a purified penicillinase from *B. cereus* 569 that had retained very little cephalosporinase activity (Abraham & Newton, 1956), indicates that cephalosporin C can form a complex with a β -lactamase from this organism in the absence of Zn^{2+} , although Zn^{2+} is necessary for enzymic hydrolysis of cephalosporin C to occur at a significant rate. This situation could be analogous to that with carboxypeptidase, whose zinc-free apoenzyme can form complexes with certain substrates but is unable to catalyse their hydrolysis (Coleman & Vallee, 1962).

Whether Zn^{2+} is incorporated into the active centre of the cephalosporinase or whether a change in conformation at the active centre results from a reaction with Zn^{2+} at another part of the molecule is not revealed by the present data. The question arises whether the cephalosporinase and penicillinase activities are associated with a single protein whose conformation is changed by complex-formation with zinc. Purification of the crude enzyme from *B. cereus* 569 under conditions such that its cephalosporinase activity is retained may be necessary before this question can receive a satisfactory answer.

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Studies on the Biosynthesis of the Corrin Ring of Vitamin B₁₂

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The similarity in structure between the corrin ring of vitamin B₁₂ and the porphyrin nucleus of haem has stimulated numerous workers to examine the similarities in the biosynthetic pathways (Bukin & Pronyakova, 1960; Shaposhnikov & Finogenova, 1963; Bardi *et al.* 1958). In the present communication we discuss the identity of the next stable intermediate after porphobilinogen. Our evidence suggests that uroporphyrinogen is an intermediate, and that porphobilinogen does not form a corrole ring directly. Previous evidence for the contrary may have resulted from the choice of experimental material by past workers.

When choosing a micro-organism for the investigation of the biosynthesis of something that is formed in minute amounts, as with vitamin B₁₂, one naturally favours organisms that have an extra-

ordinary ability to synthesize the desired compound. Thus propionibacteria, nocardia and actinomycetes have gained popularity as experimental material. However, these micro-organisms have a drawback for studies involving the question of the nature of the first tetrapyrrolic vitamin B₁₂ intermediate, namely they all have the capacity to make haem enzymes. The question is raised whether a porphyrin formed by such organisms is a primordial haem molecule, or a primordial cobamide coenzyme.

Structural considerations make it apparent that no porphyrin occurring in the biosynthetic sequence after uroporphyrinogen can be considered as possible intermediates of vitamin B₁₂. The previous observation that vitamin B₁₂-producing organisms also produce coproporphyrin III tells us little beyond the fact that the organism has the capacity

to accumulate haem precursors under the conditions employed. There is no way of knowing if there is a branch at uroporphyrinogen.

At the outset of the present work it was therefore decided to avoid the ambiguities that arise with organisms that have multiple tetrapyrrole end products. The bacteria in the genus *Clostridium* seem to offer nearly ideal biosynthetic capabilities. They are known to synthesize reasonable quantities of vitamin B₁₂ derivatives, and to date they have not been found to contain haem enzymes (Lascelles, 1964). They have never been observed to accumulate porphyrins. Since there is only one end product for δ -aminolaevulate in the clostridia it is assumed that any tetrapyrrole formed by the organisms from δ -aminolaevulate is a good candidate for consideration as a vitamin B₁₂ intermediate.

Experimental. A culture of *Clostridium tetanomorphum* strain H1 was supplied by Dr H. A. Barker, and *Clostridium perfringens* by Dr. H. W. Seeley. They were grown in the media described by Barker *et al.* (1960), and by Meister, Sober & Tice (1951), respectively.

The work of Shemin, Corcoran, Rosenblum & Miller (1956) and that of Schwartz, Ikeda, Miller & Watson (1959) demonstrating that δ -aminolaevulate and porphobilinogen are active intermediates of vitamin B₁₂ were carried out with actinomycetes. It seemed desirable at the outset therefore to determine whether the clostridia followed the pattern demonstrated in the earlier work. The results obtained with δ -amino[¹⁴C]laevulate agree with the previous work. An experiment was also carried out to test the premise that clostridia do not make haem. This was done with ⁵⁹Fe. No detectable haem was found. Thus the preliminary evidence showed that clostridia use δ -aminolaevulate to make vitamin B₁₂ and that vitamin B₁₂ and its derivatives are the sole end product of δ -aminolaevulate metabolism in this organism.

Experiments were then undertaken (see Table 1) to see if this organism could form and accumulate porphyrins under conditions where it could not make the complete B₁₂ molecule. Accordingly *C. tetanomorphum* cell suspensions were incubated in two types of media. These were basically the same as Lascelles (1956, 1964) has used in her studies on porphyrin synthesis by the Athiorhodaceae. Mixture I contained glycine and succinate, and mixture II contained δ -aminolaevulate and fumarate. The incubations were carried out in Thunberg tubes at 37° under N₂. After I₂ oxidation, it was apparent that relatively large amounts of tetrapyrrole had been formed during the incubation with δ -aminolaevulate (Burnham, 1965). In addition, it was possible to see other pigmented compounds. Cells incubated with glycine and succinate did not make porphyrins.

