Proof that Acetic Acid is Extruded During Biosynthesis of Vitamin B_{12} in *Pseudomonas denitrificans*

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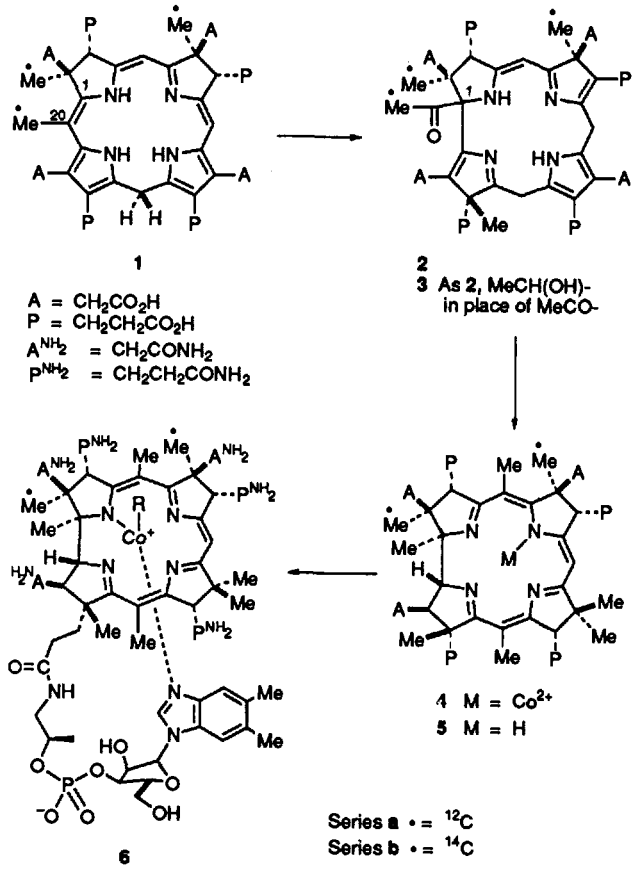
^{14}C-Labelling experiments establish that the ring-contraction process required for the biosynthesis of vitamin B_{12} in *P. denitrificans* results in elimination of acetic acid whereas a possible alternative fragment, acetaldehyde, is not formed.

Propionibacterium shermanii when grown essentially anaerobically (microaerophillically) biosynthesises vitamin B_{12} **6a**. A late intermediate on the pathway to the vitamin in *P. shermanii* is known to be cobyrinic acid **4a** having a structure based on the corrin macrocycle that is also present in vitamin B_{12} **6a** itself. The macrocycle of cobyrinic acid **4a** is biosynthesised in many steps from precorrin-3A **1a** and by inspection one of these steps has to involve a ring-contraction.[1] Appropriate labelling experiments firmly established that as a result of this contraction, C-20 and its attached methyl group of precorrin-3A **1a** are eliminated as acetic acid.[2,3]

During the past 5 years, there has been remarkable progress in elucidating the biosynthetic pathway to vitamin B_{12} with the outcome that the problem is now solved.[4,5] All these recent experiments were carried out using the aerobic organism, *Pseudomonas denitrificans*. Here also, precorrin-3A **1a** is an early precursor of vitamin B_{12} but in this organism the first intermediate having the corrin macrocycle is not cobyrinic acid **4a** but its cobalt-free analogue, hydrogenobyrinic acid **5a**. Once again C-20 and its attached methyl group of precorrin-3A **1a** have been lost before hydrogenobyrinic acid **5a** is formed, a process initiated by the ring-contraction step that produces[4,5] precorrin-4[6] **2a**. At this point, the two-carbon fragment is exposed as the acetyl group at C-1 of **2a**.

There is chromatographic evidence[7] that acetic acid is formed as precorrin-4 **2a** is carried forward along the biosynthetic pathway but rigorous proof is essential. In addition, whilst an attractive mechanism can be written[5] for the hydrolytic removal of the C-1 acetyl group as acetic acid, an equivalent sequence could afford acetaldehyde if a later enzyme could act as a reductase in addition to its presently established role to convert the ketone **2a** into the corresponding alcohol **3a**. Such a possibility cannot be ignored when at least seven enzymes involved in the biosynthesis of coenzyme B_{12} are di- or multi-functional.[4] There is little doubt that, under the incubation and subsequent handling conditions, adventitious oxidation of some of this putative acetaldehyde, which would be present in minute amounts, could occur to yield acetic acid. Therefore the plan was to carry out two experiments, one designed to isolate acetic acid and the other to isolate acetaldehyde in each case as a crystalline derivative.

