]AP·AP (**17a**) had been specifically incorporated into uro'gen-III (1) in the presence of deaminase–cosynthetase to label C-5 and C-15 equally.[21,25]

This specific incorporation of AP·AP without PBG into uro'gen-III was also found[21] with the enzymes from *E. gracilis*. The uro'gen-III formed was again enzymically converted in situ into protoporphyrin IX, and [*bridge-methylene*-^{13}C]AP·AP (**17b**) led to [5,15-$^{13}C_2$]protoporphyrin IX (**22b**), whereas [*amino-methylene*-^{13}C]AP·AP (**17c**) gave [10,20-$^{13}C_2$]protoporphyrin IX (**22c**). The sites of labeling were read directly from the ^{13}C NMR spectra of the corresponding methyl esters using the earlier unambiguous assignments.[26]

These experiments conclusively prove that, in the presence of deaminase–cosynthetase, two molecules of unrearranged AP·AP (**17**) can combine and rearrange in a highly specific manner to generate uro'gen-III (**1**). It was now necessary to determine how much of this uro'gen-III arises by competing nonenzymic reactions, and this demanded (a) a method for isolation of the corresponding uroporphyrins from the reaction mixture, not a trivial problem since any excess pyrromethane could be chemically converted into porphyrins with extreme ease, and (b) a reliable analysis for the four isomeric uroporphyrins.

Isolation and Quantitative Analysis of Uroporphyrin Isomers by Liquid Chromatography (High Pressure). Treatment of the reaction products from enzymic or chemical runs with an excess of I_2 both aromatized the uro'gens to uroporphyrins and destroyed unchanged pyrromethanes. The product(s) from the latter were shown not to be converted chemically into porphyrins during subsequent workup.

The mixture of uroporphyrins, isolated as esters, was then decarboxylated (at the acetic acid side chains) with hot acid[30] to the corresponding mixture of coproporphyrin isomers. A two-stage LC separation of the tetramethyl esters allowed the four coproporphyrin isomers to be collected separately and assayed quantitatively by UV–vis spectroscopy (and by counting if radioactive).

With this solid base of reliable methods available, the problem of chemical vs. enzymic formation of uro'gen-III (**1**) from AP·AP (**17**) could finally be settled.

Elimination of Significant Chemical Formation of Uro'gen-III. Incubation of [^{14}C]AP·AP (**17a**) at pH 7.2 for 16 h *without enzyme* followed by the above workup gave an ~30% yield of uroporphyrins containing 68% type I and 28% type IV, the remainder (~4%) being types II and III. When a strictly parallel experiment was run but with the addition of purified deaminase–cosynthetase, the isomer ratio was dramatically altered; type-III formation vastly increased (to 54%) at the expense of type-I formation (now only 15%).

These results[31] show clearly that deaminase–cosynthetase *does* catalyze the formation of uro'gen-III when unrearranged AP·AP (**17**) is provided. In addition, the doubly $^{13}C_2$-labeled form of AP·AP (**17d**), Scheme XI, was synthesized[17,20] from [2,11-$^{13}C_2$]PBG (**13**), diluted with unlabeled PBG, and incubated with purified deaminase–cosynthetase to produce copro-

(21) A. R. Battersby, D. A. Evans, K. H. Gibson, E. McDonald, L. N. Mander, and J. Moron, *J. Chem. Soc., Chem. Commun.*, 768 (1973).

(22) A. R. Battersby and E. McDonald, *Philos. Trans. R. Soc. London, Ser. B*, **273**, 161 (1976).

(23) Pyrromethane (**20**) was included in the set even though it cannot be involved in the biosynthesis. Its structure requires *both* PBG units to have rearranged, and this contrasts with *single* rearrangement established in earlier sections for type-III porphyrin biosynthesis. Nevertheless, a small incorporation of radioactivity occurred; no reliance can be placed on such small values, and we shall comment on their probable origin later.

(24) R. Bonnett and A. F. McDonagh, *Chem. Commun.*, 237 (1970).

(25) A. R. Battersby, Special Lectures, *Int. Congr. Pure Appl. Chem., 23rd*, **5**, 1 (1971).

