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BIOSYNTHESIS OF THE PIGMENTS OF LIFE[1]

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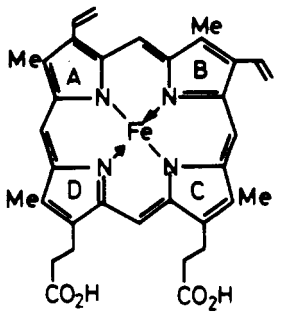
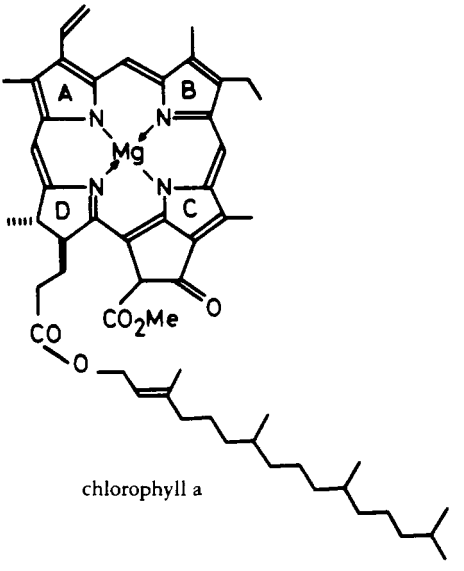
ABSTRACT.—Chlorophyll can be chosen as the fundamental pigment of life. Related to it is protoheme, the red pigment of blood. These substances are found to be biosynthesized from the same parent substance called uroporphyrinogen-III. This paper covers research over a period of 19 years, which has gradually revealed the details of Nature's pathway to uroporphyrinogen-III.

This lecture will survey our work on the biosynthesis of the pigments of life, which has extended now for about 19 years. We started around 1968 in this field, so it will be quite impossible to tell the entire story in so brief a space. The plan then is to give enough background information as I go through the story to allow the latest work to be understood and appreciated. What this means is that the first 10 or 12 years of work will be covered extremely quickly. Only the results from that stage will be presented; I will not be able to say exactly how those results were obtained or at best only rather briefly. So it must be remembered that all the synthetic support—all the enzymology, the kinetics, the spectroscopy, and so on—have been rigorously done even though little is said about them. You will not even be seeing the tip of the iceberg; all you will be seeing in this survey section is the top of the tip of the iceberg. Then having described all the fundamental work, which carries us to somewhere around 1977, I will give a more detailed description of some of the latest developments. Then in my second lecture, I will build on all this knowledge in discussing vitamin B_{12}.

The fundamental pigment of life is chlorophyll. Without this pigment, life on this planet, at least as we know it, would not exist. A second example is protoheme, the red pigment of blood, and the structures of both pigments are shown in Scheme 1. Both are



protohaem



chlorophyll a

SCHEME 1.

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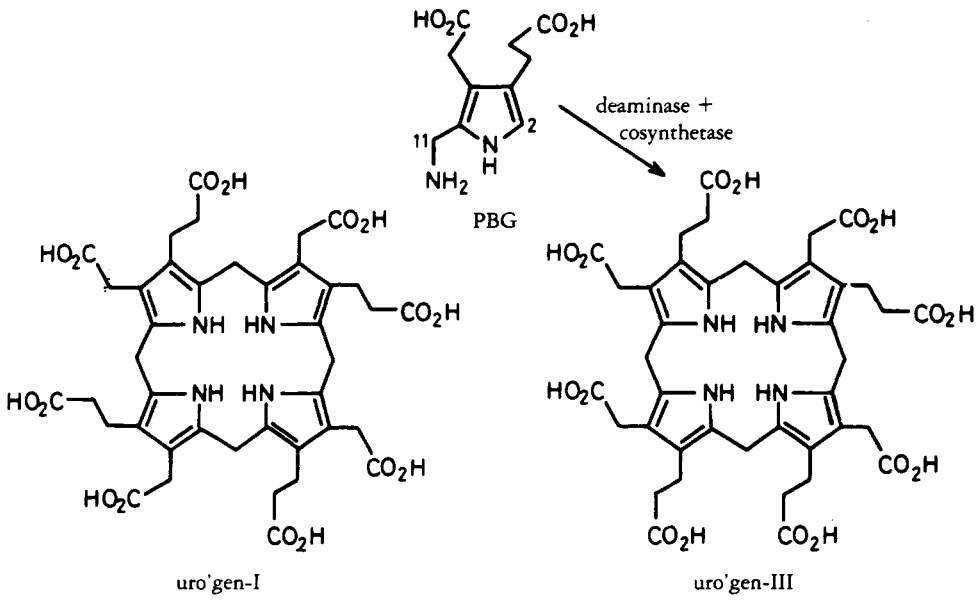
[2]Recipient of the Varro E. Tyler Distinguished Lectureship Award.

macrocyclic, and their macrocycles are composed of five-membered nitrogenous rings, of which there are four, connected by four one-carbon bridges. Protoheme has iron sequestered by the macrocycle, whereas chlorophyll holds magnesium. It can be seen that both macrocycles are fully conjugated systems, which accounts for their beautiful color. Finally, we see a number of functional groups around the periphery of the macrocycle, and two of special interest are the two propionate groups that are adjacent on the macrocyclic periphery. In the case of chlorophyll, one propionate group has been disguised by being ring-closed onto the main macrocycle, but it is still obviously a propionate residue.

Scheme 2 shows what was known at the beginning of our studies in 1968, this knowledge arising from the efforts of several research groups (1) in the 1950s, especially those of Bogorad, Granick, Neuberger, Rimington, and Shemin. It was discovered that protoheme, chlorophyll, and the cytochromes are biosynthesized from the same parent molecule called uro'gen-III. My second lecture will show that vitamin B_{12} is also made from this same parent substance. It was known that the four pyrrole rings of uro'gen-III are derived from one building block called porphobilinogen (PBG) carrying acetate and propionate side chains on a pyrrole ring. Two enzymes have to work cooperatively in order to make uro'gen-III; one is called deaminase and the other cosynthetase. There was no knowledge in 1968 about how the two enzymes carry out this remarkable biosynthesis.