[2,7,20-*methyl*-$^{14}C_3$]Precorrin-3A **1b** was synthesised enzymically[8] from 5-aminolaevulinic acid and [*methyl*-^{14}C]-*S*-adenosyl-L-methionine (SAM) and carefully purified.† It was then incubated with an enzyme preparation[10] from an engineered strain of *P. denitrificans* [SC510Rif^r(pXL253)]



in which all the enzymes needed for production of hydrogenobyrinic acid **5b** from precorrin-3A **1b** are overproduced; these are CobG, CobJ, CobM, CobF, CobK, CobL and CobH.

To the major part of the incubation mixture was added a substantial amount of unlabelled acetic acid as carrier to trap any radioactive acetic acid which could only be present in a minute amount. The total acetic acid was then isolated, converted into its crystalline *p*-bromophenacyl ester and purified to constant ^{14}C-activity. Hydrogenobyrinic acid **5b** was isolated from the minor portion of the incubation mixture.

The experiment was repeated and at the end of the incubation a substantial quantity of unlabelled acetaldehyde was injected into the sealed incubation vessels before they were opened. The acetaldehyde was then isolated as its dimedone derivative which was subsequently converted into the anhydro-form[11] for rigorous purification by chromatography and multiple recrystallisation.

† Purification was carried out after aerial oxidation and esterification of precorrin-3A **1b** to give its stable didehydro octamethyl ester. The pure ester was then hydrolysed and the octa-acid was added directly to the incubation mixture where it is known[9] to be enzymically reduced back to precorrin-3A.

Table 1 ^{14}C-Labelling experiments in *P. denitrificans*

	Radiochemical yield (%)	
	Expt. 1	Expt. 2
Acetic acid (as *p*-bromophenacyl ester)	9.7	*a*
Acetaldehyde (as anhydro dimedone derivative)	*a*	< 0.16
Hydrogenobyrinic acid **5b**	2.4	5.2

[a] Not isolated.

Table 1 shows that the amount of [^{14}C]acetaldehyde isolated following ring contraction was negligible. In contrast, a good yield of [^{14}C]acetic acid was produced which was rigorously identified. For equivalent amounts of hydrogenobyrinic acid **5b** formed, the ^{14}C-activity in the acetaldehyde derivative (still diminishing when it was judged unnecessary to purify further) was < 0.8% of that in the acetic acid. The fact that less hydrogenobyrinic acid **5b** was formed than corresponds to the amount of isolated acetic acid is a reflection of the less than quantitative conversions in the enzymic steps needed[4] to reach **5b** following the elimination of acetic acid.

These experiments thus firmly establish that during the biosynthesis of vitamin B_{12} **6a** in *P. denitrificans*, C-20 and its attached methyl group present in precorrin-3A **1a** are eliminated as acetic acid as was the case in *P. shermanii*.[2,3]

Experimental

Enzymic Synthesis of [2,7,20-methyl-$^{14}C_3$]*Trimethylisobacteriochlorin* (*TMIBC*).—This was carried out by the method described elsewhere[8] using *S*-adenosyl-L-methionine (SAM) (14 mg), *S*-adenosyl-L-[*methyl*-^{14}C]methionine (0.2 cm^3 of commercial acidic solution, 25 μCi cm^{-3}) and 5-aminolaevulinic acid (11 mg). This yielded the TMIBC octamethyl ester (1.77 mg; 3.52 × 10^6 dpm).

Enzymic Conversion of [2,7,20-methyl-$^{14}C_3$]*TMIBC into Hydrogenobyrinic Acid and Capture of Acetic Acid.*—The foregoing TMIBC ester (1.77 mg) was stirred with 2 mol dm^{-3} aqueous piperidine (0.44 cm^3, 18 °C, 48 h) then diluted with pH 7.7 Tris.–HCl buffer (0.1 mol dm^{-3} Tris.–HCl, 1 mmol dm^{-3} EDTA) to 11 cm^3. This was divided among 108 tubes, each containing the same Tris.–HCl buffer (0.6 cm^3), enzyme cofactors (0.1 cm^3 containing 0.73 mg NADH, 0.73 mg NADPH and 0.36 mg SAM in Tris.–HCl buffer) and SC510Rif^r(pXL253)[10] cell-free extract (0.08 cm^3). The tubes were sealed and incubated at 30 °C for 20 h.