(26) It is important at this stage to deal with Frydman's suggestion[27] that the work outlined so far on AP·AP (**17**) may not be leading to protoporphyrin IX (**22**) as stated but to an unnatural isomer, protoporphyrin XIII. His view was based on the known *chemical* formation by rearrangement of some uro'gen-IV from AP·AP[21,27] which could then be converted enzymically via copro'gen-IV into protoporphyrin XIII; the terminal enzymes of Scheme I are known[2] to act on these unnatural isomers. This in *not* the explanation of the results in the main text, and our product is unquestionably protoporphrin IX (**22**) on the following grounds: (a) the recently proved structure[28,29] of protoporphyrin XIII has a plane of symmetry which renders C-10 and C-20 equivalent (cf. **22**); the *four different* ^{13}C signals observed from the bridge carbons of the labeled biosynthetic products **22b** and **22c** could not have arisen from this isomer; (b) protoporphyrin IX and protoporphyrin XIII dimethyl esters are separable by LC, and the product from the enzymic experiments was distinguished in this way from isomer XIII and identified with isomer IX.

(27) R. B. Frydman and B. Frydman, *FEBS Lett.*, **52**, 317 (1975).

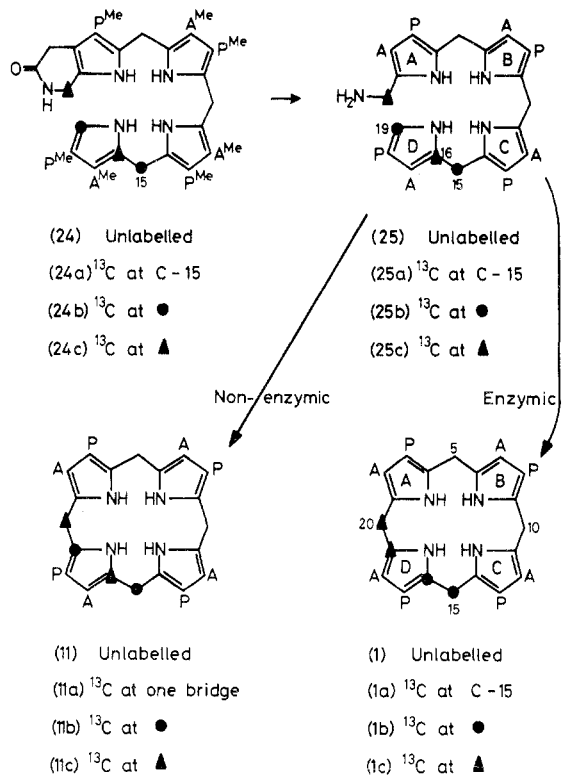
(28) Structure: A. R. Battersby, L. Mombelli, and E. McDonald, *Tetrahedron Lett.*, 1037 (1976); H. M. G. Al-Hazimi, A. H. Jackson, D. J. Ryder, G. H. Elder, and S. G. Smith, *J. Chem. Soc., Chem. Commun.*, 188 (1976).

(29) Synthesis: G. Buldain, J. Hurst, R. B. Frydman, and B. Frydman, *J. Org. Chem.*, **42**, 2953 (1977); A. R. Battersby, A. D. Hamilton, E. McDonald, and O. H. Wong, in preparation.

(30) "Porphyrins and Metallophyrins", K. M. Smith, Ed., Elsevier, Amsterdam, 1975, p 825.

(31) A. R. Battersby, D. G. Buckley, E. McDonald, and D. C. Williams, *J. Chem. Soc., Chem. Commun.*, 115 (1977).

Scheme XII



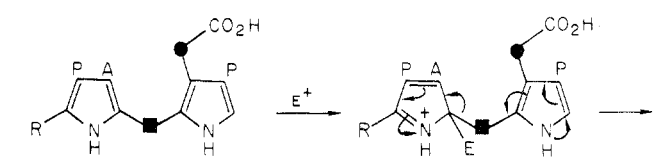
porphyrin III by the same steps as above. The ^{13}C NMR signal unambiguously from C-15 of the corresponding ester was a 72-Hz doublet centered on a smaller singlet.[32] It followed that the labeling pattern around C-15 was that shown in Scheme XI for uro'gen-III (**1d** and **1e**) and the signal from C-5 was consistent with the illustrated arrangement around ring B. The ratio of the split to unsplit signals from C-15 was in agreement with an *intramolecular* rearrangement of ring D *exactly as had been found earlier for PBG itself.* Powerful evidence was thus added from another angle showing that the conversion of AP·AP into uro'gen-III involves an enzyme-mediated rearrangement.

