The Metabolism of 2-Furoic Acid by *Pseudomonas* F2

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1. *Pseudomonas* F2 isolated by enrichment culture on 2-furoic acid and grown with it as carbon source oxidized the compound with a Q_{O_2} of 170 μ l./mg. dry wt./hr. and the overall consumption of 2.5 μ moles of oxygen/ μ mole of substrate. 2. In the presence of 1 mM-sodium arsenite, oxygen uptake was restricted to 0.54 μ mole/ μ mole of 2-furoate oxidized, with the formation of 0.86 μ mole of 2-oxoglutarate/ μ mole of 2-furoate. 3. Cell suspensions, disrupted in a French pressure cell and centrifuged at 27 000g, yielded supernatants capable of catalysing the slow oxidation of 2-furoate (0.17 μ mole/mg. of protein/hr.). 4. Fractionation of 27 000g supernatants at 200 000g yielded a soluble enzyme fraction capable of catalysing the oxidation of 2-furoate only in the presence of added 200 000g pellet or of Methylene Blue. 5. The 2-furoate-stimulated uptake of oxygen or the anaerobic reduction of Methylene Blue by dialysed 27 000g supernatant required the addition of ATP and CoA, and the rate of oxygen uptake was further enhanced by the addition of magnesium chloride and NAD⁺. 6. The role of ATP and CoA in the formation of 2-furoyl-CoA was demonstrated by the accumulation of 2-furoylhydroxamic acid in the presence of hydroxylamine. 7. Dialysed 200 000g supernatant, treated with Dowex 1, required the addition of ATP, CoA and Methylene Blue before it could oxidize 2-furoate to 2-oxoglutarate, which was trapped in unitary stoichiometric yield as its phenylhydrazone. Magnesium chloride and NAD⁺ were not stimulatory in this system. The oxidation of 2-furoate to 2-oxoglutarate was not inhibited by substrate analogues, metal ion-chelating agents, thiol-active compounds or inhibitors of cytochrome-mediated electron transport. 8. No evidence was obtained for the intervention of 2,5-dioxovalerate as an intermediate in 2-oxoglutarate formation.

The biochemical importance of the furan ring is amply demonstrated by the wide range of naturally occurring and clinically important compounds that include this cyclic structure (Dean, 1963). Furan-2,5-dicarboxylic acid is found in human urine (Flaschentrager & Abel Wahhab, 1960) and is probably a product of carbohydrate metabolism. Furanocoumarins are present in certain Leguminosae, and complex polyacetylenic compounds with furan rings are found in *Chrysanthemum* spp. (Johnson, 1965). A partially saturated furan ring may also be a rather infrequent polymeric unit of lignin (Freudenberg, 1965). Griseofulvin and morphine, naturally occurring compounds of considerable medical importance, are both complex furans.

Until recently the manner by which furans are degraded in the soil received little attention. Reported microbial transformations of furans include the accumulation of L-glutamate in the growth media by *Pseudomonas* spp. growing with 2-furoic acid (furan-2-carboxylic acid) as sole source

of carbon (Kakinuma & Yamatodani, 1964), the oxidation of 2,3,4,5-tetrahydrofuran-3-ol to the corresponding ketone by extracts of a *Corynebacterium* capable of growth on the alcohol (Rosenberg & Holmes, 1965), and the fermentation of furfural (2-formylfuran) to furfuryl alcohol (2-hydroxymethylfuran) by *Saccharomyces* spp. (Morimoto, Hirashima & Ohashi, 1968).

Metabolic transformations of the synthetic diuretic compound 2-[N-(4'-chloro-3'-sulphamoyl-benzenesulphonyl) - N - methyl]aminomethyl - 2 - methyltetrahydrofuran (I) that occur when it is administered to rats result in an opening of the tetrahydrofuran ring by way of a lactone intermediate (II) to give a carboxylic acid (III) (Meng & Kroneberg, 1967).

Previous communications (Jones & Trudgill, 1967; Trudgill, 1968) described experiments that showed that *Pseudomonas* F2 grows with 2-furoic acid as sole source of carbon, and that oxidation products of it enter the tricarboxylic acid cycle as

2-oxoglutarate. In the present work we have elucidated the pathway whereby 2-furoate is metabolized to 2-oxoglutarate, and have shown the obligatory requirement for formation of 2-furoyl-CoA before the essential hydroxylation step can occur.

MATERIALS AND METHODS

Maintenance and growth of organisms. *Pseudomonas* F2 was isolated from woodland soil near Aberystwyth by elective culture in medium containing 2-furoic acid as sole source of carbon. Stock cultures were maintained on nutrient-agar slopes at 2°.

Cells were grown at 30° in media containing (per l.): KH_2PO_4 , 6g.; $(\text{NH}_4)_2\text{SO}_4$, 1g.; carbon source, 1.5g.; yeast extract, 0.1g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4g.; its pH was adjusted to pH 7.1 with 5M-NaOH. A 10l. batch of medium in a New Brunswick Microferm vessel (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.) was inoculated with 500 ml. of a freshly grown culture, aerated by the passage of sterile air at 10l./min., and stirred at 500 rev./min. Cells in the late-exponential phase of growth were harvested in a Sharples Super centrifuge and washed once by resuspension in phosphate buffer (KH_2PO_4 , 1g./l.; Na_2HPO_4 , 2g./l., pH 7.1, followed by centrifugation at 3° and 13000g for 15 min.

Disruption of cells. Washed cells, resuspended in buffer (1.5 × cell vol.) were disintegrated by passage through a Hughes (1951) press, by exposure at 0° to the output of an MSE 500 W ultrasonic disintegrator at an average frequency of 20 kcyc./sec. (0.4 coulomb of output energy being used/20 ml. of cell suspension), or by passage through a French pressure cell (Milner, Lawrence & French, 1950) with a pressure difference at the orifice of 5000 lb./in.².

Preparation of extracts. Broken-cell suspensions were clarified by centrifugation at the g forces and for the times indicated at 0° in the Sorvall RC2.B refrigerated centrifuge (Ivan Sorvall Inc., Norwalk, Conn., U.S.A.), and, where stated, further fractionated by centrifugation in a Martin Christ Omega II preparative ultracentrifuge (Martin Christ, 3360 Osterode/Harz, W. Germany). The protein concentrations of extract fractions were determined by the method of Gornall, Bardawill & David (1949).