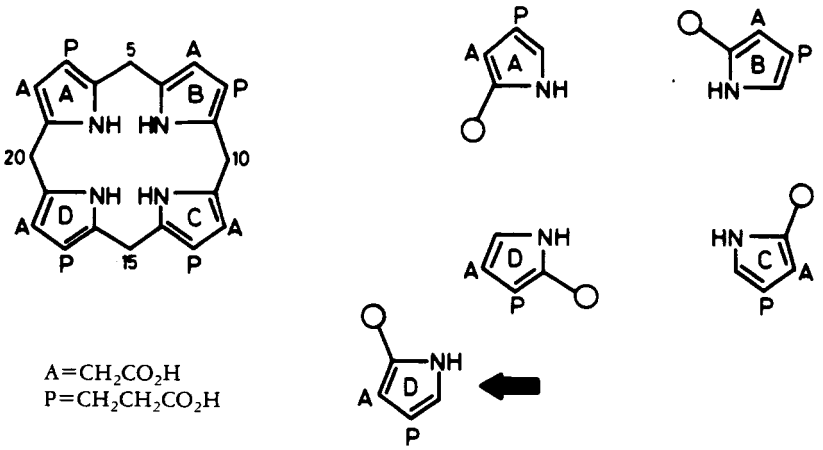
SCHEME 2.

Uro'gen-III is a particularly interesting substance. It is colorless because the pyrrole rings are joined by methylene groups; it is not a conjugated system. Nor does uro'gen-III complex metal ions; considerable structural modification is necessary before the macrocycle will complex the metals seen in the earlier structures. But the most interesting feature of uro'gen-III is that it is not the structure expected to be formed. If four PBG units are joined head-to-tail, then the acetate and propionate groups would run around the periphery of the macrocycle in sequence. But inspection of the structure of uro gen-III shows that Nature does not do that. Some rearrangement, or possibly several rearrangements, must occur to produce the unexpected structure of uro'gen-III.

That was the first problem we set for ourselves. How do the enzymes deaminase and

cosynthetase convert PBG into uro'gen-III? It should be noted in passing that if PBG is treated with deaminase alone, then uro'gen-I, the unrearranged structure, is eventually formed. (It will be clear later why I have emphasized "eventually.")

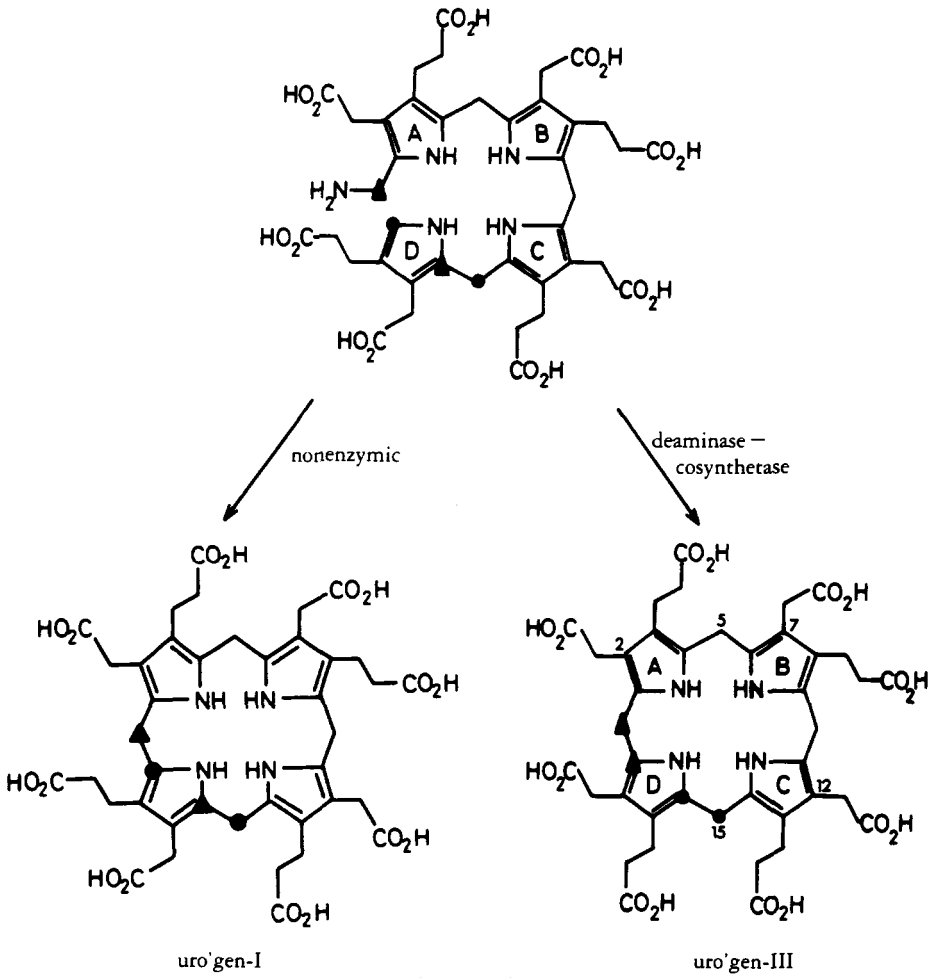
How is uro'gen-III biosynthesized? It is an unusual problem because four molecules of the same precursor (PBG) are used to produce uro'gen-III. In principle, there are 24 ways in which this can be done. To find out which one Nature uses, we had to develop a new approach based on ^{13}C-labelling at position 11 and also at position 2 of PBG in combination with ^{13}C nmr. These experiments (2,3) showed us exactly how the four building blocks are put together, and the results are illustrated in Scheme 3. We found that ring A and its attached carbon, ring B and its attached carbon, and ring C together with its attached carbon are all built as intact units into uro'gen-III. However, the PBG unit forming ring D undergoes intramolecular rearrangement such that the two ^{13}C-labelled atoms become joined together. So the results at this stage clearly proved that the biosynthesis of uro'gen-III involves one rearrangement affecting only ring D, and that rearrangement is intramolecular.