Nevertheless, even these experiments do not prove that free AP·AP is a biosynthetic precursor which is normally dimerized by the enzymes. Indeed, when short incubation times with the enzyme were used, the incorporation of AP·AP (**17**) into uro'gen-III (**1**) was reduced (see also following section). Eventually it became clear that two molecules of AP·AP might be reacting *chemically* to produce the unrearranged tetrapyrrole system, a *bilane* (**25**), Scheme XII, which, as it is formed, could be *enzymically* transformed into uro'gen-III. The key experiments with synthetic bilane (**25**) which interlock with those on AP·AP are described after a discussion of other work in the pyrromethane area.

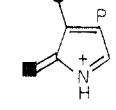
Related Work with Pyrromethanes. During the course of the foregoing researches (1969–1976), incorporation studies with pyrromethanes were reported from other laboratories. The Frydmans[33] employed enzymes from wheat germ, and paper chromatography was used for separation of the isomeric porphyrins prior to radioassay. They found[34] relative to controls that the AP·AP (**17**) in the presence of PBG was converted (~1% of pyrromethane used) by deaminase alone into uro'gen-I (**11**), but they did not observe incorporation of AP·AP (**17**) into uro'gen-III with deaminase–cosynthetase using a short incubation (1 h). In the light of the results collected in the previous sections and in the following one, it seems probable that the decisive differences between their experiments and ours were *duration* of the run and *concentration* of AP·AP. With the short time and low concentration employed, the formation of unrearranged bilane would be slow, and the resulting incorporations into uro'gen-III too small to be detected when superimposed on the large blank.

When the rearranged [^{14}C]pyrromethane PA·AP (labeled **19**) was incubated with deaminase–cosynthetase, together with PBG, radioactivity (~0.7% of incubated amount) appeared in uro'gen-III (**1**). This result was interpreted as indicating that the rearrangement step to produce the type-III system occurs as the first pyrromethane is formed. Later, Scott[35] incubated the "headless" [^{14}C]pyrromethane (**23**) with PBG and deaminase–cosynthetase and found 0.15–0.89% yield of radioactivity in the type-III porphyrins. This was thought to prove that type-III porphyrin synthesis is controlled at the pyrromethane level.

The evidence summarized in the previous sections and in the following one is now overwhelming that this is not so. With current knowledge, these small transfers of radioactivity from rearranged pyrromethanes into uro'gen-III could reasonably be explained as arising from minor chemical side reactions.[36] It should be noted that the amounts of radioactivity found in the type-III porphyrins in both these studies with rearranged pyrromethanes are of the same order (<1%) as



i, R = CH_2NH_2, with •
ii, R = H, with ■



iii

sequences which together produce ca. 1% of substances from these acrobatic pyrromethanes which are convertible into uro'gen-III would account for the results.

(32) A. R. Battersby, D. W. Johnson, E. McDonald, and D. C. Williams, *J. Chem. Soc., Chem. Commun.*, 117 (1977).

(33) (a) B. Frydman, S. Reil, A. Valasinas, R. B. Frydman, and H. Rapoport, *J. Am. Chem. Soc.*, **93**, 2738 (1971); (b) R. B. Frydman, A. Valasinas, and B. Frydman, *Biochemistry*, **12**, 80 (1973); (c) R. B. Frydman, A. Valasinas, H. Rapoport, and B. Frydman, *FEBS Lett.*, **25**, 309 (1972).

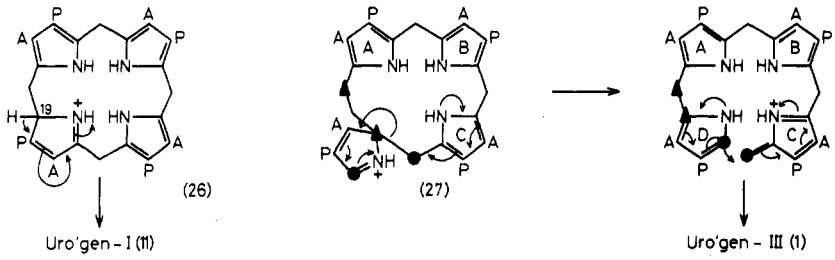
(34) See also J. Plusec and L. Bogorad, *Biochemistry*, **9**, 4736 (1970), who had isolated, from enzymic experiments inhibited by hydroxylamine, a pyrromethane either of structure **17** or this with NH_2 replaced by NHOH. This product, when PBG was added, was converted into uro'gen-I (**11**) by deaminase from spinach.

