Biochem. J. (1976) **158**, 223–229
Printed in Great Britain

The Biochemical Pathway for the Breakdown of Methyl Cyanide (Acetonitrile) in Bacteria

By JOHN L. FIRMIN* and DAVID O. GRAY
Department of Botany and Biochemistry, Westfield College, Kidderpore Avenue, Hampstead, London NW3 7ST, U.K.

(*Received* 29 *January* 1976)

[2-^{14}C]Methyl cyanide (acetonitrile) is metabolized to citrate, succinate, fumarate, malate, glutamate, pyrrolidonecarboxylic acid and aspartate. Non-radioactive acetamide and acetate compete with ^{14}C from methyl cyanide, and [2-^{14}C]acetate and [2-^{14}C]methyl cyanide are metabolized at similar rates, giving identical products. This evidence, combined with the inhibitory effect of fluoroacetate and arsenite on methyl cyanide metabolism, indicates that the pathway is: methyl cyanide → acetamide → acetate → tricarboxylic acid-cycle intermediates. The pathway was investigated in a species of *Pseudomonas* (group III; N.C.I.B. 10477), but comparison of labelling patterns suggests that it also exists in several higher plants.

Little is known of the biochemistry of covalent organic cyanides in living systems, though the simplest such compound, methyl cyanide (acetonitrile), can probably be utilized by as many species as can ionic cyanide. Thus surveys carried out by Strauss (1956) and by Firmin (1973) show that of 11 taxonomically diverse higher plants, six definitely metabolize [^{14}C]methyl cyanide and four others may do so.

Most authors (Strauss, 1956; Williams, 1959) agree that the cyanide residue of organic nitriles is finally hydrolysed:

$$CN \longrightarrow CONH_2 \xrightarrow{NH_3} CO_2H \longrightarrow CO_2$$

Some, however, believe that it must first be split off from the rest of the carbon skeleton as HCN. This idea originated with Lang (1894), who showed that dogs treated with aliphatic cyanides excreted additional thiocyanate, a detoxication product of HCN. The hypothesis explains the relative toxicities of a series of ω-fluoroalkyl cyanides in mice (Pattison, 1953) and the growth-promoting properties of certain 2,4-dichlorophenoxyalkyl cyanides in wheat seedlings (Fawcett *et al.*, 1955). It was accepted by Strauss (1956), the only previous worker to study methyl cyanide metabolism in higher plants, who reported transfer of ^{14}C from the cyanide moiety to CO_2 and pectin, but rejected acetate as a possible intermediate because the saponifiable (bound fatty acid) fraction did not become significantly labelled in *Pisum sativum*, an argument invalidated by the sporadic labelling of this fraction in *Zea mays*.

* Present address: Department of Genetics, John Innes Institute, Colney Lane, Norwich, NR4 7UH, U.K.

The early literature, reviewed by Williams (1959), suggests that covalent cyanides may also be hydrolysed directly and the corresponding acids certainly accumulate when indol-3-ylmethyl cyanide and 2,4-dichlorophenoxymethyl cyanide are incubated with wheat tissue (Fawcett *et al.*, 1955). Mimura *et al.* (1969) have suggested that methyl cyanide itself is metabolized in this way, but the only supporting evidence was the temporary accumulation of amide, not specifically acetamide, by a bacterium (*Corynebacterium nitrophilus* nov. sp. C-42) growing on a medium containing methyl cyanide.

Thus the theories of covalent cyanide metabolism are based on conflicting and circumstantial evidence. The purpose of this paper is to show, for the first time, that methyl cyanide at least does undergo direct hydrolysis; the evidence comes from a species of *Pseudomonas* isolated for its ability to utilize the nitrile as sole carbon source.

Experimental

Paper chromatography

Descending chromatograms were run on Whatman 3MM paper in the following solvents: A, phenol/water (3:1, w/v); B, phenol/water (100:39, w/v); C, phenol/90% (v/v) formic acid/water (100:10:29, w/v/v); D, butan-1-ol/acetic acid/water (90:10:29, by vol.); E, butan-1-ol/propionic acid/water (92:47:61, by vol.); F, organic phase of 2-methylbutan-2-ol/90% (v/v) formic acid/water (8:1:4, by vol.).