Gas-exchange reactions. Gas exchanges catalysed by whole-cell suspensions or cell extracts were measured in Warburg manometers at 30°; for those involving uptake of O_2 , the centre wells contained strips of Whatman no. 542 filter paper and 0.1 ml. of 20% (w/v) KOH.

Buffers. Phosphate buffer, pH 7.1, was obtained by dissolving 2g. of anhydrous Na_2HPO_4 and 1g. of anhydrous KH_2PO_4 in deionized water to a final volume of 1l. Other buffers were obtained by adjusting solutions of KH_2PO_4 at appropriate concentrations to the desired pH value by addition of 5M-NaOH solution. All buffer concentrations are expressed as concentrations of phosphate.

Chromatography. One-dimensional paper chromatography of free carboxylic acids was used chiefly to examine products of the oxidation of 2-[carboxy-¹⁴C]furoic acid by whole cells and soluble protein extracts of *Pseudomonas* F2. For descending chromatography on Whatman no. 1 papers solvent A [eucalyptol-propan-1-ol-formic acid (5:5:2, by vol.), then plus water to saturation] was used, and for ascending chromatography solvent B [phenol-formic acid-water (500:13:167, w/v/v) (Kornberg, 1958)] and solvent C [butan-1-ol-formic acid (9:1, v/v), then plus water to saturation (Magasanik & Umbarger, 1950)] were used. T.l.c. of furans was carried out on Kieselgel G (E. Merck A.-G., Darmstadt, Germany) developed with solvent D (benzene). Chromatography of furans and 2,4-dinitrophenylhydrazones of keto acids was performed on Whatman no. 1 paper in solvent E [butan-1-ol-ethanol-(NH_4)₂CO₃ buffer, pH 10 (40:11:19, by vol.) (Dagley, Fewster & Happold, 1952)] and on Kieselgel G in solvent F [ethyl formate-light petroleum (b.p. 60–80°)-propionic acid (14:26:3, by vol.) (Ronkainen, 1963)]. T.l.c. of hydroxamic acids was carried out on Kieselgel G developed with solvent G [benzene-dioxan-acetic acid (90:25:4, by vol.)] and solvent H [benzene-methanol-acetic acid (45:8:4, by vol.) (Petrowitz & Pastuska, 1962)].

Detection of compounds on chromatograms. Furfural, 2-furoate and 2-oxoglutarate were detected by direct examination under u.v. light (principal emission 254 nm.) from a Hanovia portable u.v. lamp (Hanovia Ltd., Slough, Bucks.), or by the more sensitive technique of spraying the chromatograms with 0.05% (w/v) 2',7'-dichlorofluorescein in ethanol or 2% Rhodamine 6G in acetone followed by u.v. examination. Keto acids were detected by spraying chromatograms with 0.1% (w/v) DNP-hydrazine in 2M-HCl, heating them at 50° for 10 min. and observing the intense yellow spots formed. Hydroxamates were detected as their purple ferric iron complexes after spraying of the chromatograms with 5% (w/v) FeCl_3 in ethanol.

Spectrophotometry. The u.v. and visible spectra were recorded in the Unicam SP. 800 spectrophotometer (Unicam Instruments Ltd., Cambridge). Kinetic studies at constant wavelength were also performed in the Unicam SP. 800, with the exception of those in which phenylhydrazone formation was being followed, when the Hitachi Perkin-Elmer model 139 u.v.-visible spectrophotometer (Hitachi Ltd., Tokyo, Japan) fitted with a photomultiplier attachment was used. This latter instrument was also used for checking absorption maxima and for the production of calibration curves.

Radioactivity measurements. (a) Scintillation counting. The ¹⁴CO₂ evolved in Warburg flasks was absorbed by Hyamine hydroxide 10-X [benzyltrimethyl-(p-1,1,3,3-tetramethylbutylcresoxyethoxyethyl)ammonium hydroxide]. In each centre well was placed a strip of Whatman no. 1 paper (4 cm. × 2 cm.) that carried 30 μl. of m-Hyamine hydroxide in methanol (Passman, Radin & Cooper, 1956); at the end of the reaction period the paper was transferred to a counting vial containing 75 mg. of 2,5-diphenyloxazole and 4.5 mg.

of 1,4-bis-(5-phenyloxazol-2-yl)benzene dissolved in 15 ml. of toluene, which was then counted in a Packard Tri-Carb model 314 EX liquid-scintillation spectrometer.

(b) Thin-layer and paper chromatograms. These were scanned directly for radioactive zones with a Berthold-Desaga thin-layer scanner fitted with a 2π windowless gas-flow counting head carrying a 1 mm.-wide slit (Berthold, 7547 Wildbad Fernruf., Germany).

(c) Planchet assay. 2-[carboxy- ^{14}C]furoate, 2-oxo[^{14}C]glutarate and succinate were assayed in a proportional counter (Nuclear-Chicago model 4306 automatic gas-flow system; Continental Distributors Ltd., London S.W.7). The counts were corrected for background and machine efficiency.

(d) Radioautography. Radioactive spots were located by exposing the dried chromatograms to fast X-ray film (Kodirex; Kodak Ltd., Kirkby, Lancs.).