SCHEME 3.

Gradually, clues began to be assembled that the four PBG units are probably first assembled head-to-tail in an unrearranged fashion to produce a bilane. In order to test this possibility, we synthesized two samples of the aminomethylbilane (Scheme 4), one carrying ^{13}C labels at the sites marked with circles and the other at the sites marked with triangles (4,5). These syntheses allowed the very important experiment of treating this bilane with the two enzymes, deaminase and cosynthetase. You can imagine our delight when the synthetic bilane was converted into uro'gen-III and ^{13}C-spectroscopy demonstrated that in each case the two labelled atoms had been bonded together by an intramolecular process (4,5).

However, a problem arose when we studied the rate at which the bilane is converted enzymically into uro'gen-III. This rate was approximately 1/15 of the rate at which the natural starting material, PBG, was converted enzymically into uro'gen-III. In order to understand the reasons for this difference, we studied carefully what happens when the bilane is treated with each of the two enzymes separately (6,7). Cosynthetase left the aminomethylbilane largely unchanged, but deaminase accelerated the ring closure of the bilane to produce eventually uro'gen-I (see Scheme 2). It should be understood that this bilane ring closes non-enzymically in solution to form uro'gen-I; this happens spontaneously, but deaminase accelerates the formation of uro'gen-I. The critically important observation was made at this stage that in the enzymic experiments there was a

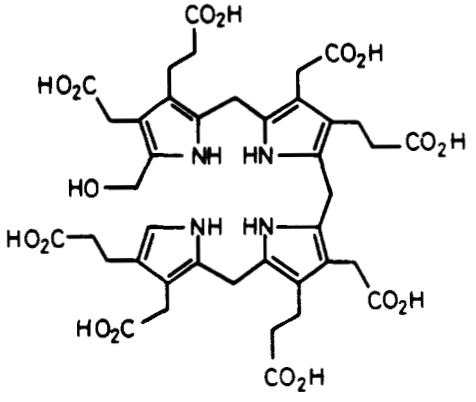


SCHEME 4.

lag phase before the rate reached the full rate. Does the action of deaminase on the natural precursor, PBG, cause a similar lag? Indeed, the lag was even more pronounced showing that some intermediate is being produced. Suitable experiments showed that this intermediate ring closes non-enzymically to produce uro'gen-I and, importantly, is cyclized by cosynthetase to form the rearranged uro'gen-III. Clearly, we must determine the structure of this intermediate.

This was achieved by synthesizing PBG labelled with carbon-13 at the aminomethyl group followed by enzymic conversion of the labelled PBG into the intermediate. ^{13}C-nmr spectroscopy then showed unequivocally (8,9) that the intermediate is the unrearranged hydroxymethylbilane shown in Scheme 5. We then synthesized this bilane (8,9) which made it readily available, and the natural and synthetic samples were shown to be identical substrates for cosynthetase alone, leading to the formation of the rearranged product, uro'gen-III.

All this work had led, by 1979, to an understanding of the roles of the two enzymes involved (10) in the biosynthesis of uro'gen-III. Deaminase is not a ring-closing enzyme, which it had always been thought to be; it is the assembly enzyme that builds the open-chain hydroxymethylbilane. Cosynthetase is the ring-closing and rearranging enzyme that operates on hydroxymethylbilane to make uro'gen-III, the natural precursor of all the pigments of life.

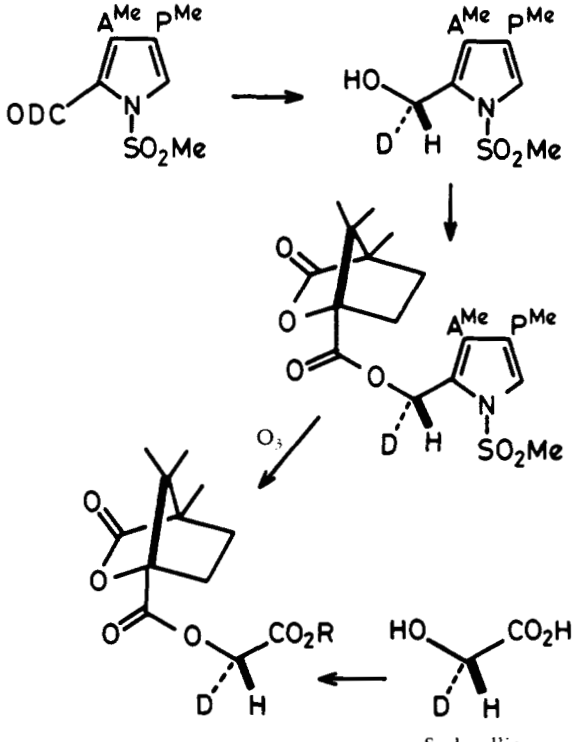


hydroxymethylbilane

SCHEME 5.

We have now surveyed all the essential knowledge from our earlier research to allow us to look in greater detail at our latest advances. One such step forward concerns the stereochemistry of action of deaminase. Our long-term goal is to work out the stereochemical changes occurring at each of the four methylene groups of the hydroxymethylbilane which are derived from the aminomethylene group of PBG. In the present paper we concentrate on the hydroxymethyl group of the bilane.