Chromatograms were dried for 24h at 30°C before treatment with the appropriate detecting reagent. Amino acids were located by dipping the papers

through 0.2% (w/v) ninhydrin (indanetrione hydrate) in ethanol and heating at 50°C. Organic phosphates and acids were detected with 0.004 M-$FeCl_3$ in acetone followed by 0.33 M-ammonium thiocyanate in acetone (Firmin & Gray, 1974). Sugars were revealed by using an alkaline silver reagent (Trevelyan *et al.*, 1950) and acetamide with a modified ferric hydroxamate test (Firmin & Gray, 1975).

Radioactive substrates

These were supplied by The Radiochemical Centre, Amersham, Bucks., U.K., and were normally stored in aqueous solution at −20°C.

[2-^{14}C]Methyl cyanide (11.3 mCi/mmol) had a chemical purity, determined immediately before use, exceeding 99%, as measured by g.l.c. on columns (0.4 cm × 200 cm long) of (*A*) 15% Apiezon L on Chromosorb W (80–100 mesh) eluted with N_2 at 60°C, and (*B*) 8% Carbowax 20M + 2% (w/w) KOH on the same support, eluted with N_2 at 150°C. The cyanide preparation contained less than 0.01% of [^{14}C]acetamide. This was shown by chromatographing samples containing carrier acetamide (200 μg) on paper in solvent E. The acetamide zone was located by comparison with standards, and its ^{14}C content was measured after elution.

Sodium [2-^{14}C]acetate (radiochemical purity > 98%) and $NaH^{14}CO_3$ had specific radioactivities of 56 mCi/mmol and 42 mCi/mmol respectively.

Isolation and culture of Pseudomonas sp. group III

A modified minimal medium (Cruickshank *et al.*, 1965) was used, containing 0.1% (v/v) methyl cyanide as sole carbon source, together with K_2HPO_4 (7 g/l), KH_2PO_4 (3 g/l), $MgSO_4,7H_2O$ (100 mg/l), $(NH_4)_2SO_4$ (1 g/l), $CaCl_2$ (10 mg/l), $FeSO_4,7H_2O$ (2.5 mg/l), $ZnSO_4,7H_2O$ (2.5 mg/l), $MnSO_4,3H_2O$ (2.5 mg/l) and H_2SO_4 (0.25 mg/ml). It was sterilized by autoclaving at 69 kN/m^2 (10 lb/in^2) for 30 min, in sealed bottles to avoid loss of nitrile. The bacterium obtained by exposing this medium to laboratory air and plating out on the same mixture containing 2% (w/v) BDH agar was shown to be in pure culture and identified as a *Pseudomonas* species, belonging to group III as defined by Shewan *et al.* (1960) (N.C.I.B. 10477). The organism was maintained as a routine in the above liquid medium at 4°C and subcultured at monthly intervals.

The mean generation time, measured by nephelometry, was 1.8 h in continuously shaken well aerated liquid medium at 25°C. The optimum methyl cyanide concentration for growth was 0.05–0.1%; 1.0% (v/v) completely inhibited cell division.

Bacterial cultures: incubation with labelled substrates

Typically, cultures of *Pseudomonas* were grown at 20°C in 200 ml batches of well-aerated minimal medium, containing 0.05% (v/v) methyl cyanide, until their turbidity was 50% of its stationary-state value. The cells were then harvested by centrifugation (35000*g*, 5 min, 15°C) and resuspended in 30 ml of minimal medium containing no carbon source. They were immediately exposed to the labelled substrate (13–75 μCi) at 20–25°C, usually for 3 min, re-centrifuged and fixed with 70% (v/v) ethanol (15 ml). Experiments were duplicated and accompanied by appropriate autoclaved cell controls. Further details are given in the Table legends.

Identification of labelled compounds in bacterial extracts

The ethanolic cell extracts were initially fractionated as shown in Scheme 1, solutions being evaporated as a routine *in vacuo* at 20–50°C.