The porphyrin was extracted from the suspension and was purified. It was identified as follows. On chromatography with Eriksen's (1958) solvent, the unknown porphyrin moved with the same *R_F* as uroporphyrin. The unknown porphyrin was esterified and chromatographed in the solvent system of Chu & Chu (1957) and it chromatographed with *R_F* identical with that of uroporphyrin octamethyl ester. The absorption spectrum of the ester (λ_{max} . 502, 535, 572 and 626 m μ) was compared with those of authentic samples of other porphyrin esters and it showed absorption characteristics identical with uroporphyrin. On the basis of this evidence it is believed that the tetrapyrrole produced by *C. tetanomorphum* when incubated in the presence of δ -aminolaevulate is uroporphyrin. Similar experiments have been carried out with *C. perfringens* cell suspensions, and also with broken cell preparations of both micro-organisms, and in all cases uroporphyrin is the only porphyrin observed.

The other pigmented compound(s) produced in addition to uroporphyrin is currently under investigation. The formation of it tends to be erratic, whereas uroporphyrin formation is very regular. Its absorption spectrum resembles that of partially oxidized porphyrinogen, i.e. tetrahydroporphyrin, or dipyrromethene. However, the observed chemical properties are not consistent with those described by Mauzerall & Granick (1958) for such compounds.

Discussion. None of the evidence obtained in the present work indicates the point at which cobalt is incorporated. Suggestions have been made that ring-closure may occur around the cobalt atom (Bonnett *et al.* 1955). Porra & Ross (1965), however, have demonstrated that cell-free extracts of *C. tetanomorphum* catalyse the incorporation of cobalt into porphyrins, indicating that an enzyme exists which will catalyse the incorporation of cobalt into

Table 1. Porphyrin formation by clostridia

The incubation system contained 15 mg. of *C. tetanomorphum* or 5 mg. of *C. perfringens*, 125 μ moles of potassium phosphate buffer, pH 7.5, 5 μ moles of MgCl₂, 12.5 μ moles of fumarate, δ -aminolaevulate as indicated, cells and water to give a final volume of 2.5 ml. Incubation was at 37° under N₂ for 48 hr.

δ -Aminolaevulate added (μ mole/ml.)	Porphyrin formed (μ g./ml.)	
	With <i>C. tetanomorphum</i>	With <i>C. perfringens</i>
0	0	0
0.05	3.1	1.5
0.1	5.7	1.9
0.2	10.4	2.0
0.4	13.6	2.5
0.8	13.3	2.1

macrocyclic tetrapyrroles and strongly suggesting that in the natural system the cobalt is incorporated into preformed tetrapyrrole.

The present evidence indicates that uroporphyrinogen is the first macrocyclic tetrapyrrole intermediate of vitamin B₁₂. It is suggested that the next step in the formation of the corrin ring is the reopening of the macro-ring with the expulsion of the γ -methene bridge carbon as methyl group, followed by ring-closure. Mauzerall (1960) has demonstrated that ring-opening does occur with uroporphyrinogens. The new compound, a methyl-substituted corrole, would not be expected to possess physical or chemical properties described for an unsubstituted corrole (Johnson & Kay, 1965). The substitution at the α -position of the A ring prevents the formation of a fully conjugated system as in corrole. Thus the characteristic Soret band and the typical red fluorescence would not be observed. A compound of the type suggested would, however, most likely resemble a tetrahydroporphyrin, or a di- or tri-pyrrolylmethene. Since material with these absorption characteristics is sometimes found when clostridia are incubated with δ -aminolaevulic acid it is suggested that this material may represent the next biosynthetic intermediate of vitamin B₁₂.

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An Early Intermediate in the Biosynthesis of Biotin: Incorporation Studies with [³⁵S]Sulphate

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In a previous study on biotin biosynthesis, an unknown biotin vitamer, which was characterized as an amino acid, was shown to be present in the culture medium of *Phycomyces blakesleeanus* (Eisenberg, 1963). A compound with a similar R_F was also reported in culture filtrates of *Escherichia coli* (Dhyse & Hertz, 1958) and *Corynebacterium xerosis* (Genghof, 1956). This compound has now been obtained in high yields from the culture medium of a biotin auxotroph of *Penicillium*

chrysogenum, and it becomes labelled when the organism is grown in the presence of [1,7-¹⁴C₂] pimelic acid (M. A. Eisenberg, unpublished work). Since pimelic acid is a direct precursor of biotin (Eisenberg, 1962; Elsford & Wright, 1962), the isotope-incorporation studies suggested the possibility of this vitamer being an intermediate in biotin synthesis. To obtain more information about the chemical composition of this unknown vitamer, the question whether sulphur is present in the