Clearly, we have to synthesize PBG stereospecifically labelled at the aminomethyl group (11). Placing a hydrogen isotope stereospecifically at that position in the pyrrole field calls for a special approach involving attachment of a strong electron withdrawing

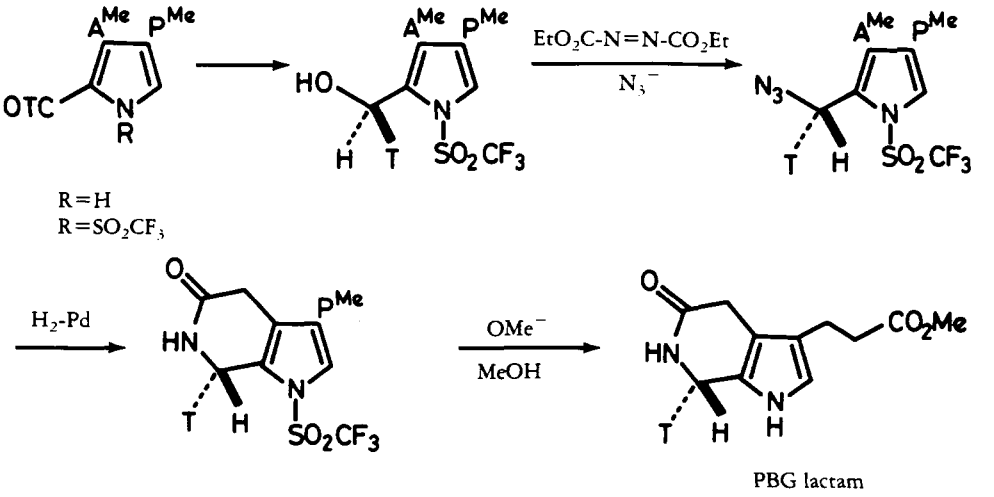


S-glycollic acid

SCHEME 6.

group to the nitrogen atom of the pyrrole system (Scheme 6). This scheme also shows how the chiral center was set up using pinyl borane and how the product was correlated by degradation with a standard sample of monodeuterio-*S*-glycollic acid (kindly given to us by our good friend Professor Duilio Arigoni). Scheme 6 shows that we can generate and handle a chiral CHD center attached to a pyrrole ring (12).

It was necessary for the enzymic experiments to use tritium labelling rather than deuterium, and the way this was achieved (12) is shown in Scheme 7. I will return later to establish the absolute configurations illustrated in Scheme 7.



SCHEME 7.

Now we have two considerable problems to solve. The hydroxymethylbilane is an unstable compound, and its ring closes non-enzymically very easily, indeed with a half-life of about four minutes at pH 7, to give uro'gen-I. This is an acid-catalyzed reaction and so by raising the pH to about pH 12, the hydroxymethylbilane is stable for several hours. Because the enzyme will not work at pH 12, we immobilized deaminase on a gel column. The labelled PBG was then passed through the column, where hydroxymethylbilane was rapidly generated, and was stabilized by collection in pH 12 solution below the column. One can accumulate a considerable amount of hydroxymethylbilane in that way (Figure 1). The next problem is how to isolate this highly hydrophilic material from the alkaline solution. This was solved by silylation of the product in situ using *t*-butyldimethylsilyl chloride, which silylated the hydroxyl group as expected but also sufficient of the side-chain carboxyl groups to make the molecule extractable by solvent. As a final step, we planned to degrade this product to the illustrated derivative of glycollic acid carrying a tritium label (Scheme 8).

The aim in the foregoing degradation was to produce a substance from which labelled glycollic acid could be derived because an enzyme is available, called glycollate oxidase, which stereospecifically oxidizes glycollate to the corresponding aldehyde, called glyoxylate. So an enzymic assay of the configuration of the product from Scheme 8 should be possible. As it turned out, there were major difficulties with this assay because of kinetic isotope effects, but these were eventually overcome by trapping the glyoxylate in situ as it was formed using hydroxylamine, which yielded the stable oxime of glyoxylic acid (12).

Thus, it was possible at this stage to determine the absolute stereochemistry of the product synthesized in Scheme 7. It was ozonized to give glycine, which was converted into glycollic acid using nitrous acid, a process involving retention of configuration. Fi-

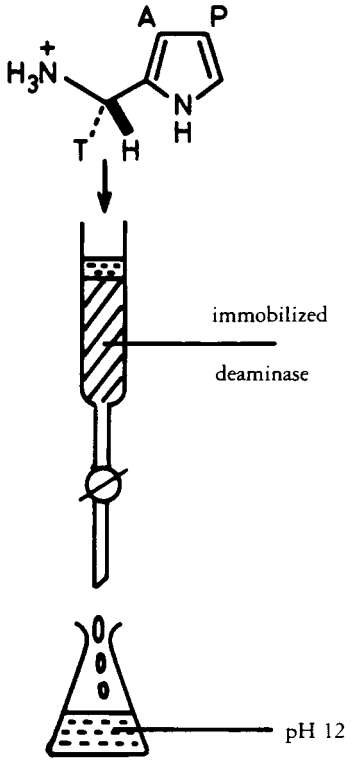
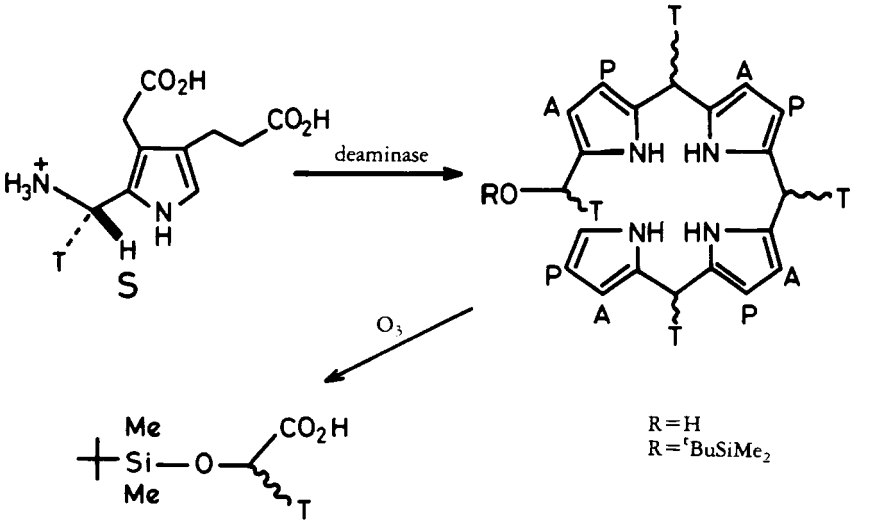