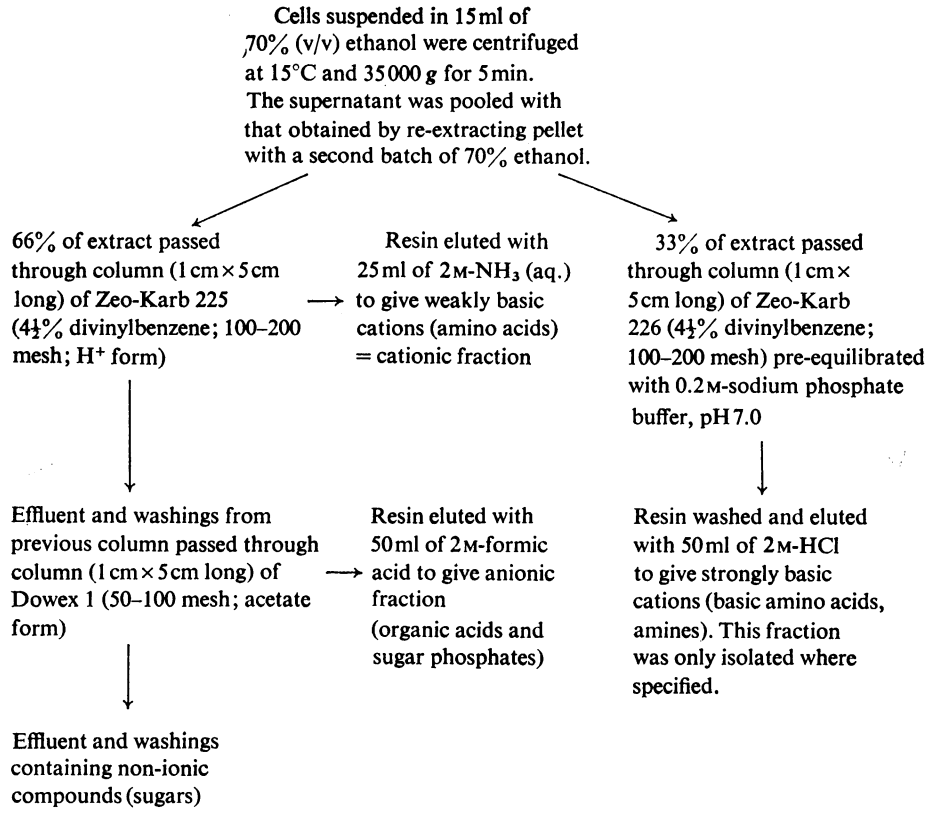
The amino acid fraction was separated into its components by two-dimensional paper chromatography in solvents A and D, the organic acids in solvents C and F, and the sugars in solvents B and E. Radioactive compounds were detected by radioautography, the exposure time usually being 6 weeks. Acetamide appeared in the 'sugar' fraction, but was so volatile that it could only be detected on radioautograms if unlabelled carrier (250 μg/chromatogram) was first added to the cell extract (Firmin & Gray, 1975).

The basic amino compounds were separated on a column (0.4 cm × 200 cm long) of the weak cation-exchange resin, Zeo-Karb 226 (4.5% divinylbenzene) of mean particle diameter 27 μm. This column was eluted, at 21°C, with 0.1 M-sodium phosphate buffer, pH 7.0, containing 0.9 M-$NaNO_3$ and 0.5 M-KNO_3, at a flow rate of 12.5 ml/h. Radioactive compounds were detected by scintillation counting, at an overall efficiency of 85%, after diluting the eluate 1:1 with water (see the next section).

Labelled substances were always identified by comparison with internal standards, mostly detected as described under 'Paper chromatography'. However, basic amino compounds in column effluents were revealed with naphthaquinonesulphonic acid (Blau & Robson, 1957).

Measurement of ^{14}C

Spots on paper chromatograms were normally cut out and counted for radioactivity directly by liquid-scintillation spectrometry in 5 ml of toluene containing 0.5% (w/v) 2,5-diphenyloxazole and 0.03% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene (Rogers *et al.*, 1966), the overall counting efficiency being 57%. Alternatively, the concentrated eluate from the paper, like other aqueous solutions, was counted after mixing with KL 372 scintillator fluid (Koch–Light, Colnbrook, Bucks., U.K.), giving a



Scheme 1. *Fractionation procedure*

net efficiency of 92%. All counts are given in absolute terms, as they have been corrected for quenching, counter efficiency and background. Approximate standard errors are: 0.01 nCi, 10%; 0.1 nCi, 6%; 1 nCi, 2%; 10nCi, 1%.