Preparation of 2-[carboxy- ^{14}C]furoate. The dehydration of [1- ^{14}C]pentose sugars to [^{14}C]furfural followed by oxidation of the latter compound produces 2-furoate carrying ^{14}C only on the carboxyl group (Bonner & Roth, 1959). A solution of 100 mg. of D-[1- ^{14}C]ribose (80 μC) and 1 g. of NaCl in 3 ml. of 3 M-HCl was overlaid with 5 ml. of benzene and dehydrated to [carbonyl- ^{14}C]furfural by boiling it under reflux for 5 hr. (Schoenmann, 1961). The benzene layer, which contained the [carbonyl- ^{14}C]furfural, was evaporated to 0.2 ml. at 50° under a stream of N_2 and applied as a 15 cm. band to a Kieselgel G plate, which was then developed with solvent *D*. The [carbonyl- ^{14}C]furfural band (R_F 0.27) was detected by spraying the plate with 0.2% (w/v) Rhodamine 6G in acetone and examining it under u.v. light and transferred to a stoppered tube, and the [carbonyl- ^{14}C]furfural was extracted by six shakings each with 3 ml. of diethyl ether. Water (0.2 ml.) was added to the pooled ethereal extracts and the ether evaporated at room temperature under a stream of N_2 . The residual aqueous [carbonyl- ^{14}C]furfural solution was cooled to 0°, stirred continuously with a micro magnetic stirring bar (1 mm. $\times$ 10 mm.) and subjected to a Cannizzaro alkali dismutation by the slow addition (10 min.) of 0.2 ml. of 10 M-NaOH (Geissman, 1944). After the reaction mixture had been maintained at 0° for a further 60 min. it was diluted to 5 ml. with water and the unwanted [^{14}C]furfuryl alcohol removed by extraction with six 3 ml. portions of diethyl ether. The aqueous phase was acidified with 6 M-HCl and the 2-[carboxy- ^{14}C]furoic acid was extracted with diethyl ether and finally purified by chromatography of the dried ether extract as a 10 cm. band on Whatman no. 1 paper developed with solvent *G*. The 2-[carboxy- ^{14}C]furoic acid, which was visible under u.v. light, was located precisely by radioautography and eluted with phosphate buffer, pH 7.1. The final product, yield 2.2%, specific radioactivity 94.2 $\mu\text{C}/\text{m-mole}$, was judged to be radiochemically pure since it co-chromatographed with carrier 2-furoic acid as a single radioactive spot in solvents *B* (R_F 0.89), *C* (R_F 0.87), *E* (R_F 0.27) and *F* (R_F 0.31).

Preparation of 2,5-dioxovaleric acid. This was prepared as described by Dagley & Trudgill (1965) and purified by ion-exchange chromatography on Dowex 1 (X10; Cl^- form) as described by Singh & Adams (1965).

Preparation of diazomethane. Solutions of diazomethane in diethyl ether were prepared from *N*-methyl-*N*-nitrosotoluene-*p*-sulphonamide (Diazald) by the method of de Boer & Backer (1954).

Preparation of 2-furoylhydroxamic acid. For assay purposes a standard solution was prepared; 11.2 mg. (100 μmoles) of 2-furoic acid in 1 ml. of methanol was esterified by the addition of excess of diazomethane solution in diethyl ether. The methyl ester was converted into the hydroxamic acid by addition of 4.5 ml. of 3.5 M-NaOH and 2 M-hydroxylamine hydrochloride to give a final volume of 10 ml. For chromatographic purposes a solution of 2-furoylhydroxamic acid in methanol was prepared from the methyl ester by the method of Thompson (1951).

Other materials. Furfuralacetone (furfurylidenebut-3-en-2-one) and *N*-methyl-*N*-nitrosotoluene-*p*-sulphonamide were from Aldrich Chemical Co., Milwaukee, Wis., U.S.A. Alcohol dehydrogenase (EC 1.1.1.1), catalase (EC 1.11.1.6), L-glutamate dehydrogenase (EC 1.4.1.3), L-lactate dehydrogenase (EC 1.1.1.27), ATP and GSH were from C.F. Boehringer und Soehne G.m.b.H., Mannheim, W. Germany. $\alpha\alpha'$ -Bipyridyl, *p*-chloromercuribenzoic acid, iodoacetamide and 2-oxoglutaric acid were from British Drug Houses Ltd., Poole, Dorset. Benzo[*b*]furan-2-carboxylic acid and tetrahydrofurfuryl alcohol were from Eastman Organic Chemicals, Rochester, N.Y., U.S.A. Ethyl formate, eucalyptol, 2,7'-dichlorofluorescein, 2-furoic acid, furfural, heptane, hydroxylamine hydrochloride, NaN_3 , tetrahydrofuran and Tiron (disodium 1,2-dihydroxybenzene-3,5-disulphonate) were from Hopkin and Williams Ltd., Chadwell Heath, Essex. Sodium pyridine-3-sulphonate was from K & K Laboratories Inc., Plainview, N.Y., U.S.A. MS antifoam emulsion was from Midland Silicones Ltd., Barry, Glamorgan. ADP, CoA, NAD^+ , NADP^+ , NADH, NADPH, phenylhydrazine hydrochloride and thiamin pyrophosphate were from Sigma Chemical Co., St Louis, Mo., U.S.A.

Solvents. Diethyl ether was dried over sodium wire and redistilled before use. All other solvents were of A.R. or equivalent grade.

Determination of materials. 2,5-Dioxovalerate solutions were assayed in Warburg flasks by oxidative decarboxylation with H_2O_2 as described by Dagley & Trudgill (1965). 2-Furoate concentrations in aqueous solutions were calculated from their observed E_{245} values by using a value of $10.8 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ for the molar extinction coefficient, which had been determined experimentally with a sample of the compound that had been twice recrystallized from acetone-light petroleum (b.p. 60–80°). 2-Oxoglutarate was determined: (1) as its DNP-hydrazone by the method of Friedemann & Haugen (1943); (2) by measuring the decrease in E_{340} in a 1 cm.-light-path cell after the addition of a sample of the solution to phosphate buffer, pH 7.1, 0.1 mg. of L-glutamate dehydrogenase, 0.5 μmole of NADH and 0.15 m-mole of NH_4Cl in a total volume of 3 ml. (2,5-dioxovalerate is not utilized as a substrate by L-glutamate dehydrogenase); (3) by measuring the increase in E_{320} in phosphate buffer, pH 6.5, in the presence of an excess of phenylhydrazine. Measurements obtained with a solution of 2-oxoglutarate that had been standardized by the enzymic assay procedure gave the molar extinction coefficient $11.7 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ for the phenylhydrazone.

Determination of enzyme activities. 2,5-Dioxovalerate dehydrogenase (2,5-dioxovalerate-NADP oxidoreductase, EC 1.2.1.-) (Dagley & Trudgill, 1965; Singh & Adams, 1964, 1965) was assayed by observing the increase in E_{340} that occurred when the test extract (5–10 mg. of protein) was added to a 1 cm.-light-path cell that contained, in 3 ml., 60 μmoles of phosphate buffer, pH 7.1, 5 μmoles of NADP^+

and 8 μ moles of sodium 2,5-dioxovalerate. The assays were performed at 24° under anaerobic conditions achieved as described by Hodgson & McGarry (1968).