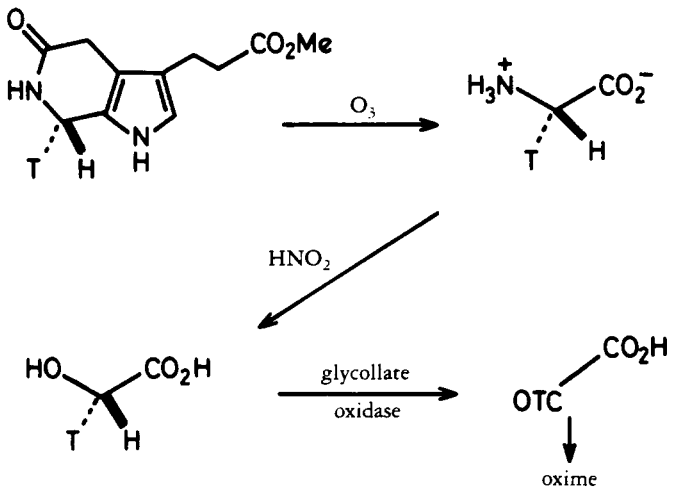
FIGURE 1. Use of immobilized deaminase on a gel column for the generation of hydroxymethylbilane.

nally, the glycollic acid was assayed using glycollate oxidase, which proved the configurations shown in Scheme 9. We also synthesized the enantiomer of that shown in Scheme 7 simply by starting with the enantiomeric pinene at the outset; the final absolute configuration was determined analogously to Scheme 9.

The stage was now set for the key experiment. The 11*S*-$^3H_1$-PBG was converted by deaminase into the hydroxymethylbilane that was isolated and degraded as above.



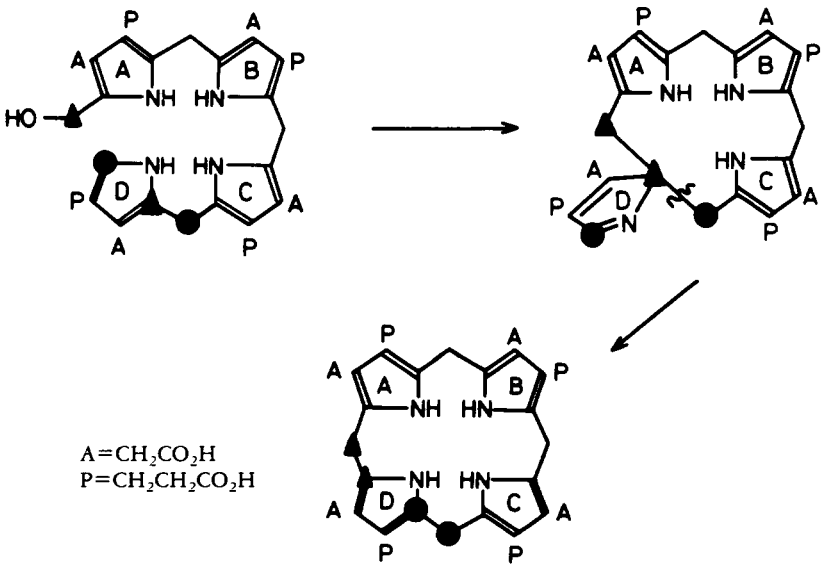
SCHEME 8.



SCHEME 9.

Assay of the final glycollic acid showed it to possess the *S* configuration. So the enzymic process from PBG to hydroxymethylbilane runs with overall retention of configuration at the hydroxymethyl group (12). This result was confirmed by showing that 11*R*-$^{3}H_{1}$-PBG is enzymically converted into hydroxymethylbilane with the *R* configuration at the $HOCH_2$ group. This is an important result, not only for knowledge of deaminase but also for our future studies on the mechanism of action of cosynthetase.

It is with this fascinating enzyme cosynthetase that I plan to round off this first lecture. Cosynthetase catalyzes the conversion of the hydroxymethylbilane into uroporphyrinogen-III (uro'gen-III). Scheme 10 summarizes some of the extensive ^{13}C-labelled experiments we have carried out to study the mechanism of this conversion. An intramolecular rearrangement is involved, and all our results limit the number of possible mechanisms for this process to two. We will consider just one of those mechanisms, which involves the intermediacy of the spiro intermediate shown in Scheme 10. This



SCHEME 10.

idea, in a somewhat modified form from that shown, was first proposed by Mathewson and Corwin (13); they used a heavily protonated form of the spiro system to increase its flexibility, but the key idea was there.

Our plan was to gain evidence for or against the spiro intermediate by the synthetic approach, and the first problem was to find out whether the macrocyclic portion of the spiro intermediate could be built. There was considerable doubt whether it could exist because on models it seems to be rather tightly packed. Scheme 11 shows the last step of the synthesis, an acid-catalyzed ring closure, which produced the macrocycle (14). To our delight, the product crystallized, and an X-ray structure showed that this macrocycle is indeed very tightly puckered (Figure 2). Two pyrrole rings are pointing up, and the one at the back is pointing down.

Let us now consider the synthesis of a spiro system that is structurally very closely related to the proposed spiro intermediate in Scheme 10. The molecule chosen was the spiro lactam system shown in Scheme 12. The difficult part of this synthesis is to construct a pyrrolenine with attached pyrrolomethyl groups. Eventually, after many other

