The Formation of ω -Cyclohexyl-Fatty Acids from Shikimate in an Acidophilic Thermophilic Bacillus

A NEW BIOSYNTHETIC PATHWAY

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Labelled acetate, phenylalanine and shikimic acid were fed to *Bacillus acidocaldarius*. A high proportion of the ^{14}C incorporated from acetate and shikimate was recovered in methyl esters from the cell lipids, but such recovery of ^{14}C from phenylalanine was low. Only the ^{14}C from shikimate was selectively incorporated into 11-cyclohexylundecanoate and 13-cyclohexyltridecanoate. Degradation of these cyclohexyl-fatty acids showed that shikimate was incorporated as an intact C_7 unit.

From hot acid springs at Pisciarelli (Agnano) near Naples we isolated (De Rosa *et al.*, 1972) an aerobic spore-forming bacillus that tolerates pH from 2 to 5 at 50–70°C. The morphology, the pH and temperature requirements and the nutritional characteristics closely resemble those of several isolates designated as *Bacillus acidocaldarius* by Darland & Brock (1971), and we believe that the Agnano organism belongs to the same group. Pursuing suggestions that such resistant organisms may have special membrane assemblies that can maintain the organizational integrity of the cell contents (Brock, 1967), we investigated the lipid components, which proved to be unusual.

From the non-saponifiable lipids we isolated (De Rosa *et al.*, 1971a) a mixture of pentacyclic triterpene hydrocarbons (about 0.3% of cell dry wt.), mainly hopene-*b* [hop-22(29)-ene], which is synthesized *de novo* by the organism from mevalonate (M. De Rosa, A. Gambacorta, L. Minale & J. D. Bu'Lock, unpublished work). From the saponifiable lipids about 3% of the cell dry weight was recovered as saturated fatty acids, the two major components of which were characterized as 11-cyclohexylundecanoate and 13-cyclohexyltridecanoate (De Rosa *et al.*, 1971b). In biosynthetic terms such acids must represent products of chain extension, presumably by the usual (malonyl-CoA) fatty acid synthetase route, of a 'starter' unit that is not identifiable *a priori*.

Conceivable biosynthetic pathways would include (a) terminal cyclization of a pre-formed $n\text{-C}_{17}$ or $n\text{-C}_{19}$ fatty acid precursor, (b) formation of a reduced C_9 (cyclohexylpropionate) or C_7 (cyclohexanecarboxylate) precursor from phenylalanine, or (c)

formation of a precursor such as cyclohexanecarboxylate from shikimate; in the present paper we describe experiments supporting the existence of a pathway of type (c).

Experimental

Radiochemicals

Sodium [$1\text{-}^{14}\text{C}$]acetate (61 mCi/mmol) and [$\text{U-}^{14}\text{C}$]phenylalanine (513 mCi/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. [$\text{U-}^{14}\text{C}$]shikimic acid (1.9 mCi/mmol) was purchased from New England Nuclear Corp., Boston, Mass., U.S.A.

Preparation of 12-phenyl-*n*-dodecane

Authentic 12-phenyl-*n*-dodecane was prepared by the Friedel–Craft condensation of benzene with dodecyl chloride (75% yield) and subsequent Huang Minlon reduction of the ketone (m.p. 43°C; M^+/ϵ 260; λ_{max} , 240 nm). The 12-phenyl-*n*-dodecane was purified by silica-gel column chromatography (70% yield) and its purity was checked by g.l.c. (liquid at room temp.; M^+/ϵ 246; λ_{max} , 229 nm).

Instrumental details

The ^{14}C -labelled samples, weighed on a Cahn electrobalance, were dissolved in 10 ml of Bray's (1960) solution and the radioactivity was measured by a Beckman LS-250 liquid-scintillation system (efficiency 88–91% by internal standardization). The

insoluble samples (bacteria and BaCO_3) were added to the scintillator solution in the presence of Packard Cab-O-Sil and counted for radioactivity at an efficiency of 88%. All g.l.c. measurements were done on a Carlo Erba Fractovap model GV apparatus. Mass spectra were obtained with an AEI MS9 double-focus spectrometer, n.m.r. spectra with a Varian HA100 spectrometer in CCl_4 solution with tetramethylsilane as an internal standard and u.v. spectra with a Bausch and Lomb Spectronic 505 apparatus.