(35) A. I. Scott, K. S. Ho, M. Kajiwara, and T. Takahashi, *J. Am. Chem. Soc.*, **98**, 1589 (1976).

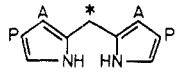
(36) The most probable side reactions are known ones.[37] For example, the rearranged pyrromethanes i and ii could fragment after electrophilic attack (e.g., $E^+ = H^+$ or E^+ derived from PBG) to generate a labeled PBG equivalent (iii). This could react with PBG to yield labeled AP·AP, eventually to produce a trace of labeled unrearranged bilane ready for conversion into uro'gen-III. But this is purely illustrative; *any* chemical

(37) G. W. Kenner, A. H. Jackson, and D. Warburton, *J. Chem. Soc.*, 1328 (1965).

Scheme XIII



we found (see Scheme IX) for the three rearranged aminomethylpyrromethanes, including the "wrong" isomer PA·PA (**20**). Finally, none of the foregoing



(23) ^{14}C at *

weakly radioactive type-III porphyrins from experiments with rearranged pyrromethanes has been degraded to locate the labels.

Proof That Rearrangement Occurs at the Tetrapyrrole Level. The crystalline lactam ester **24** (Scheme XII) was synthesized and alkaline hydrolysis then gave the bilane (**25**), corresponding to unrearranged, head-to-tail joining of four PBG units.[38] This cyclized chemically (without enzyme) at pH 7.2 to give virtually pure (>95%) uro'gen-I (**11**), isolated as uroporphyrin I. However, when the bilane (**25**) was incubated at pH 7.2 with purified deaminase–cosynthetase, a striking result was obtained;[38] now the products after aromatization were uroporphyrin III (70%) derived from 1 and uroporphyrin I (30%) derived from **11**. Müller et al.[39] prepared the same bilane by a different method, and at a late stage in our researches they reported it was transformed by deaminase–cosynthetase from *P. shermanii* into a uro'gen mixture having a type-III:type-I ratio of 16:84. It is true that the proportion of type-III isomer was lower than above (perhaps due to using less enzyme than in our experiments), but both sets of results strongly suggested that the unrearranged bilane (**25**)[40] is a key precursor in the biosynthesis of uro'gen-III (**1**). The following experiments with specifically labeled bilanes have established that status beyond doubt.

[15-^{13}C]Bilane (**25a**) was synthesized[38] and was converted by the deaminase–cosynthetase system into a product consisting of 80% uro'gen-III (**1a**) and 20% uro'gen-I (**11a**) as shown by LC analysis of the corresponding coproporphyrin esters. The coproporphyrin III ester was shown by NMR to be labeled specifically at C-15, and the proof was made rigorous by unambiguous synthesis of [15-^{13}C]coproporphyrin III tetramethyl ester to act as standard for the NMR studies. This result[38] established *specific* incorporation of the bilane (**25a**) into uro'gen-III (**1a**), and the final proof of *intact* incorporation required doubly labeled samples of the bilane.

Accordingly, the two [$^{13}C_2$]bilanes (**25b** and **25c**) were prepared;[42] the enrichment at each labeled site was 90 atom %, so that 81% of the molecules were doubly labeled as illustrated in Scheme XII. Each sample was diluted with unlabeled bilane (ca. three parts), and the two mixtures so obtained were incubated separately with purified deaminase–cosynthetase. The illustrated location of the bridge label in the resultant samples of uro'gen-III (**1b** and **1c**, Scheme XII) was proved by the same decarboxylation and NMR method used above.[43] Most importantly, ^{13}C NMR showed in each case a 72-Hz doublet for the major resonance from the labeled bridge carbon;[42] the two ^{13}C atoms in each case have therefore become directly bonded as illustrated for **1b** and **1c** via an intramolecular reaction. The relative sizes of the split and unsplit ^{13}C NMR signals from C-15 of the coproporphyrin ester corresponding to **1b** and similarly for C-20 of **1c** provide a sensitive check on the proportions of doubly and singly labeled species. Those found corresponded to there having been no change in the content of either during the enzymic conversion of bilanes (**25b** and **25c**) into uro'gen-III (**1b** and **1c**).