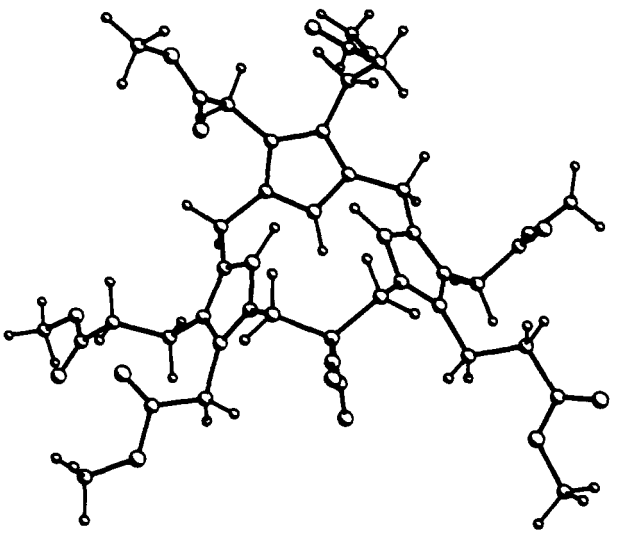
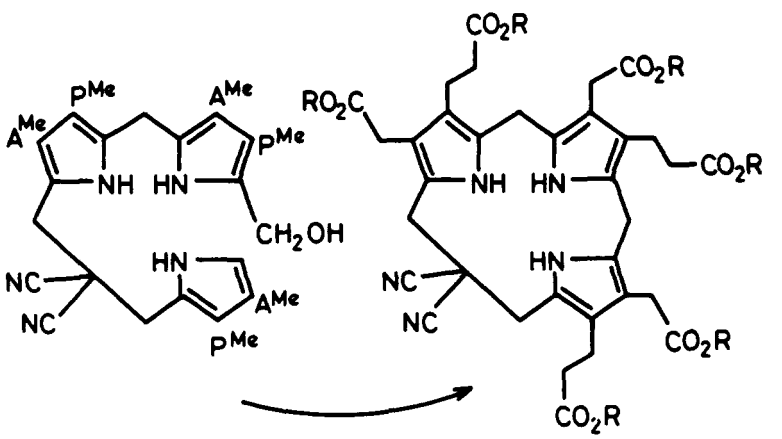
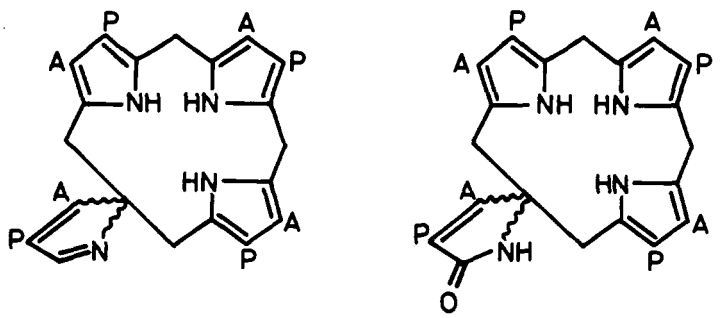


FIGURE 2. X-ray structure of the macrocycle of proposed spiro intermediate in the formation of uro'gen-III.



R=Me

SCHEME 11.



proposed spiro intermediate

synthetic (2 isomers)

SCHEME 12.

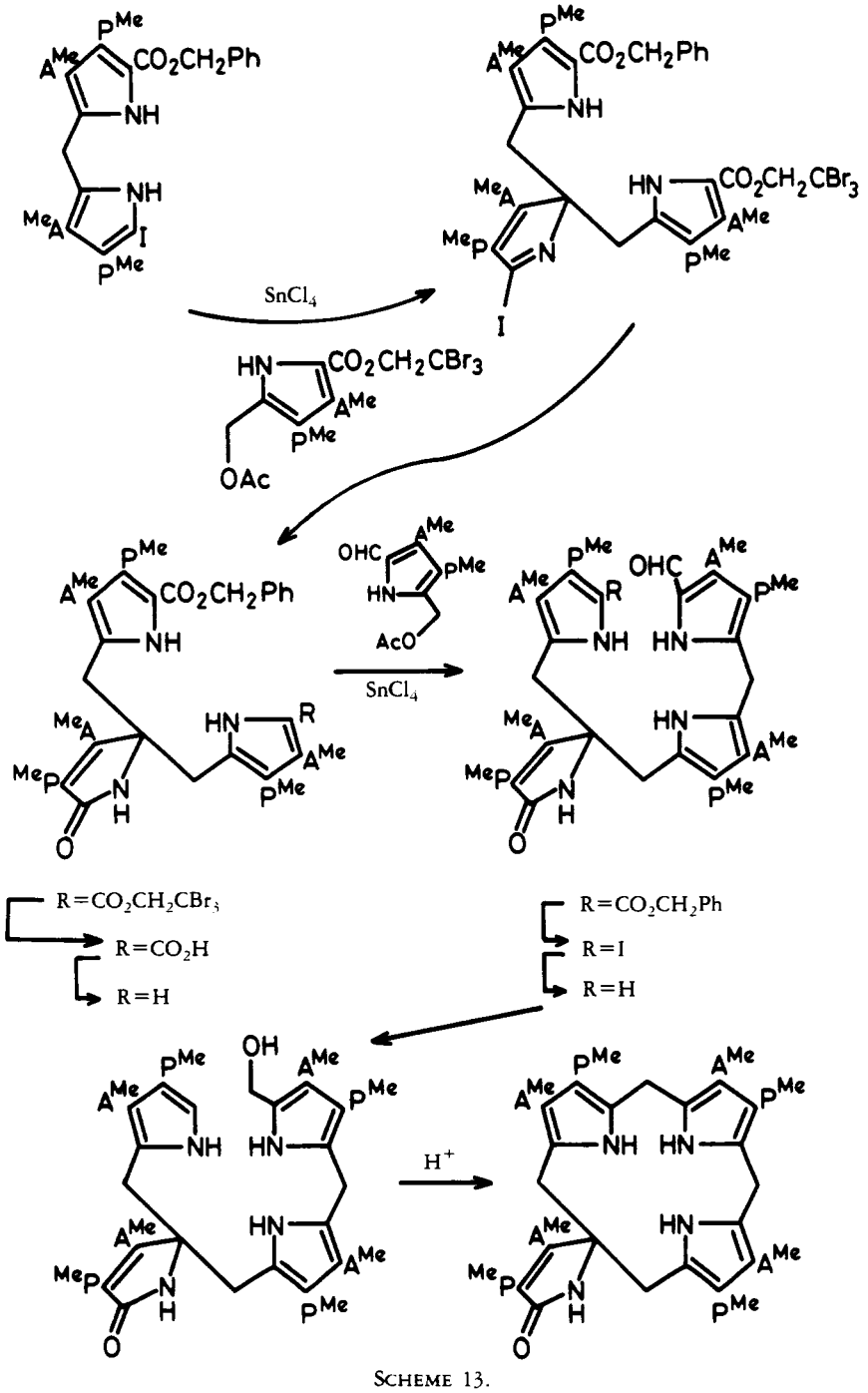
approaches had been tried, a new reaction (see start of Scheme 13) was found that allowed the ready construction of the required molecules (15). What a fantastic difference such a finding makes to a project; we can now go forward to tackle the main synthetic target.