Results

Metabolites of methyl cyanide

Time-course experiments (Fig. 1) showed that when *Pseudomonas* sp. was supplied with [2-^{14}C]-methyl cyanide, label first appeared in glutamate, aspartate and succinate and then in all the chemically stable amino and organic acids associated with the tricarboxylic acid cycle. Fig. 1 suggests that glutamate, aspartate and succinate, at least, are close to the point of entry of ^{14}C, whereas pyrrolidonecarboxylic acid has the kinetic characteristics of a secondary product. Fig. 2(*a*) illustrates the greater number of compounds that became labelled when the cells were briefly exposed to [2-^{14}C]methyl cyanide of higher specific radioactivity. Sugar phosphates then also became significantly labelled, but the sugar fraction did not (Table 2*a*).

Some label entered substances, such as lysine and methylamine, in the strongly basic cationic fraction, but less than 0.001% of that supplied appeared in ethylamine after 120min under the experimental conditions of Fig. 1. Thus the cyanide was not significantly reduced to the corresponding amine.

Evidence that acetamide and acetate are intermediates in methyl cyanide breakdown

The nature of the labelled products, demonstrated in *Pseudomonas* in six diverse experiments, suggested that carbon from methyl cyanide entered the tricarboxylic acid cycle. Thus direct hydrolysis of the nitrile to acetamide and acetate seemed probable.

Pseudomonas supplied with [2-^{14}C]methyl cyanide was shown to accumulate labelled acetamide by radioautography of two-dimensional 'sugar'-fraction chromatograms, loaded with carrier acetamide. The amide only became slowly saturated with ^{14}C, but accounted for at least 6.0% of that originally supplied after 250min incubation (Table 1).

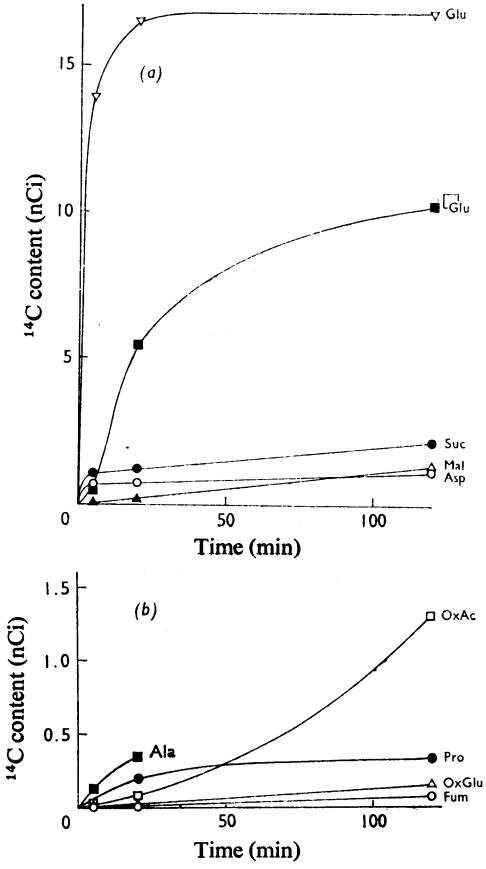


Fig. 1. *Time-course of radiocarbon accumulation by metabolites of* [2-^{14}C]*methyl cyanide in Pseudomonas sp.*

(*a*) Results for the major metabolites; (*b*) results for related compounds that accumulate less ^{14}C. Each result represents the metabolite extracted from an 8ml sample of an unconcentrated culture, which had been incubated with 4μCi of ^{14}C at 20°C. No attempt was made to remove residual unlabelled methyl cyanide in this experiment. Key: Glu, 5-pyrrolidone-2-carboxylic acid; Fum, fumarate; Mal, malate; OxAc, oxaloacetate; OxGlu, 2-oxoglutarate; Suc, succinate.

Unlabelled acetate and acetamide both appeared to compete very effectively with [^{14}C]methyl cyanide (Table 2). For example, unlabelled acetate decreased the ^{14}C in the amino acid fraction 24-fold (Table 2*a*) and that in intermediates of the tricarboxylic acid cycle by an average of 13-fold (Table 2*b*). Chromatography and radioautography showed that the relatively high radioactivity of the non-ionic fraction in the '+ acetamide' culture was due to [^{14}C]acetamide itself. Similar trapping of ^{14}C by the large acetate pool could not be demonstrated, as the compound was lost during fractionation and chromatography.