RESULTS

Oxidation of substrates by whole cells. Jones & Trudgill (1967) reported that *Pseudomonas* F2 grew with 2-furoate as carbon source. The mean generation time at 30° was 2½ hr., and freshly harvested cells oxidized 2-furoate with the consumption of 2.5 μ moles of oxygen/ μ mole of 2-furoate oxidized and a Q_{O_2} (μ l. of oxygen/mg. dry wt./hr.) of 170. Benzo[b]furan-2-carboxylic acid, furfuraldehyde, 3-hydroxytetrahydrofuran and tetrahydrofurfuryl alcohol were not oxidized by cells grown with 2-furoate, nor would they support growth of the organism.

Pentose sugars, saccharic acids and L-hydroxyproline, which are oxidized by pathways involving 2,5-dioxovalerate as an intermediate in certain *Pseudomonas* spp. (Dagley & Trudgill, 1965; Singh & Adams, 1964), did not support growth of *Pseudomonas* F2.

Accumulation of 2-oxoglutarate by whole cells. Whole cells oxidized sodium 2-furoate at a decreased rate (40 μ l. of oxygen/mg. dry wt./hr.) in the presence of 1 mM-sodium arsenite with a stoichiometry of approx. 0.5 μ mole of oxygen/ μ mole of 2-furoate oxidized, with the concomitant formation of approx. 1 μ mole of 2-oxoglutarate/ μ mole of 2-furoate (Table 1). The 2-oxoglutarate was identified by its ability to serve as a substrate for L-glutamate dehydrogenase and by a comparison of its DNP-hydrazone with authentic 2-oxoglutarate DNP-hydrazone; this showed that: (1) in alkali they had identical absorption spectra in the range 350–600 nm.; (2) after chromatography in solvents *E* and *F* they had identical R_F values, and each corresponding spot gave the same colour change

when sprayed with 2% (w/v) potassium hydroxide in 90% (v/v) ethanol.

Direct conversion of 2-furoate into 2-oxoglutarate by whole cells. That 2-oxoglutarate accumulated by whole cells oxidizing 2-furoate in the presence of mM-sodium arsenite arises directly from the C₅ skeleton of 2-furoate, and not by an extended sequence of reactions that involves fragmentation of the 2-furoate molecule and subsequent formation of 2-oxoglutarate from one or more fragments, is indicated by the results of experiments with 2-[carboxy-¹⁴C]furoate. When whole cells (3.1 mg. dry wt.) oxidized 10.56 μ moles (0.059 μ C) of 2-[carboxy-¹⁴C]furoate in the presence of 2 mM-sodium arsenite, the 2-oxoglutarate that accumulated was radioactive. T.l.c. in solvent *F* of DNP-hydrazones of the keto acid reaction products revealed a single yellow DNP-hydrazone band that had the same R_F (0.12) as authentic 2-oxoglutarate DNP-hydrazone and corresponded to the only radioactive region detected on scanning of the plate on the Berthold-Desaga scanner. The radioactive DNP-hydrazone band was eluted with ethyl acetate. Samples were assayed for 2-oxoglutarate by the procedure of Friedemann & Haugen (1943) and for radioactivity by plating out on 22 mm. discs of lens tissue, which were air-dried and counted in the proportional counter. The 2-[carboxy-¹⁴C]furoate solution was assayed by measuring E_{245} , and its radioactivity was determined by plating samples on to lens-tissue discs, which were then dried under an i.r. lamp before being counted. The specific radioactivity (c.p.m./ μ mole) of the 2-oxo[¹⁴C]glutarate eluted from the plate [$12\,600 \pm 556$ (5)] corresponded very closely to that of the 2-[carboxy-¹⁴C]furoate used as substrate [$12\,682 \pm 856$ (4)]. Values are given as means $\pm$ S.D. with the number of tests in parentheses.

When 2 μ moles of 2-oxo[¹⁴C]glutarate, obtained from the previous experiment with whole cells, were purified as the free acid by paper chromatography

Table 1. *Stoichiometry of 2-furoate oxidation by Pseudomonas F2 in the presence of 1 mM-sodium arsenite*

The main compartments of Warburg flasks contained, in 2.0 ml., 42 μ moles of phosphate buffer, pH 7.1, washed cells (6.4 mg. dry wt.) and 2 μ moles of sodium arsenite. The centre wells contained 0.1 ml. of 20% (w/v) KOH. After equilibration at 30° reactions were started by the addition of sodium 2-furoate from the side arms. At the cessation of 2-furoate-stimulated O₂ uptake, cells were removed by centrifuging at 12000g for 15 min. at 0°. Residual sodium 2-furoate was assayed by measuring E_{245} and 2-oxoglutarate formed was assayed by the L-glutamate dehydrogenase assay as described in the Materials and Methods section. Uptake of O₂ (0.59 μ mole of O₂/mg. dry wt. of cells hr.) in the absence of substrate was subtracted from the values in column C.

2-Furoate added (μ moles) <i>A</i>	2-Furoate used (μ moles) <i>B</i>	O ₂ uptake (μ moles) <i>C</i>	2-Oxoglutarate formed (μ moles) <i>D</i>	Ratio <i>C/B</i>	Ratio <i>D/B</i>
3.24	3.24	1.65	2.74	0.51	0.85
6.48	6.38	3.58	5.60	0.56	0.88
9.72	9.64	5.50	8.32	0.57	0.86

with solvent *A*, the only radioactive region of the chromatogram (R_F 0.47) corresponded to the only acid spot present in the reaction sample and to authentic 2-oxoglutaric acid. The 2-oxo[^{14}C]-glutarate was excised as a 3 cm.-diameter disc and its radioactivity assayed directly in the proportional counter (431 c.p.m., corrected for background but not for instrument efficiency because of the non-standard counting procedure). It was quantitatively eluted with 0.01M-sodium acetate buffer, pH 4.2, and transferred to a Warburg flask in a final volume of 1.9 ml. of the same buffer. Oxidative removal of C-1 from the 2-oxo[^{14}C]-glutarate by addition of 0.1 ml. of 0.54M- H_2O_2 from the flask side arm as described by Singh & Adams (1964) yielded $^{14}\text{CO}_2$ (318 c.p.m.). Catalase (0.1 mg.) was added to the aqueous phase to decompose any residual H_2O_2 , and the flask contents were freeze-dried, redissolved in 0.1 ml. of 70% (v/v) ethanol and chromatographed adjacent to 2-oxoglutarate and succinate markers with solvent *A*. Succinate, the only detectable compound, was excised as a 3 cm.-diameter disc, counted directly and found to be virtually unlabelled (15 c.p.m.).