Organism and culture conditions

An isolate of *Bacillus acidocaldarius* from hot springs in the volcanic area of Naples, described by De Rosa *et al.* (1972), was used.

The medium contained (g/100 ml of water): yeast extract (Difco), 0.1; glucose, 0.1; $(\text{NH}_4)_2\text{SO}_4$, 0.01; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; KH_2PO_4 , 0.01; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.001; FeCl_3 , 0.0001; the pH was adjusted to 2.6 with 0.2 M-potassium citrate buffer, pH 2.6. The organism was grown in 25-litre batches at 60°C with aeration (0.5 litre of air/min per litre of culture volume) and agitation in a 30-litre fermenter (Terzano). The culture vessel was inoculated by adding 3 litres of a 12 h broth culture.

The labelled substrate, sodium [$1\text{-}^{14}\text{C}$]acetate (0.10 mCi), [$1\text{-}^{14}\text{C}$]phenylalanine (0.25 mCi) or [$1\text{-}^{14}\text{C}$]shikimic acid (0.050 mCi), was added to the cultures at the beginning of the exponential phase. For shikimic acid, the addition was accompanied by phenylalanine (0.1 g/litre) and tryptophan (0.1 g/litre) to minimize metabolic demands upon the shikimate pool. Cells were harvested in the stationary growth phase (20 h incubation) by continuous-flow centrifugation, then they were washed with 0.1 M-NaCl and freeze-dried (yield: 4–12 g of dried cells from 25 litres of culture).

Extraction and saponification of lipids

The dried cells were extracted continuously (Soxhlet) for 12 h with chloroform-methanol (1:1, v/v). The lipid extract was evaporated and treated with KOH in methanol (10%, w/v; 40 ml/100 mg of lipids) under reflux; after 6 h the methanol was removed *in vacuo* and the remaining solution was washed with diethyl ether to remove neutral products, then acidified with 2 M-HCl and extracted three times with diethyl ether. The extract was methylated with an excess of diazomethane to give crude fatty acid methyl esters.

Purification and fractionation of fatty acid methyl esters

Preliminary purification of the crude esters was carried out on a silica-gel column [about 10 g of

silica (Merck chromatographic grade)/100 mg of esters], elution being first with cyclohexane to remove residual hydrocarbons and then with diethyl ether in cyclohexane (1:49, v/v) to remove the methyl esters. A steel capillary column (80 m long; diethylene glycol succinate; 220°C; carrier gas N_2 at 1 ml/min) was used to check the composition of the mixture after each batch-culture experiment.

Methyl esters were separated by preparative g.l.c. on a 2 m column (internal diam. 10 mm) packed with 25% diethylene glycol succinate on Chromosorb P 30/60 and operated at 180°C with N_2 at 130 ml/min. From the effluent three peaks were collected manually; the first peak contained a mixture of iso- and anteiso- C_{17} methyl esters, the second peak contained methyl 11-cyclohexylundecanoate and the third peak contained methyl 13-cyclohexyltridecanoate.

Degradation of [^{14}C]fatty acid methyl esters

A mixture of fatty acid methyl esters (102 mg) from cells grown in the presence of [$1\text{-}^{14}\text{C}$]shikimic acid, as recovered from the silica-gel column (see above), was placed together with 100 mg of 10% palladium-charcoal in a Carius tube. The tube was sealed and then heated at 260°C for 48 h. After cooling, the tube was opened and ether was added. Filtration to remove the catalyst and evaporation to dryness yielded a mixture of ^{14}C -labelled hydrocarbons (54 mg) separable by analytical g.l.c. [2 m glass column packed with 1% SE-30 on Chromosorb W-sil (100–120 mesh) operating at 180°C] into three major peaks that were identified by direct g.l.c. and mass spectrum comparisons with authentic samples as (i) an iso- and anteiso- C_{16} hydrocarbon mixture, (ii) 10-phenyl-*n*-decane and (iii) 12-phenyl-*n*-dodecane (third peak).