The sequence used is shown in Scheme 13. Initially to our surprise, the final product was a mixture of two isomeric and separable spiro lactams. Why should there be two? The reason is that the macrocyclic part of these molecules is in a locked, puckered conformation. It is then clear that there are two different ways in which the lactam ring can be fused to the macrocycle; so there are two diastereoisomers (15).

We can now carry out the critical enzymic experiments. Cosynthetase converts the hydroxymethylbilane uniquely into uro'gen-III with inversion of ring D. If our synthetic materials are indeed close analogues of the real spiro intermediate, then one of those two isomeric lactams should lock into the active site of the enzyme system and block it (Scheme 12). The results could not have been better. One of the two synthetic lactams had no detectable effect on cosynthetase, whereas the other one blocked it very effectively at a concentration of around 10^{-7} molar. This result (15) gives very strong support for the spiro system shown in Scheme 10 being a true intermediate of the pathway to uro'gen-III.

One further aspect of our work on cosynthetase is the study of molecular recognition: What are the important functions of the spiro intermediate which are recognized by the enzyme? The plan was to synthesize the two isomeric spiro lactams shown in Scheme 14, which lack the acetate and propionate groups on the lactam ring. In addition, we hydrolyzed the esters of the dinitrile from Scheme 11; in so doing, one of the nitrile groups was also hydrolyzed to give a nitrile acid as expected for such a dinitrile. These three macrocycles shown in Scheme 14 were then tested as inhibitors of cosynthetase, and none of them had any detectable effect on the enzyme. It is, thus, clear that a full set of binding groups is needed in order to get strong binding to the enzyme (15). Perhaps not surprisingly, the acetate and propionate groups on the five-membered spiro ring turn out to be very important in the binding process to the enzyme.

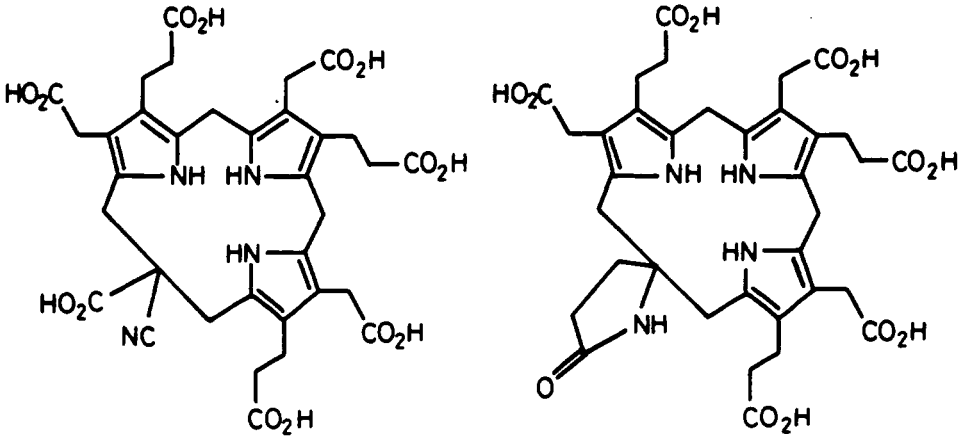
Finally, let us look at the fragmentation process involved in the conversion of the spiro intermediate into uro'gen-III. As shown in Scheme 15, this fragmentation could occur in two very similar ways. That marked III is the one Nature uses to produce uro'gen-III, whereas that marked I would finally yield uro'gen-I. The only difference between the two alternatives is that for cleavage III there is a propionate group adjacent to the spiro system, whereas for cleavage I an acetate group is adjacent to the spiro system. The natural enzymic process is completely selective for III over I. Is this entirely as a result of enzymic control or could there be an intrinsic preference for one way over the



SCHEME 13.

other when an enzyme is not involved? It is a question of interest in relation to evolution.

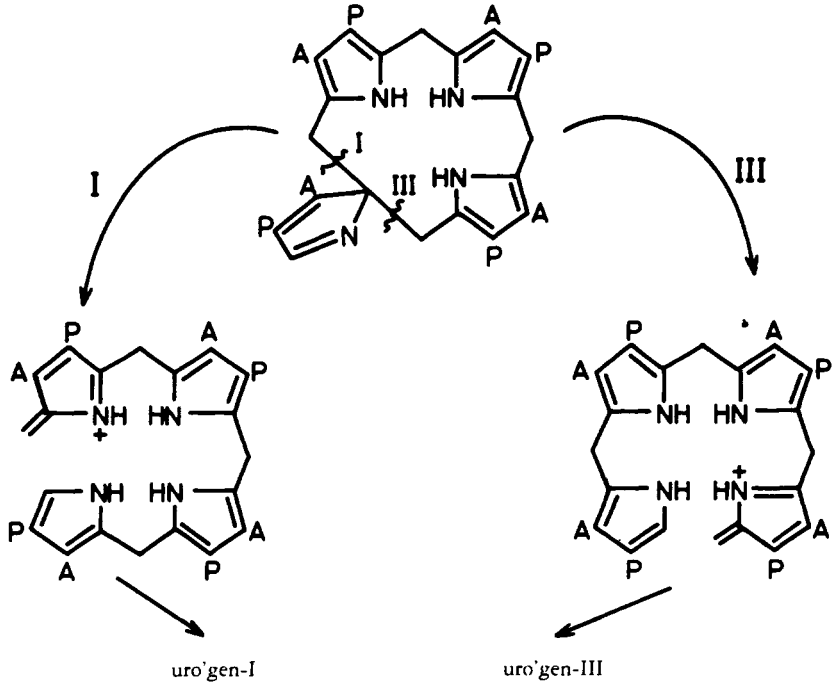
This question was studied by synthesis (16) of the model pyrrolenine shown in Scheme 16, which reproduces exactly the arrangement of these three rings in the spiro intermediate. This product fragmented with extreme ease by treating it with a trace of acid in methylene chloride. Scheme 16 shows the two possible directions of fragmentation, and the fragments then recombine to yield mainly the two illustrated tripyrroles.