Metabolism of 2-furoate by cell extracts. Early attempts to obtain active cell extracts were not successful (Jones & Trudgill, 1967). In the present work, disruption of the cells by ultrasonic treatment failed to yield active extracts. Though disruption of cells by passage through the Hughes (1951) press yielded active preparations, the more convenient French pressure cell (Milner *et al.* 1950) was used as a routine throughout this work. The turbid supernatants obtained by centrifuging disrupted-cell suspensions at 27 000g and 0° for 10 min. were capable of oxidizing 2-furoate slowly with the

consumption of 116 nmol of oxygen/mg. of protein/hr. This activity was enhanced more than threefold by the addition of 1 mM-Methylene Blue to reaction systems in Warburg flasks (Table 2). Further fractionation of the low-speed supernatant at 200 000g and 0° for 60 min. yielded a virtually inactive supernatant that could be activated tenfold by the addition of the resuspended pellet and 13-fold by Methylene Blue. Phenazine methosulphate and 2,6-dichlorophenol-indophenol were inactive in this system. These experiments suggested that soluble enzymes catalysing transformations of the 2-furoate molecule have an obligatory requirement for an insoluble electron-transport system that can be adequately replaced by Methylene Blue. Activation of the rate of oxygen uptake by the turbid 27 000g supernatant by Methylene Blue suggests that the optimum combination of soluble enzymes and electron-transport system is not achieved in these preparations.

Cofactor requirements for 2-furoate oxidation by cell extracts. When 27 000g supernatant was dialysed against phosphate buffer, pH 7.1, for 16 hr. at 3° the rate of endogenous oxygen uptake was decreased from 107 to 18 nmol/mg. of protein/hr. and 2-furoate-stimulated oxidation was totally abolished. Activity towards 2-furoate was regained at a rate three times that of the undialysed preparation when ATP, CoA, NAD^+ and magnesium chloride were added. The ATP and CoA were responsible for most of this reactivation (Table 3).

2-Furoyl-CoA, an intermediate in 2-furoate oxidation. The aqueous-ethanol-soluble material from a reaction system in which 95 mg. of extract protein (dialysed 27 000g supernatant) was incubated with 3.3 m-moles of phosphate buffer, pH 7.1, 100 μmoles

Table 2. *Oxidation of 2-furoate by extract fractions of Pseudomonas F2, and the requirement for an electron acceptor system*

The main compartments of Warburg flasks contained, in 2.0 ml., 42 μmoles of phosphate buffer, pH 7.1, cell extract fractions and electron acceptors (2 μmoles) as stated. Centre wells contained 0.1 ml. of 20% (w/v) KOH. After equilibration at 30° reactions were started by the addition of 2.5 μmoles of 2-furoate from the side arms.

Values for O_2 uptake were corrected for the uptake of O_2 in the absence of substrate.

Broken-cell fraction	Electron acceptor added	O_2 uptake (nmol/mg. of protein/hr.)
27 000g supernatant*	—	0
27 000g supernatant	—	116
27 000g supernatant	Methylene Blue	397
200 000g supernatant	—	9
200 000g pellet	—	0
200 000g supernatant plus 200 000g pellet	—	94
200 000g supernatant	Methylene Blue	121
200 000g supernatant	2,6-Dichlorophenol-indophenol†	0
200 000g supernatant	Phenazine methosulphate	3

* Cell extract boiled before incubation.

† The 2,6-dichlorophenol-indophenol was not bleached during the experimental period (70 min.).

Table 3. *Oxidation of 2-furoate by dialysed 27 000g supernatant in the presence of added cofactors*

The main compartments of Warburg flasks contained, in a volume of 2.0 ml., 560 μ moles of phosphate buffer, pH 7.1, 16 mg. of crude or dialysed 27 000g supernatant, 2.5 μ moles of Methylene Blue, 10 μ moles of ATP, 2.5 μ moles of NAD⁺, 0.13 μ mole of CoA and 5 μ moles of MgCl₂, with omissions as indicated. The centre wells contained 0.1 ml. of 20% (w/v) KOH. The manometers were equilibrated at 30°, and the reactions were started by the addition of 10 μ moles of sodium 2-furoate from the side arms.

Uptake of O₂ in the absence of 2-furoate was subtracted in each case.

Broken-cell fraction	Omission	O ₂ uptake (nmoles/mg. of protein/hr.)
27 000g supernatant	All cofactors	151
Dialysed 27 000g supernatant	All cofactors	0
Dialysed 27 000g supernatant	None	436
Dialysed 27 000g supernatant	ATP	64
Dialysed 27 000g supernatant	CoA	129
Dialysed 27 000g supernatant	NAD ⁺	284
Dialysed 27 000g supernatant	MgCl ₂	385

of ATP, 1.3 μ moles of CoA, 25 μ moles of NAD⁺, 50 μ moles of MgCl₂, 10 μ moles of Methylene Blue and 4 m-moles of freshly neutralized hydroxylamine hydrochloride in the presence of 100 μ moles of sodium 2-furoate at 30° for 3 hr., was chromatographed on Kieselgel G plates with solvent *G* and solvent *H*. When the plates were sprayed with 5% (w/v) ferric chloride in ethanol, authentic 2-furoyl-hydroxamic acid gave an intense purple colour reaction, as did a compound in the reaction fraction that had the same *R_F* as the standard in both solvents. This compound was absent from reaction systems that were stopped at zero time, or from which sodium 2-furoate was omitted.

The authentic and reaction hydroxamic acids were eluted from thin-layer plates with diethyl ether and redissolved in *m*-hydrochloric acid, and their u.v. spectra were recorded. Clear areas of the plates were similarly eluted to act as blanks. The identical λ_{max} values of 253.5 nm. represent a bathochromic shift in the 2-furoic acid λ_{max} value of 245 nm. resulting from the protonation of the hydroxamic acid in *m*-hydrochloric acid solution. The purple chromophores produced by the addition of 5% (w/v) ferric chloride in 2*M*-hydrochloric acid to the eluted authentic and reaction hydroxamates had identical spectra over the range 400–700 nm., with λ_{max} 545 nm.