In trial runs, pure methyl 11-cyclohexylundecanoate and methyl 13-cyclohexyltridecanoate when subjected to this dehydrogenation procedure gave in very good yields (60%) respectively 10-phenyl-*n*-decane [M^+/e 218; base peak m/e 91 (tropylium)] and 12-phenyl-*n*-dodecane (M^+/e 246; base peak m/e 91), also characterized by n.m.r. [^1H multiplet at $\delta=7.10$ due to aromatic protons, ^2H triplet ($J=7\text{ Hz}$) at $\delta=2.58$ due to methylene attached to the aromatic ring, a broad singlet centred at $\delta=1.28$ due to the remaining $-\text{CH}_2-$ groups and a distorted triplet at $\delta=0.93$ (^3H) attributable to a terminal methyl group] and u.v. spectroscopy (λ_{max} , 229 nm). In the same reaction conditions methyl palmitate similarly afforded *n*-pentadecane (M^+/e 212).

The radioactive mixture of hydrocarbons (54 mg) was diluted with carrier (12-phenyl-*n*-dodecane, 484 mg) and placed together with a tenfold excess of KMnO_4 (5.4 g) in four Carius tubes, which were then sealed and heated at 200°C for 12 h. After being cooled, the tubes were opened and 1 M-HCl was

added. The acidic aqueous solutions were combined and extracted three times with diethyl ether. The extract was concentrated and chromatographed by preparative t.l.c. [two 20cm×20cm precoated plates of Merck silica gel F254; eluent diethyl ether–light petroleum (b.p. 40–70°C) (1:1, v/v)] to give crystalline benzoic acid, which was recrystallized from water to constant radioactivity (121 mg).

Schmidt degradation of [^{14}C]benzoic acid

Schmidt decarboxylation of benzoic acid (61 mg) was performed in a standard manner (Colowick & Kaplan, 1957). The evolved CO_2 was recovered as BaCO_3 which was centrifuged, washed with decarbonated water to neutrality and dried over P_2O_5 under vacuum at 90°C (85 mg). The aniline formed (45 mg) was extracted with ether from the reaction mixture after the addition of 1M-NaOH, and its purity was checked by t.l.c. (on silica gel) and by u.v. spectroscopy.

Results

In three separate experiments, growing cultures of *B. acidocaldarius* were incubated with [^{14}C]acetate, [^{14}C]phenylalanine, and [^{14}C]shikimate, and the fatty acids were recovered from the harvested cells as a purified methyl ester fraction, which was then resolved into (a) methyl iso- and anteiso-heptadecanoate, (b) methyl 11-cyclohexylundecanoate and (c) methyl 13-cyclohexyltridecanoate, as

described in the Experimental section. In typical cells these three fractions comprise respectively 27, 27 and 31% of the total methyl esters. The distribution of the recovered radioactivity in the three experiments is summarized in Table 1. The total incorporation from [^{14}C]acetate into the dried cells was comparatively low (1.4%) when compared with the incorporations from [^{14}C]phenylalanine (14.8%) and [^{14}C]shikimate (24.1%), and apart from any metabolic differences this is ascribed to the rapid removal of acetic acid from the medium, at 60°C and pH 2.6, in the fermenter air stream. Consequently it is more significant to compare the three substrates as lipid precursors on the basis of the total radioactivity incorporated into the cells. On this basis the proportions of the cell activity recovered in the methyl esters were 69% for the [^{14}C]acetate experiment, less than 0.5% for [^{14}C]phenylalanine and 37% for the [^{14}C]shikimate.

Results after the g.l.c. separation of the labelled methyl esters from the three experiments are given in Table 2. From [^{14}C]acetate the specific radioactivities of the cyclic C_{19} and C_{17} esters were one-fifth to one-half that of the branched C_{17} esters and from [^{14}C]phenylalanine all three ester fractions had about the same (low) radioactivity, but label from [^{14}C]shikimate was very selectively incorporated into the cyclic esters, relative to which the branched C_{17} ester fraction had only some 0.5% of the specific radioactivity.

The hydrocarbon mixture obtained by palladium–charcoal treatment of the mixed methyl esters from

Table 1. Incorporation of ^{14}C -labelled precursors by *B. acidocaldarius*

Incorporation of ^{14}C from the indicated precursors into whole cells (freeze-dried) and into the methyl esters prepared from the cell lipids was measured. For details see the text.