Table 1. *Time-course of acetamide production from* [2-^{14}C]*methyl cyanide in Pseudomonas sp.*

An unconcentrated culture (60ml) having a turbidity of 50% of its stationary-state value was incubated with 15μCi of [^{14}C]methyl cyanide at 20°C. Samples (10ml) were fixed in ethanol (25ml) containing carrier acetamide (500μg). A sample (50%) of each non-ionic fraction obtained was chromatographed on paper in solvent E, which was able to separate acetamide from all other labelled substances present. The acetamide spot, located by comparison with standards, but not itself treated with the hydroxamate reagent, was excised and eluted with 70% (v/v) ethanol (15ml) before being counted for radioactivity. Recovery of the acetamide cannot have exceeded 38% (Firmin & Gray, 1975), and the value given in the results is corrected for this 62% loss.

Incubation time with [^{14}C]methyl cyanide (min)	^{14}C in acetamide (nCi)
0.08	0.015
0.33	0.039
1.33	0.097
5.33	1.28
50	3.09
250	28.5

Table 2. *Effect of unlabelled acetamide and acetate on the labelling of metabolites of* [2-^{14}C]*methyl cyanide in Pseudomonas sp.*

A bacterial suspension, concentrated as described, was divided into 15ml portions. Water (1ml) was added to each containing, where appropriate, 400μmol of either unlabelled acetamide or unlabelled sodium acetate: [^{14}C]methyl cyanide (25μCi, 2.2μmol) was introduced 1min later and the cells were incubated for 3min at 20°C before analysis. The results given in Table 2(*b*) correspond to the whole anionic fraction, though only half was chromatographed.

(*a*) Effect on the major fractions

	^{14}C present in each fraction (nCi)		
	Untreated culture	Culture + acetamide	Culture + acetate
Cationic fraction	845	46.6	35.3
Anionic fraction	283	14.7	14.8
Non-ionic fraction	5.19	19.8	4.02

(*b*) Effect on individual organic acids

	^{14}C present in each compound (nCi)		
	Untreated culture	Culture + acetamide	Culture + acetate
Citrate/isocitrate	6.84	2.54	1.59
Malate	24.4	1.75	1.43
2-Oxoglutarate	2.22	0.080	0.158
Succinate	38.4	3.82	2.54
Fumarate	14.1	0.95	0.794