The spectral and chromatographic identity of the hydroxamic acid isolated from the enzymic reaction with the authentic 2-furoylhydroxamic acid, and the requirement for ATP and CoA in its formation, demonstrated it to be derived from 2-furoyl-CoA. Its formation in reaction systems with dialysed 27 000g supernatant from which NAD⁺ and Methylene Blue were omitted and in which no consumption of oxygen occurred, and conversely, the lack of oxygen uptake when ATP and CoA were omitted led us to the conclusion that formation of 2-furoyl-CoA is a necessary prelude to any oxidative attack on the furan ring.

Anaerobic reactions with Methylene Blue as electron acceptor. Because of the very high rate of endogenous reduction of Methylene Blue by 27 000g supernatant under anaerobic conditions, this preparation could not be used directly for such studies. Dialysis of the 27 000g supernatant for 18 hr. at 3° against phosphate buffer, pH 7.1, almost eliminated this endogenous activity and the effect of added 2-furoate could then be studied satisfactorily. Reactions were performed with 33 μ M-Methylene Blue. At this concentration the compound is still predominantly in the monomeric α -form (Schubert & Levine, 1955) and has a molar extinction coefficient at 665 nm. of $4.18 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. The 2-furoate-stimulated reduction of Methylene Blue that occurred under anaerobic conditions was shown to be dependent on the addition of ATP and CoA, but not NAD⁺ or magnesium chloride (Fig. 1). The rather long and somewhat variable lag period in this assay system is believed to be due to traces of oxygen in the 'anaerobic' cuvette that caused an initial reoxidation of reduced Methylene Blue as it was formed.

Stoichiometry of formation of 2-oxoglutarate from 2-furoate by cell extracts. The rate of oxidation of 2-oxoglutarate by 200 000g supernatant was significantly less than that by 27 000g supernatant. Though this rate could be further decreased by dialysis of the 200 000g supernatant for 18 hr. at 3° it was not completely eliminated even in the presence of 5 mM-sodium arsenite, 1 mM-sodium pyridine-3-sulphonate or 1 mM-cadmium sulphate. Dialysis of 200 000g supernatant against 1 mM-EDTA followed by incubation with 2-oxoglutarate in the presence of 1 mM-EDTA at 30° produced the lowest rate of oxidation of 2-oxoglutarate (0.028 μ mole/mg. of protein/hr.). Though none of these manipulations significantly inhibited 2-furoate oxidation, the low rate (0.04–0.10 μ mole of 2-furoate oxidized/mg. of protein/hr.) required that 2-oxoglutarate oxidation be completely eliminated if precise stoichiometry

Fig. 1. Reduction of Methylene Blue in the presence of 2-furoate, catalysed by dialysed 27000g supernatant from disrupted *Pseudomonas* F2. Reaction mixtures, in 1cm.-light-path cuvettes, contained 560 μ moles of phosphate buffer, pH 7.1, 4.2 mg. of 27000g supernatant (dialysed for 18 hr. at 3° against phosphate buffer, pH 7.1), 0.1 μ mole of Methylene Blue, 10 μ moles of ATP, 0.13 μ mole of CoA, 5 μ moles of $MgCl_2$ and 2.5 μ moles of NAD^+ , with omissions as indicated. Antifoam emulsion (2 μ l.) was added to prevent foaming, and cuvettes were gassed with N_2 as described by Hodgson & McGarry (1968). Reactions, at 24°, were started by adding 10 μ moles of 2-furoate, which had been previously gassed with and stored under N_2 , and followed by measuring the decrease in E_{665} due to reduction of methylene blue. (a) Complete system; (b) 2-furoate omitted; (c) ATP and CoA omitted; (d) NAD^+ and $MgCl_2$ omitted.

were to be achieved. This did not prove possible, so an alternative approach was employed.

When dialysed 200000g supernatant was incubated with 2-furoate in the presence of the necessary cofactors and phenylhydrazine the formation of a keto acid phenylhydrazone could be followed by measuring the increase in E_{320} . The identity of this keto acid as 2-oxoglutarate was established by addition of L-glutamate dehydrogenase to the reaction system containing an excess of ammonium chloride in the presence of a constant concentration of NADPH, maintained by a generating system in which the glucose 6-phosphate dehydrogenase was capable of forming NADPH at a rate 100-fold greater than that of production of 2-oxoglutarate from 2-furoate, the rate-limiting step of NADPH oxidation during L-glutamate formation. The diversion of 2-oxoglutarate to L-glutamate stopped the formation of 2-oxoglutarate

Fig. 2. Formation of 2-oxoglutarate phenylhydrazone from 2-furoate by 200000g supernatant from *Pseudomonas* F2. The basal reaction mixture for the observation of phenylhydrazone formation contained (in 6.4 ml.) 1.9 m-moles of phosphate buffer, pH 6.5, 25.2 mg. of protein, 40 μ moles of ATP, 0.26 μ mole of CoA, 0.2 μ mole of Methylene Blue and 50 μ moles of phenylhydrazine. For simple observation of phenylhydrazone formation, 3 ml. of this mixture was removed into each of two cuvettes, one of which served as a blank, the reaction being started in the other by the addition of 2 μ moles of 2-furoate. This procedure ensured that the endogenous increase in E_{320} (0.03/min.) was identical in both cuvettes. The extinction was recorded at 2 min. intervals in the Hitachi model 139 spectrophotometer fitted with a photomultiplier attachment. The slit width was maintained at 2 mm. Adjustments to achieve zero extinction of the blank were made with the photomultiplier amplifier controls. Cuvettes were withdrawn from the spectrophotometer after each reading and gently agitated for 20 sec. to maintain aerobic conditions. $\square$, Complete system; $\circ$, 2-furoate absent. For reactions in which the effect of L-glutamate dehydrogenase was being investigated the basal reaction mixture also contained, in a total volume of 6.4 ml., 20 μ moles of glucose 6-phosphate, 1 μ mole of $NADP^+$, 0.1 mg. of glucose 6-phosphate dehydrogenase and 1.2 m-moles of NH_4Cl . Reactions were started by the addition of 2 μ moles of 2-furoate, and 0.2 mg. of L-glutamate dehydrogenase was added as indicated (GDH). $\blacktriangle$, Complete system, glutamate dehydrogenase added at 12 min.; $\blacksquare$, Complete system, glutamate dehydrogenase added at 20 min.; $\bullet$, 2-furoate absent, glutamate dehydrogenase added at 12 min.

phenylhydrazone (Fig. 2). At pH 6.5 the total increase in E_{320} was directly proportional to the amount of 2-furoate added. A comparison of the increases in E_{320} observed on addition of known amounts of 2-furoate with a spectrophotometric

Table 4. *Stoichiometry of 2-furoate oxidation and 2-oxoglutarate phenylhydrazone formation by 200 000g supernatant from an extract of Pseudomonas F2*

The reaction system for the spectrophotometric observation of phenylhydrazone formation was set up as described in Fig. 2. Reactions were started by addition of amounts of 2-furoate as indicated and followed at 24° until increase in E_{320} ceased. The amounts of 2-oxoglutarate phenylhydrazone formed were determined by reference to a calibration curve prepared as described in the Materials and Methods section.