It will probably be helpful to give the yields in this work.[38,42] The bilane **24b**, 10.5 mg, underwent chemical cyclization (no enzyme) to give uro'gen-I (**11b**), 47% yield, which as the derived crystalline coproporphyrin I ester was shown to be 96% pure type I.[44] In the above enzymic run with the same bilane, 21 mg, the combined yield of uro'gen-III (**1b**) and uro'gen-I (**11b**) was 45%, the ratio of the two being 8:2, respectively;[42] the corresponding coproporphyrin esters were isolated crystalline.

The foregoing combined results prove intact incorporation of unrearranged bilane (**25**) *into uro'gen-III* (**1**) *and inversion of ring D by an intramolecular process exactly as found earlier for PBG* (**10**).

So it is now certain that the rearrangement occurs at the tetrapyrrole level, and the biosynthesis of the natural (type III) porphyrins, chlorins, and corrins involves the following steps. Four PBG units (**10**) are joined together head to tail[45] and the resultant bilane

(38) A. R. Battersby, E. McDonald, D. C. Williams, and H. K. W. Würziger, *J. Chem. Soc., Chem. Commun.*, 113 (1977).

(39) H.-O. Dauner, G. Gunzer, I. Heger, and G. Müller, *Z. Physiol. Chem.*, **357**, 147 (1976).

(40) It is most interesting that a substance having the expected properties of the unrearranged bilane (**25**) was isolated[6,41] by treatment of PBG with deaminase in presence of NH_4^+ ions. When this bilane was produced in ^{14}C-labeled form and was incubated[6] with deaminase-cosynthetase, but with the addition of a ca. 200 molar excess of PBG, no incorporation could be detected into uro'gen-III after turning over ca. 27% of the PBG. It seems probable that these competitive conditions did not sufficiently overcome the parallel chemical cyclization to type I.

(41) R. C. Davies and A. Neuberger, *Biochem. J.*, **133**, 471 (1973).

(42) A. R. Battersby, C. J. R. Fookes, E. McDonald, and M. J. Meegan, *J. Chem. Soc., Chem. Commun.*, 185 (1978).

(43) Rational synthesis of [20-^{13}C]coproporphyrin III tetramethyl ester provided the comparison standard.

(44) The ^{13}C signal from its bridge label was, as expected, a 6-Hz doublet corresponding as earlier to unchanged three-bond separation of the two ^{13}C labels.

(45) The pyrromethane AP·AP (**17**) is envisaged as an enzyme-bound intermediate, but the low incorporations found when the incubation time was short and/or the concentration of AP·AP was low indicate that the bound form does not exchange significantly with AP·AP added to the medium.

(25), bound covalently or by physical forces to the enzyme system, is then converted into uro'gen-III (1) by an intramolecular rearrangement which directly affects only ring D and the two carbons which become C-15 and C-20. The nature of the intermediate between the bilane and uro'gen-III remains to be established, and this leads to the concluding section.

Prospect. In the presence of deaminase alone, and also nonenzymically, the cyclization of bilane (**25**) occurs at C-19 to produce **26**, leading to uro'gen-I (**11**). We suggest that in the presence of cosynthetase cyclization occurs at C-16 rather than at C-19 (Scheme XIII). The postulated attack at C-16 would produce the spiro intermediate **27**; the labeling arising from **25b** and **25c** is shown. Fragmentation and cyclization again as illustrated would generate uro'gen-III.[46] The spiro system is related to that proposed by Mathewson and Corwin[47] in 1961, and its intermediacy is consistent with all the work summarized above. Notice that both **26** and **27** contain a new sp^3 center, and they differ less in shape than may appear from a planar diagram.