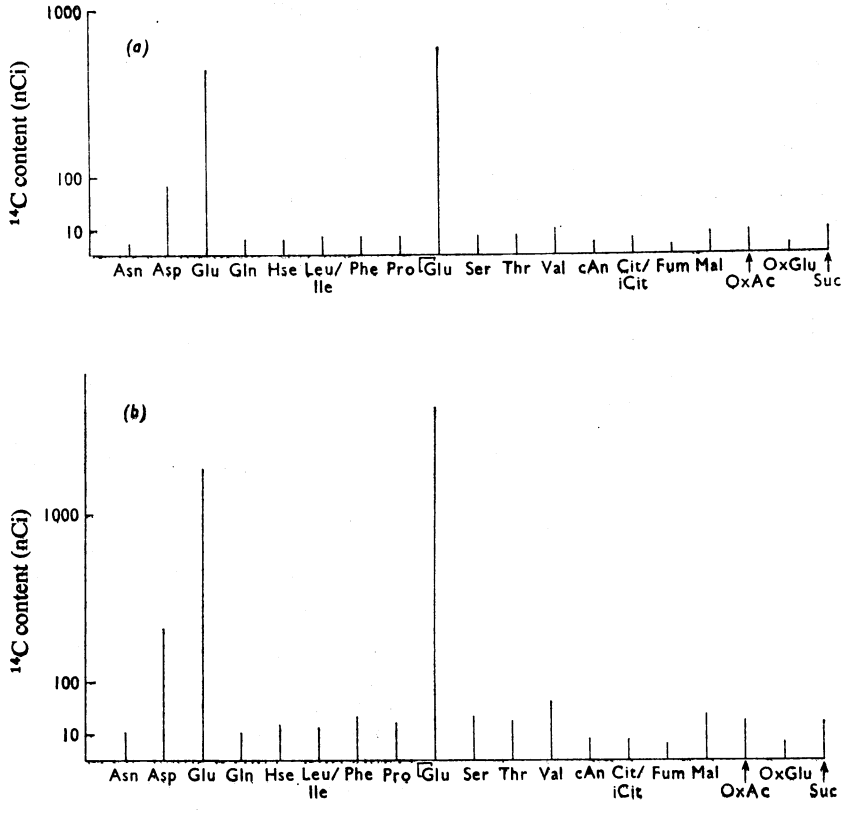


Fig. 2. *Comparison of labelling of metabolites of* [2-^{14}C]*methyl cyanide* (*a*) *and* [2-^{14}C]*acetate* (*b*) *in Pseudomonas sp.*

Each result represents the metabolite extracted from 5ml of a concentrated bacterial suspension after this had been incubated with 17μCi of ^{14}C for 3min at 20°C. The height of the line representing each compound is proportional to the square root of its radiocarbon content, to allow extreme results to be shown on the same diagram. The intermediates shown in (*b*) contain more radiocarbon, since the acetate supplied had a specific radioactivity five times greater than that of the methyl cyanide. Key: Hse, homoserine; ⊏Glu, 5-pyrrolidone-2-carboxylic acid; cAn, *cis*-aconitate; Cit, citrate; iCit, isocitrate; Fum, fumarate; Mal, malate; OxAc, oxaloacetate; OxGlu, 2-oxoglutarate; Suc, succinate.

Finally, radioautograms showed that [2-^{14}C]acetate and [2-^{14}C]methyl cyanide were metabolized to exactly the same 94 products, and Fig. 2 illustrates that the relative labelling of the major ones was similar in the two cases. Comparison of Fig. 2(*a*), Fig. 1 and Table 2(*b*) shows that the percentage of ^{14}C in the intermediates assayed was very sensitive to experimental conditions and this emphasizes the agreement between Figs. 2(*a*) and 2(*b*).

Further evidence that carbon from methyl cyanide enters the tricarboxylic acid cycle

The tricarboxylic acid-cycle inhibitors, fluoroacetate and arsenite, strongly suppressed the transfer of label from [2-^{14}C]methyl cyanide to all products revealed by radioautography (Table 3). Moreover, fluoroacetate increased the relative labelling of citrate, confirming that aconitate hydratase was genuinely inhibited in this experiment. The effect of malonate on methyl cyanide metabolism was not investigated, since, even at 100mM, it stimulated rather than inhibited bacterial growth.

Tracer studies (Table 4) showed that C-2 of acetate entered cycle intermediates and other low-molecular-weight metabolites at a rate comparable (70%) with that for C-2 of methyl cyanide, whereas carbon from bicarbonate was utilized 112 times less rapidly. This confirms that the methyl carbon atom

Table 3. *Effect of inhibitors of the tricarboxylic acid cycle on labelling of metabolites of* [2-^{14}C]*methyl cyanide in Pseudomonas sp.*

An unconcentrated culture, having a turbidity of 30% of its stationary-state value, was divided into 40ml portions. Sterile water or sterile inhibitor solution (1 ml) was added to give final concentrations of sodium arsenite and fluoroacetic acid of 3 mM and 5 mM respectively: these values inhibited growth by approx. 90%. After 3h at 20°C, each sample was centrifuged and the cells were resuspended in a medium containing inhibitor, where appropriate, but no unlabelled methyl cyanide. [^{14}C]-Methyl cyanide (25 μCi) was added immediately and the cells were incubated, harvested and analysed as usual.

	^{14}C present (% of that found in the same intermediate in the uninhibited culture)	
Compound	Arsenite-inhibited	Fluoroacetate-inhibited
Citrate/isocitrate	1.1	34.1
2-Oxoglutarate	2.8	7.5
Malate	0.4	4.9
Fumarate	3.0	7.0
Succinate	1.0	2.7

Table 4. *Relative rates of metabolism of different carbon sources by Pseudomonas sp.*

Concentrated cultures were divided into 15 ml batches and the appropriate labelled substrate (50 μCi in 1.3 ml for *a* and 13 μCi in 0.5 ml for *b*) was added to each. The relative rates of metabolism given in the text have been calculated by adjusting these results for the different specific radioactivities of the three substrates.