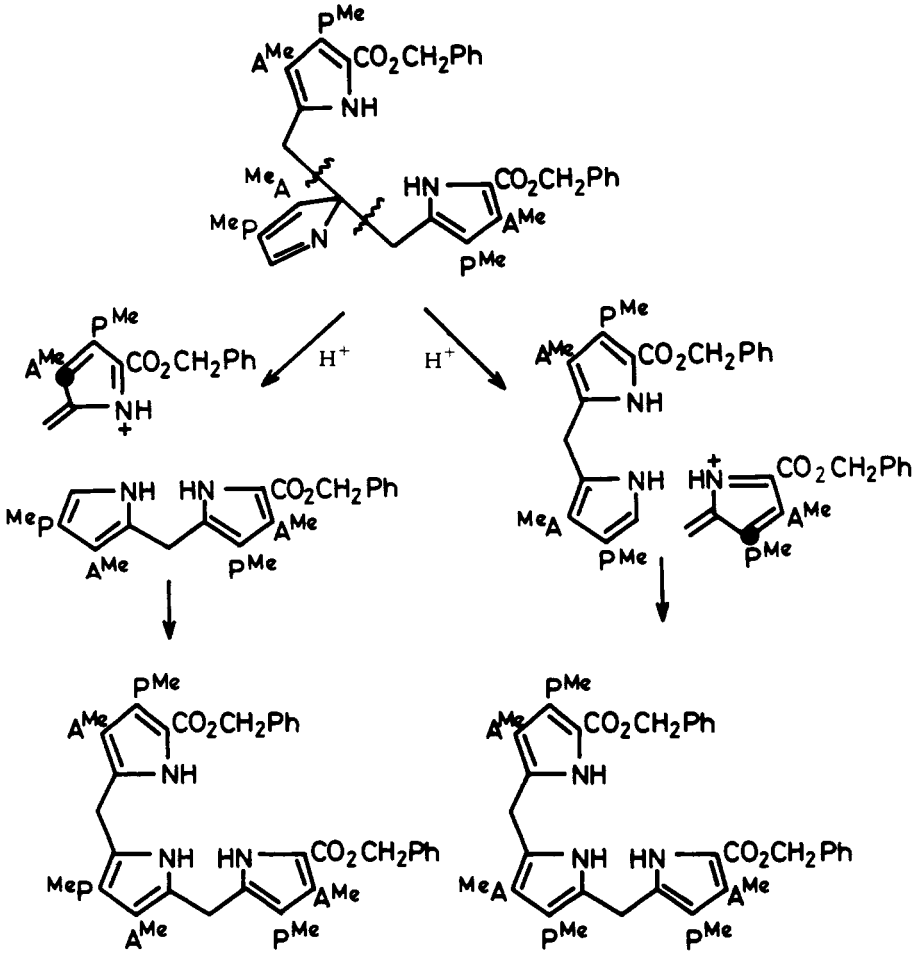
SCHEME 14.

The interesting finding was that the product resulting from the "natural" fragmentation adjacent to the propionate group is favored over the alternative. The reason for this regio-selectivity may be that fragmentation in the favored direction places a positive charge on the carbon carrying a propionate group. This is a more favorable arrangement than that resulting from the alternative mode, where the positive charge appears on a carbon carrying an acetate residue. Whatever the explanation is, the intrinsic favoring of the "natural" direction of fragmentation is quite clear (16).

Let us summarize what has been achieved (17). We now know that deaminase is the assembly enzyme and that it assembles the hydroxymethylbilane starting with ring A and ending with ring D to produce the bilane covalently bonded to the enzyme. Deaminase is not a ring-closing enzyme; its function is to build and release into the



SCHEME 15.



(minor products from crossover)

SCHEME 16.

medium the hydroxymethylbilane. The structure of this product has been confirmed by total synthesis. It has been proved also that as PBG is converted into the bilane, there is overall retention of stereochemical configuration at the hydroxymethyl center. The hydroxymethylbilane is the substrate for cosynthetase that causes cyclization with concomitant inversion of the terminal ring D by an intramolecular process. Further, we know from experiments involving the synthesis of two isomeric, very close analogues of the proposed spiro intermediate, that one of these analogues strongly inhibits cosynthetase, whereas the other does not. This result provides strong evidence in favor of the spiro system being the intermediate Nature uses to perform the conjuring trick of converting the hydroxymethylbilane into uro'gen-III. Finally, the last few experiments we considered demonstrated that for non-enzymic rearrangement of a suitable model system, there is an intrinsic favoring of what I called the "natural" direction of fragmentation. When uro'gen-III has been built, the pathway leads forward to the pigments of life, such as protoheme, chlorophyll, and vitamin B_{12}, the last being our topic for tomorrow.

ACKNOWLEDGMENTS

I wish to record the names of my excellent colleagues in the recent Cambridge group whose work I described in some detail. They are Drs. A. Abell, P.C. Anderson, M.G. Baker, C.J. Hawker, S. Jendrzejewski, W. Neidhart, P.R. Raithby, J.-R. Schauder, and W.M. Stark, together with my senior colleagues Drs. G.J. Hart and F.J. Leeper. These are the scientists who have achieved what has been described to you, and they deserve enormous credit. I take a special pleasure on this formal occasion of the Varro E. Tyler Distinguished Lecture to pay my warmest tribute to this group of colleagues and to acknowledge my debt to them. These are the recent members of the Cambridge team, but we should not forget all the outstanding earlier members on whose shoulders the present group stands.

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