2-Furoate added (μ mole)	$+\Delta E_{320}$	2-Oxoglutarate phenylhydrazone formed (μ mole)
0.075	0.280	0.071
0.112	0.410	0.108
0.150	0.584	0.152

Fig. 3. Cofactor requirements for the formation of 2-oxoglutarate from 2-furoate. The basal reaction mixture contained (in 6.4 ml.) 1.9 m-moles of phosphate buffer, pH 6.5, 21 mg. of protein [200 000g supernatant, dialysed for 18 hr. against phosphate buffer, pH 7.1, adjusted to pH 8 and treated with Dowex 1 (X10; Cl⁻ form)], and 50 μ moles of phenylhydrazine. The following components (μ moles/cuvette) were either present initially or added after 10 min. as indicated: ATP, 20 μ moles; CoA, 0.13 μ mole; NAD⁺, 2.5 μ moles; MgCl₂, 5 μ moles; Methylene Blue, 0.1 μ mole. Reactions, at 28°, were started by adding 0.15 μ mole of 2-furoate. ■ Complete system; □, NAD⁺ and MgCl₂ omitted; ●, ATP omitted (added at 10 min.); ○, CoA omitted (added at 10 min.); △, Methylene Blue omitted (added at 10 min.).

calibration curve for the 2-oxoglutarate phenylhydrazone established a stoichiometry of 1:1 between 2-furoate added and 2-oxoglutarate formed (Table 4).

Formation of 2-oxoglutarate and the obligatory requirement for ATP, CoA and Methylene Blue. Significant formation of 2-oxoglutarate phenylhydrazone by dialysed 200 000g supernatant that had been treated with Dowex 1 (X10; Cl⁻ form) to remove CoA (Chantrenne, 1951) was dependent on the presence of ATP, CoA and Methylene Blue. Neither NAD⁺ nor magnesium chloride had any stimulatory effect (Fig. 3).

Specific activities of 2,5-dioxovalerate dehydrogenase in cell extracts. When 27 000g supernatants were prepared from suspension of *Pseudomonas F2* that had been disrupted by passage through the French pressure cell and 2,5-dioxovalerate dehydrogenase activities measured as described in the Materials and Methods section, results showed that the enzyme is not induced during growth on 2-furoate. Supernatant from 2-furoate-grown cells catalysed the formation of 18 nmol of NADPH/mg. of protein/min. and that from nutrient-broth-grown cells formed 16 nmol of NADPH/mg. of protein/min.

Effect of inhibitors on the conversion of 2-furoate into 2-oxoglutarate phenylhydrazone by 200 000g supernatant. The oxidation of 2-furoate to 2-oxoglutarate was resistant to inhibition by substrate analogues, metal ion-chelating agents, thiol-active compounds and electron-transport inhibitors when Methylene Blue was used as electron acceptor. The rate of formation of 2-oxoglutarate phenylhydrazone was not significantly affected by 10 mM-benzo-[b]furan-2-carboxylate, 10 mM-5-bromo-2-furoate, 5 mM- α -bipyridyl, 10 mM-EDTA, 1 mM-Tiron, 1 mM- and 10 mM-potassium cyanide, 3 mM-iodoacetamide and 10 mM-sodium arsenite, and was 13% inhibited by 3 mM-sodium azide.

DISCUSSION

Though whole cells of *Pseudomonas F2* grown with 2-furoate as carbon source were capable of oxidizing the compound, early attempts to prepare active extracts were unsuccessful (Jones & Trudgill, 1967). This failure resulted from the use of supernatants from disrupted-cell preparations that were obtained by centrifuging at 35 000g for 45 min. Such a procedure removed most of the necessary electron-transport system and inactivated the supernatant. Methylene Blue was not added to reaction systems in these early experiments.

The threefold stimulation of 2-furoate oxidation by 27 000g supernatant on the addition of Methylene Blue indicated a potential for enzymic transformation by the soluble enzymes that was limited by the

rate of electron transport to oxygen. The complete dependence of the activity of the 200 000g supernatant on added pellet material, which could be replaced by Methylene Blue, provided the justification for considering the system in two distinct parts; a sequence of reactions catalysed by soluble enzymes that oxidized 2-furoate to 2-oxoglutarate, and an obligatory electron-transport system, particulate in nature, and able to be replaced effectively by Methylene Blue. The inability of 2,6-dichlorophenol-indophenol and phenazine methosulphate to act as electron acceptors is probably due to structural incompatibilities since it cannot be explained by redox potentials.

Based on a suggestion by Singh & Adams (1965), it can be proposed that organisms capable of growth on 2-furoate might open the furan ring by a hydrolytic reaction to form 2,5-dioxovalerate, oxidation of which, catalysed by 2,5-dioxovalerate dehydrogenase, would yield 2-oxoglutarate. Although the stoichiometric yield of 2-oxo[1-¹⁴C]glutarate from 2-[carboxy-¹⁴C]furoate with no change in specific radioactivity was clear evidence for a direct conversion of 2-furoate into 2-oxoglutarate, it provided no evidence about the reaction sequence involved in this transformation. Although the accumulation of 2-oxoglutarate by whole cells and extracts of 2-furoate-oxidizing *Pseudomonas* F2 has been amply demonstrated, 2,5-dioxovalerate has not been detected as an intermediate and the low activity of the key enzyme 2,5-dioxovalerate dehydrogenase (EC 1.2.1.-) is identical in extracts from 2-furoate- and nutrient-broth-grown cells. It is noteworthy that pentose sugars, saccharic acids and L-hydroxyproline, which are all oxidized through 2,5-dioxovalerate by certain *Pseudomonas* strains (Dagley & Trudgill, 1965; Singh & Adams, 1965), are not utilized as carbon sources by *Pseudomonas* F2, and it was therefore not possible to obtain an induced 2,5-dioxovalerate dehydrogenase activity for comparative purposes.