(*a*) [2-^{14}C]Methyl cyanide/[2-^{14}C]sodium acetate comparison

	^{14}C in each fraction (nCi)	
	Culture supplied with [^{14}C]methyl cyanide	Culture supplied with [^{14}C]acetate
Cationic fraction	3724	12030
Anionic fraction	626	2436
Non-ionic fraction	10.5	87.8

(*b*) [2-^{14}C]Methyl cyanide/NaH$^{14}CO_3$ comparison

	^{14}C in each fraction (nCi)	
	Culture supplied with [^{14}C]methyl cyanide	Culture supplied with NaH$^{14}CO_3$
Cationic fraction	826	26.5
Anionic fraction	132	4.91
Non-ionic fraction	3.58	0.54

of acetonitrile must enter the cycle as acetate and cannot do so as CO_2, the only other possibility consistent with the radioautograms.

Discussion

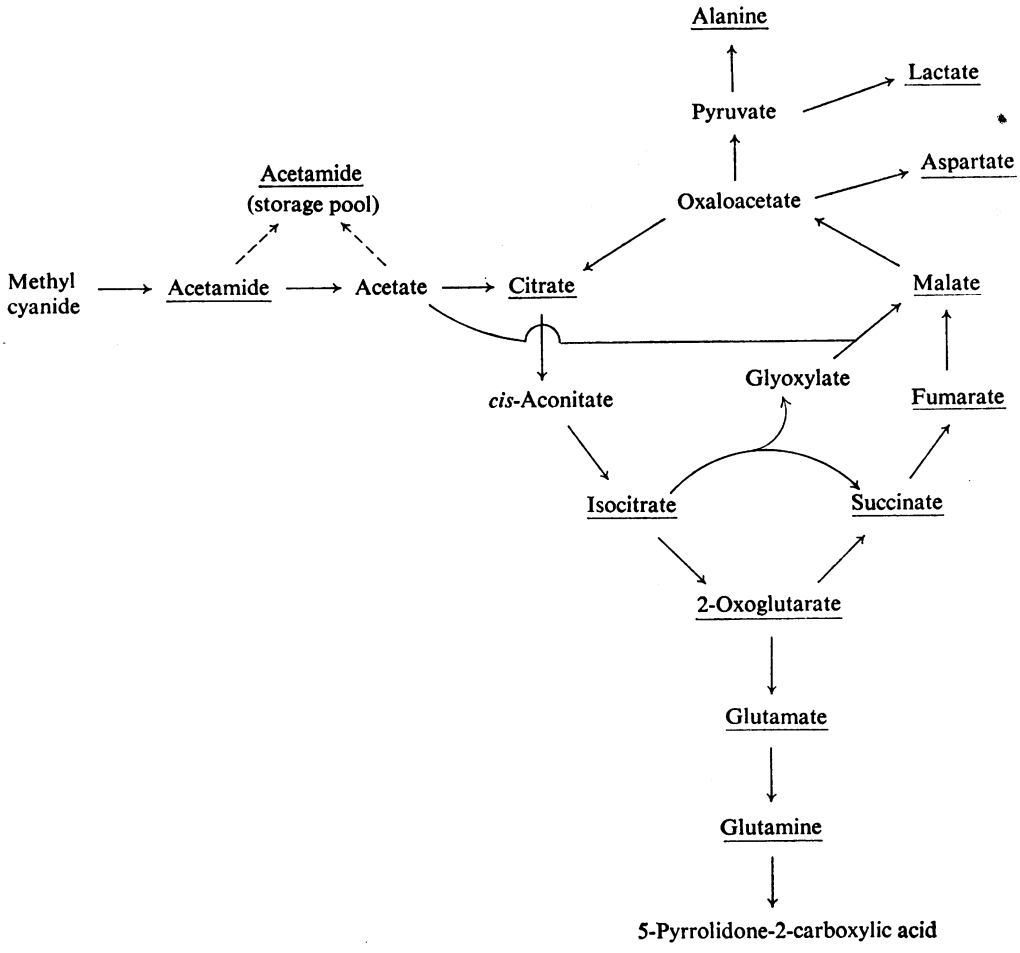
All our observations can be explained in more than one way. For example, unlabelled acetate could suppress the metabolism of methyl cyanide by inhibiting an essential enzyme and not by acting as a genuine competitor. Nevertheless, the combined evidence strongly supports the pathway shown in Scheme 2.

