The Incorporation of Formate into Nicotinic Acid by *Clostridium butylicum*By T. A. SCOTT and M. MATTEY
Department of Biochemistry, University of Leeds

(Received 31 January 1968)

Cell extracts of *Clostridium butylicum* synthesize the nicotinamide moiety of NAD from aspartate, acetate and formate (Isquith & Moat, 1966). From work with intact cells (Isquith & Moat, 1965), there is strong evidence that C-2, C-3 and C-7 of nicotinic acid are derived from C-2, C-3 and C-4 of aspartic acid, but the sites of incorporation of the carbon atoms of formate and acetate are not known. The present work describes the incorporation of [^{14}C]formate into nicotinic acid by resting cells of *Cl. butylicum*. Sodium [^{14}C]formate was purchased from The Radiochemical Centre, Amersham, Bucks. *Cl. butylicum* N.C.I.B. 9380 was obtained from The National Collection of Industrial Bacteria, Aberdeen.

Cells of *Cl. butylicum* were grown anaerobically at 37° for 36 hr. in the medium of Lampen & Peterson (1943) with the addition of biotin (0.001 $\mu\text{g./ml.}$) and *p*-aminobenzoic acid (0.05 $\mu\text{g./ml.}$). Incubation mixtures contained sodium [^{14}C]formate (11.7 μmoles), phosphate buffer, pH 7 (1250 μmoles), D-ribose (200 μmoles), adenine sulphate (25 μmoles)

and cells of *Cl. butylicum* corresponding to 20 mg. dry wt., in a total volume of 20 ml. After incubation anaerobically at 37° for 5 hr., 2N-H₂SO₄ (50 ml.) was added and the mixture was hydrolysed for 30 min. at 15 lb./in.². A sample (5 ml.) of the hydrolysate was taken for the microbiological assay of nicotinic acid by the method of Barton-Wright (1944). Unlabelled carrier nicotinic acid (100 mg.) was added to the remainder of the hydrolysate, followed by 15% (w/v) NaNO₂ (100 ml.) to destroy the amino acids. When the vigorous evolution of nitrogen had subsided, the solution was heated for 20 min. at 100°, and more 2N-H₂SO₄ was added when necessary to keep the reaction acidic. Excess of HNO₂ was then destroyed by the gradual addition of solid urea until no more nitrogen was evolved. Insoluble material was removed by centrifugation at 10000g for 15 min. The supernatant was evaporated to 4–5 ml. and placed on a column (2 cm. $\times$ 20 cm.) of Dowex 50 (X4; H⁺ form). A concave gradient of 0–4N-HCl in a total volume of 2 l. was passed through the column, and nicotinic

Table 1. The distribution of radioactivity in a sample of [^{14}C]nicotinic acid derived from *Cl. butylicum* after feeding with [^{14}C]formate

The conditions of incubation are described in the text. Nicotinic acid was degraded chemically by the method of Scott (1967). Microbiological assay showed that the hydrolysed cells of *Cl. butylicum* contained 31 $\mu\text{g.}$ of nicotinic acid; this was diluted with 100 mg. of unlabelled carrier nicotinic acid. The specific radioactivity of the sodium [^{14}C]formate was 42.8 mc/m-mole; this corresponds to an observed radioactivity of 1.996×10^8 counts/min. at infinite thickness of BaCO₃ and a counting efficiency of 15%. The experimental mean of all the carbon atoms was determined by totally burning a sample to CO₂ (Scott & Hussey, 1965). The calculated mean is derived from the values for separate carbon atoms.

Carbon atom no.	Sp. radioactivity (counts/min. at infinite thickness of BaCO ₃)		Radioactivity (% of total incorporated, based on the experimental mean)
	Observed value	Value corrected for dilution with carrier	
2	38.0	1.2×10^5	9.7
3	6.6	0.2×10^5	1.7
4	0	0	0
5	0	0	0
6	353.0	11.4×10^5	90.0
7	12.3	0.4×10^5	3.2
Experimental mean	65.2	2.1×10^5	
Calculated mean	68.3	2.2×10^5	

acid was detected in the effluent by its extinction at $260\text{m}\mu$; it appeared at a peak effluent volume of 1.5l., when the concentration of HCl was approximately 2N. The fractions containing nicotinic acid were evaporated to dryness, redissolved in water (20ml.) and placed on a column (2cm. $\times$ 20cm.) of Dowex 1 (formate form). A linear gradient of 0–0.1N-formic acid in a total volume of 1.2l. was passed through the column, and the fractions containing nicotinic acid (peak effluent volume 1.025l.) were evaporated to dryness. The residue was redissolved in 2N-HCl (10ml.) and evaporated to dryness again, and the residual nicotinic acid hydrochloride was sublimed under lowered pressure. The product was radiochemically pure on two-dimensional paper chromatography (Scott & Hussey, 1965). Samples of radioactive nicotinic acid were degraded chemically by the method of Scott (1967), and the resulting samples of CO_2 were converted into BaCO_3 (Scott & Hussey, 1965), and its radioactivity was measured at infinite thickness in a Nuclear-Chicago Corp. gas-flow counter at 15% efficiency.

The results are shown in Table 1. Of the radioactivity of the nicotinic acid, 90% was in C-6. From the data available, it was not possible to determine the amount of nicotinic acid synthesized during the incubation period; in our experience there is no

detectable change in the total nicotinic acid content of the cells after incubation under the above conditions. There is therefore a turnover of nicotinic acid, without a net increase; the approximately 200-fold dilution of radioactivity (radioactivity of C-6/radioactivity of formate) is only apparent, and the real dilution is probably less.

Evidence for the participation of *N*-formyl-aspartic acid in the biosynthesis of nicotinic acid by *Cl. butylicum* has already been reported (Scott, Bellion & Matthey, 1968). The incorporation of radioactivity from [^{14}C]formate into C-6 of nicotinic acid by *Cl. butylicum* supports the proposal that the formylation of the amino group of aspartic acid is a step in the biosynthesis of nicotinic acid in this organism.

We thank Carreras Ltd. for the purchase of radiochemicals and for financial support for M.M.

- Barton-Wright, E. C. (1944). *Biochem. J.* **38**, 314.
Isquith, A. J. & Moat, A. G. (1965). *Bact. Proc.* **65**, 74.
Isquith, A. J. & Moat, A. G. (1966). *Biochem. biophys. Res. Commun.* **22**, 565.
Lampen, J. O. & Peterson, W. H. (1943). *Arch. Biochem. Biophys.* **2**, 443.
Scott, T. A. (1967). *Biochem. J.* **102**, 87.
Scott, T. A. & Hussey, H. (1965). *Biochem. J.* **96**, 9c.
Scott, T. A., Bellion, E. & Matthey, M. (1968). *Biochem. J.* **107**, 23 p.