To the combined contents of 98 tubes was added sodium acetate trihydrate (71.6 mg, 0.53 mmol) and the pH adjusted to 1–2 by addition of H_2SO_4 (3 mol dm^{-3}). The mixture was steam distilled using a few drops of Antifoam A Emulsion (Sigma). The distillate (300 cm^3) was basified to phenolphthalein with 0.105 mol dm^{-3} potassium hydroxide (8.2 cm^3) then evaporated and dried *in vacuo*. This was treated[12] in anhydrous acetonitrile (10 cm^3) with *p*-bromophenacyl bromide (326 mg, 1.17 mmol) and 18-crown-6-ether (60 mg) and heated at 80 °C for 2 h. The residue from evaporation was purified by chromatography in benzene on a short silica column and then by preparative TLC using benzene before elution with acetonitrile. The crystalline acetate ester (1.65 × 10^5 dpm in total) was recrystallised twice from toluene and hexane to give pure product at constant activity (831 dpm mg^{-1}; 2.14 × 10^5 dpm mmol^{-1}), mp 85–86 °C as in lit.;[12] δ_H(400 MHz; C_6D_6) 7.10 (4 H, AA'BB', *J* 8.5, C_6H_4), 4.68 (2 H, s, CH_2) and 1.81 (3 H, s, Me); δ_C(100 MHz; C_6D_6) 190.7 (Me*C*O), 169.7 (CH_2*C*O), 133.2 (aromatic-C), 132.0 (2 × aromatic-CH), 129.3 (2 × aromatic-CH), 128.5 (aromatic-C), 65.7 (CH_2) and 20.1 (Me).

The solution in the remaining 10 tubes was acidified by 2 mol dm^{-3} hydrochloric acid, centrifuged and the supernatant was loaded onto a prewashed Lychroprep RP-18 column (Merck). The pigments eluted with 1:1 aqueous acetonitrile were separated by HPLC (PhaseSep Nucleosil 5ODS) and appropriate fractions were lyophilised to give hydrogenobyrinic acid (activity equivalent to 5.57 × 10^4 dpm in all 108 tubes).

The Parallel Experiment Seeking Acetaldehyde.—[2,7,20-*methyl*-$^{14}C_3$]TMIBC ester (1.07 mg, 2.1 × 10^6 dpm) was hydrolysed and incubated as above but with proportional quantities of reagents and enzymic solution in 50 tubes. Into each of 45 tubes (at 0 °C) was injected 0.2 cm^3 of a solution of acetaldehyde (50 mg) in water (9 cm^3) and the combined solution adjusted to pH 9. It was then steam distilled as above, the distillate (100 cm^3) being collected into a solution of 5,5-dimethylcyclohexane-1,3-dione (396 mg) in 1:2 aqueous ethanol (30 cm^3) and piperidine (2 drops). After 16 h, ethanol (5 cm^3) was added followed by concentrated hydrochloric acid (5 cm^3) and the mixture was heated at 100 °C for 15 min. The ethanol was evaporated and the product isolated by ether extraction and purified by repeated preparative TLC in 1% methanol and dichloromethane (50 cm^3) containing piperidine (5 drops) to give crystalline material (243 mg; 1 × 10^3 dpm mmol^{-1}). Five recrystallisations from dichloromethane and hexane gave chemically pure product (3.5 dpm mg^{-1}), mp 176–177 °C as in lit.;[11] δ_H(400 MHz; $CDCl_3$) 3.62 (1 H, q, *J* 6.4, C*H*Me), 2.34 (4 H, s, 2 × CH_2), 2.26 (4 H, s, 2 × CH_2), 1.08 (12 H, s, 4 × Me) and 1.07 (3 H, d, *J* 6.4, CH*Me*); δ_C(100 MHz; $CDCl_3$) 197.1 (2 × CO), 162.7 (2 × =C–O), 116.7 (2 × *C*=C–O), 50.9 (2 × CH_2), 40.8 (2 × CH_2), 32.1 (CH), 29.3 (2 × Me), 27.2 (2 × Me) and 21.7 (CH*Me*).

Hydrogenobyrinic acid (activity equivalent to 7.3 × 10^4 dpm in all 50 tubes) was isolated as above from the other 5 tubes.

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