Possible modifications to the sequence of reactions suggested by Singh & Adams (1965) in which 2-furoyl-CoA is formed before hydrolytic cleavage of the furan ring occurs, and in which electrons liberated by the action of 2,5-dioxovalerate dehydrogenase are donated to a cytochrome system or to Methylene Blue without the intervention of NADP⁺, could be postulated as being compatible with most of the known data. They must, however, be rejected, since addition of L-glutamate dehydrogenase completely arrests phenylhydrazine formation in the spectrophotometric assay system (Fig. 2) even though 2,5-dioxovalerate readily forms a phenylhydrazine and is not a substrate for L-glutamate dehydrogenase.

The initial reaction postulated, a hydrolytic cleavage of the furan ring, may perhaps be regarded

as unlikely in view of the pseudoaromatic nature of the ring and the fact that cleavage of aromatic compounds occurs not by hydrolysis but usually by an oxygenase (EC 1.99.2.-) attack after the introduction of hydroxyl groups at appropriate points (Evans, 1963; Dagley, Chapman, Gibson & Wood, 1964; Gibson, 1968). A consideration of the intermediates detected in the breakdown of 2-[N-(4'-chloro-3'-sulphamoylbenzenesulphonyl)-N-methyl]-aminomethyl-2-methyltetrahydrofuran by rat tissues (Meng & Kroneberg, 1967) suggests that the transformation of the tetrahydrofuran ring into a substituted 4-butyrolactone may have occurred through an initial hydroxylation of the tetrahydrofuran ring followed by a dehydrogenation. The introduction of a hydroxyl group into the analogous position in 2-furoyl-CoA to form 5-hydroxy-2-furoyl-CoA produces a molecule that, by keto-enol tautomerism, would form a lactone. This could then be attacked by a lactone hydrolase (EC 3.1.1.-) to form the CoA ester of 2-oxoglutarate.

The substrate and cofactor requirements have been investigated for a variety of hydroxylases of the mixed-function oxygenase class (EC 1.99.1.-). The incorporated oxygen atom is derived from gaseous molecular oxygen, and an electron donor, most frequently NADH or NADPH, is required so that the rejected oxygen atom can be reduced to water; the enzymes may also require FMN or FAD and in some cases Fe²⁺ ions for activity (Nagatsu, Levitt & Udenfriend, 1964; Conrad, Lieb & Gunsalus, 1965; Katagiri, Maeno, Yamamoto & Hayaishi, 1965; Trudgill, Du Bus & Gunsalus, 1966). The absence of inhibition of either the reduction of Methylene Blue (Trudgill, 1968) or the formation of 2-oxoglutarate by $\alpha\alpha'$ -bipyridyl, and the general insensitivity of the system to metal ion-chelating agents and thiol-active compounds are somewhat atypical of the mixed-function oxygenases. Further, the obligatorily aerobic nature of such a hydroxylase excludes it from consideration in the pathway of 2-oxoglutarate formation from 2-furoate.

A second type of aromatic hydroxylase has been reported in which the incorporated oxygen is obtained from water and which requires an electron acceptor, not an electron donor, so that the two hydrogen atoms can be oxidized to water. Two documented cases display characteristics bearing a marked resemblance to those of the 2-furoate-oxidizing system. Turbid low-speed supernatants from *Pseudomonas fluorescens* KB1 hydroxylate nicotinic acid to 6-hydroxynicotinic acid, the incorporated oxygen atom being derived from water and the observed consumption of oxygen being due to oxidation of the two hydrogen atoms liberated from the water (Hunt, Hughes & Lowenstein, 1958). Extracts of *Rhodotorula* MR catalyse the hydroxylation of picolinic acid to 6-hydroxypicolinic acid

Scheme 1. Proposed pathway for the oxidation of 2-furoic acid to 2-oxoglutaric acid by *Pseudomonas* F2.

aerobically in the presence of Methylene Blue with the consumption of 0.5 mole of oxygen/mole of substrate hydroxylated, or anaerobically with the reduction of Methylene Blue (Dagley & Johnson, 1963). The 2-furoate-oxidizing system exhibits all these characteristics in addition to the unusual requirement for CoA-ester formation before hydroxylation can occur. A possible alternative reaction mechanism for the hydroxylation, which if valid makes that term somewhat of a misnomer, is suggested by the close parallel that can be drawn between the formation of the postulated lactone precursor of 2-oxoglutarate and the conversion of a *trans*-2,3-dehydroacyl-CoA into a 3-oxoacyl-CoA during β -oxidation of fatty acids. Important differences are the site of attack, which is one double bond more remote in the conjugated furan ring system, and the donation of electrons to an acceptor other than NAD⁺ at the dehydrogenation step.

A proposed reaction sequence by which 2-furoate is oxidized to 2-oxoglutarate by *Pseudomonas* F2 and which is compatible with all the reported results is shown in Scheme 1. The postulated intermediates, 5-hydroxy-2-furoyl-CoA and the isomeric lactone 5-oxo-Δ²-dihydro-2-furoyl-CoA, have not yet been isolated or identified. Though elimination of the CoA is shown in this scheme as occurring at the 2-oxoglutaryl-CoA stage we have no evidence for this, and alternatively it may occur immediately before hydrolysis of the lactone. The possibility of CoA recycling being mediated by a CoA trans-

ferase, although not rigorously excluded, seems unlikely in view of the requirement for an excess of ATP for the maximum rate of 2-oxoglutarate formation by 27 000g or 200 000g supernatant.

The stimulation of oxygen uptake by 27 000g supernatant in the presence of 2-furoate on the addition of NAD⁺ and magnesium chloride (Table 3) is probably a reflection of their requirement during oxidation of 2-oxoglutarate, since they are ineffective in stimulating 2-oxoglutarate formation by 200 000g supernatant (Fig. 3).

The unidentified keto acid that formed a DNP-hydrazone giving a stable colour in alkali with λ_{max} 471 nm. and previously reported as a possible intermediate (Trudgill, 1968) has been shown to be unlabelled when 2-[carboxy-¹⁴C]furoate is used as substrate. It is therefore considered to be a product of the further metabolism of 2-oxoglutarate that is of no immediate importance.

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