Intracellular acetamide accumulates ^{14}C much more slowly than does glutamate (Table 1 and Fig. 1), thus behaving as a secondary product, which presumably acts as a store of available nitrogen. Mimura *et al.* (1969) have shown that a bacterium metabolizing methyl cyanide can accumulate up to 30% of the nitrogen supplied as amide. However, acetamide must be a primary product too, since, when added in quantity, it not only traps ^{14}C from methyl cyanide but largely prevents this label from spreading to other metabolites (Table 2). The cells must contain an amidase, but the incubation period was so short and the acetamide so effective that it, and not its hydrolysis product, was almost certainly the active competitor. Thus a small, metabolically active, pool of acetamide may lie on the main pathway and equilibrate slowly with the large storage pool. Alternatively, methyl cyanide may be hydrolysed outside the cell.

Acetamide breakdown has been described in micro-organisms (Skinner & Clarke, 1968), so the pathway proposed only involves one new enzyme, a nitrilase. This may not be specific for methyl cyanide, as higher aliphatic cyanides could be metabolized by direct hydrolysis as well, combined with α- and β-oxidation. Nevertheless, the 'splitting before hydrolysis' theory cannot yet be rejected. Fawcett *et al.* (1955) concluded that both this process and direct hydrolysis occurred simultaneously in higher-plant seedlings.

The higher plants, *Atrichum undulatum*, *Dryopteris filix-mas*, *Arum maculatum* and *Bryonia dioica*, also metabolize [2-^{14}C]methyl cyanide (Firmin, 1973) and the major labelled products, glutamate, aspartate, glutamine, asparagine, 5-pyrrolidone-2-carboxylic acid and malate are the same as those found in *Pseudomonas*. Thus the pathway described is probably present in higher as well as in lower plants.

The existing indications that a large proportion of higher plants can metabolize methyl cyanide (Strauss, 1956; Firmin, 1973) suggests that this ability confers some selective advantage and that the covalent cyanides must be of greater biological significance than previously recognized.



Scheme 2. [^{14}C]*Methyl cyanide metabolism*

All compounds underlined were positively identified in *Pseudomonas*. There were also radioactive spots in chromatographic positions corresponding to glyoxylate, oxaloacetate, *cis*-aconitate and pyrrolidonecarboxylic acid.

Thanks are due to Dr I. J. Bousfield of the National Collection of Industrial Bacteria, Torry, Scotland, who kindly identified the bacterium used here and confirmed that it was in pure culture.

References

Blau, K. & Robson, W. (1957) *Chem. Ind.* 424
Cruickshank, R., Duguid, J. P. & Swain, R. H. A. (eds.) (1965) *Medical Microbiology*, 11th edn., p. 739, E. and S. Livingstone, Edinburgh
Fawcett, C. H., Seeley, R. C., Taylor, F., Wain, R. L. & Wightman, F. (1955) *Nature* (*London*) **176**, 1026–1028
Firmin, J. L. (1973) Ph.D. Thesis, University of London
Firmin, J. L. & Gray, D. O. (1974) *J. Chromatogr.* **94**, 294–297
Firmin, J. L. & Gray, D. O. (1975) *J. Chromatogr.* **105**, 215–218
Lang, S. (1894) *Arch. Exp. Pathol. Pharmakol.* **34**, 247–258
Mimura, A., Kawano, T. & Yamaga, K. (1969) *J. Ferment. Technol.* **47**, 631–638
Pattison, F. L. M. (1953) *Nature* (*London*) **172**, 1139–1141
Rogers, L. J., Shah, S. P. J. & Goodwin, T. W. (1966) *Biochem. J.* **99**, 381–388
Shewan, J. M., Hobbs, G. & Hodgkiss, W. (1960) *J. Appl. Bacteriol.* **23**, 379–390
Skinner, A. J. & Clarke, P. H. (1968) *J. Gen. Microbiol.* **50**, 183–194
Strauss, A. A. (1956) Ph.D. Thesis, University of Illinois
Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950) *Nature* (*London*) **166**, 444
Williams, R. T. (1959) *Detoxication Mechanisms: The Metabolism of Drugs and Allied Organic Compounds*, 2nd edn., pp. 390–409, Chapman and Hall, London