How does cosynthetase divert the unrearranged bilane to exclusive type-III formation when in its absence type I is exclusively formed? We consider here two possibilities. One is that cosynthetase alters the conformation of the deaminase–bilane complex to direct cyclization of **25** at C-16. There are indications[48,49] that deaminase associates with cosynthetase, and it has been suggested[33b,50] that cosynthetase acts as a "specifier protein" in the way lactoalbumin works during the biosynthesis of lactose. The other possibility is that the bilane (**25**) is the *product* from deaminase but is then the *substrate* for cosynthetase which brings about ring closure with rearrangement to produce uro'gen-III specifically.

Work is in hand on these aspects and on the problem of the structure of the intermediate[51] between the bilane and uro'gen-III. It will be good to have the answers to the few remaining questions.

We are glad to have this opportunity to record our debt and thanks to our young colleagues whose courage in this demanding field and experimental skills made the work posible. Their names are given in the literature references. We are also grateful for financial support from the Nuffield Foundation, Science Research Council, and Roche Products.

(46) The alternative, and equivalent, fragmentation from ring A would simply regenerate an equivalent of the unrearranged bilane ready for re-formation of the spiro system **27**.

(47) J. H. Mathewson and A. H. Corwin, *J. Am. Chem. Soc.*, **83**, 135 (1961).

(48) R. B. Frydman and G. Feinstein, *Biochim. Biophys. Acta*, **350**, 358 (1974).

(49) M. Higuchi and L. Bogorad, *Ann. N.Y. Acad. Sci.*, **244**, 401 (1975).

(50) B. Middleton, Cambridge, quoted by A. R. Battersby and E. McDonald in ref 2, p 96.

(51) The only alternative to the spiro intermediate involves fission of the C-15/C-16 bond of bilane (**25**) while it is bound to the enzyme, followed by inversion of ring D with strictly no exchange with the medium of the now separated pyrrole fragment. We find this possibility less attractive.

Functional Group Manipulation Using Organoselenium Reagents

HANS J. REICH

Department of Chemistry, University of Wisconsin, Madison, Wisconsin 53706

Received March 13, 1978

The use of second, third, and fourth row elements in organic synthesis received its major modern impetus from the discovery of the Wittig olefin synthesis. Since then many applications of silicon, tin, phosphorus, sulfur, selenium, and other metalloids in organic synthesis have been proposed, and a significant number of these procedures are finding their place in routine organic synthesis.

In this Account some recent developments in the chemistry of organoselenium compounds which have synthetic potential are described, with particular emphasis on the exploitation of chemical properties of selenium which differ from those of sulfur. In addition to work at Wisconsin, research groups headed by Sharpless, Seebach, Grieco, Krief, Nicolaou, and others have been active in this area.[1]

The utility of selenium and sulfur is derived from the great ease with which a wide variety of organoselenium and organosulfur compounds can be prepared, as well as the facility of transformations of these compounds to effect useful functional group interconversion. Both nucleophilic and electrophilic reagents are readily available for the introduction of selenium. The large stabilization of carbanions provided by sulfur and selenium substituents (at least 10–15 pK_a units for PhS and PhSe, more for sulfoxides, selenoxides, and sulfones[2]) has resulted in their use as activating groups for a variety of carbon–carbon bond-forming procedures involving organometallic reagents.

The chemistry of sulfur and selenium is very closely related. The greater expense of selenium and the hazard resulting from its toxicity[3] are justifiable only

Hans J. Reich was born in 1943 in Danzig, Germany. After a B.Sc. degree at the University of Alberta and Ph.D. at UCLA with D. J. Cram, he spent 2 years doing postdoctoral work, first at Cal Tech with J. D. Roberts and then at Harvard with R. B. Woodward. In 1970, he joined the faculty at the University of Wisconsin—Madison, where he is now Associate Professor. He is an Alfred P. Sloan Fellow and has research interests in the chemistry of the organometalloids and their application to organic synthesis.

(1) For a comprehensive account of synthetic organoselenium chemistry, see H. J. Reich in "Oxidation in Organic Chemistry, Part C", W. Trahanovsky, Ed., Academic Press, New York, 1978, p 1.

(2) F. G. Bordwell et al., *J. Org. Chem.*, **42**, 326 (1977).

(3) See K. Schwartz and K. D. Pathak, *Chem. Ser.*, **8A**, 85 (1975), and D. V. Frost and D. Ingvoldstad, *ibid.*, **8A**, 96 (1975), for reviews on the beneficial and harmful effects of dietary selenium.

0001-4842/79/0112-0022$01.00/0