Precursor	Yield (g)		$10^{-5} \times \text{Radioactivity}$ (d.p.m.)	
	Cells	Methyl esters	Cells	Methyl esters
Sodium [^{14}C]acetate (0.34 mg; 0.54×10^8 d.p.m.)	3.7	0.16	74	51
[^{14}C]Phenylalanine (0.18 mg; 11.2×10^8 d.p.m.)	12.3	0.27	1660	8
[^{14}C]Shikimic acid (4.68 mg; 1.12×10^8 d.p.m.)	6.0	0.17	270	100

Table 2. Specific radioactivities of the methyl ester fractions from *B. acidocaldarius*

Methyl ester fractions from cells labelled with the indicated ^{14}C -labelled substrates (cf. Table 1) were resolved by preparative g.l.c. For details see the text.

Methyl ester fraction		$10^{-9} \times$ Specific radioactivity (d.p.m./mol)			
		Substrate ...	[^{14}C]Acetate	[^{14}C]Phenylalanine	[^{14}C]Shikimate
iso- and anteiso-Heptadecanoate			16.6	0.70	0.24
11-Cyclohexylundecanoate			8.9	0.83	41.5
13-Cyclohexyltridecanoate			3.5	0.90	41.5

the incubation with $[U-^{14}C]$ shikimate, purified and diluted tenfold with synthetic 12-phenyl-*n*-dodecane (total 540mg with 2.8×10^6 d.p.m.), had a specific radioactivity of 1.46×10^9 d.p.m./mol, and on $KMnO_4$ oxidation gave benzoic acid (50% yield) with a specific radioactivity of 1.41×10^9 d.p.m./mol, thus accounting for over 96% of the ^{14}C . Schmidt degradation of the benzoic acid showed that it was essentially uniformly labelled (like the original $[U-^{14}C]$ shikimate), the resulting $BaCO_3$ and aniline having specific radioactivities of 0.21×10^9 and 1.20×10^9 d.p.m./mol respectively, i.e. $0.97 \times$ one-seventh and $1.01 \times$ six-sevenths of the original specific radioactivity.

Discussion

Our experiments show that the major fatty acids of *B. acidocaldarius*, i.e. the C_{17} and C_{19} ω -cyclohexyl-fatty acids, arise by chain elongation from a starter unit containing the intact carbon skeleton, C_7 , of shikimate; hypothetically this would be the CoA derivative of cyclohexanecarboxylate. This C_7 unit differs from the 'benzoic acid unit' found in some microbial and plant products in that it is not formed degradatively from phenylalanine. Moreover, the results in Table 2 suggest that it is not biosynthesized from acetate to any significant extent. The existence of such a starter unit, and its formation from sugars via shikimate, were hitherto quite unsuspected.

The fatty acid composition of bacteria reflects, in general, the ability of their fatty acid synthetase systems to accept 'starter' acyl-CoA species other than acetyl-CoA, namely propionyl-, isobutyryl-, 2-methylbutyryl-, or 3-methylbutyryl-CoA (Kaneda, 1963). The branched-chain starters so characteristic of bacteria are derived from corresponding amino acids or α -oxo acids

[e.g. valine $\rightleftharpoons (CH_3)_2CH-CO-CO_2H \rightarrow$
isobutyryl-CoA];

and consequently the fatty acid composition of a species is linked with its amino acid metabolism. However, in *B. acidocaldarius* the ω -cyclohexyl-fatty acids are not related to amino acids in this way, and this explains certain features of the rather complex relationship we have found between fatty acid composition, temperature and pH. The proportion of cyclic to acyclic starter species competing for fatty

acid synthetase will in general be higher in less oxidizing conditions, and indeed at pH 3.0 and 60°C increased aeration lowers the cyclic/acyclic ratio. At high pH (5.0) such an effect predominates as the temperature is raised from 50° to 70°C, presumably because of the lowering of the O_2 transfer rate. At low pH (2.0 or 3.0) the effect is reversed and increasing temperature raises the cyclic/acyclic ratio. We presume that this reflects the greater importance of the ion-pumping mechanisms under these conditions, and feedback from these (unknown) mechanisms to the two different regions of intermediate metabolism that the two types of starter represent.

The range of bacterial species in which these ω -cyclohexyl-fatty acids occur is as yet unknown, though their presence in the total lipids of rumen bacteria (Hansen, 1967) indicates that at least one other bacterium present in the rumen flora produces this type of fatty acid. However, apart from their intrinsic significance as indicators of a new metabolic pathway, their existence as major components in at least one *Bacillus* species (albeit one from an extreme habitat) is perhaps of some geochemical interest. Related *n*-alkylcyclohexanes and *n*-alkylbenzenes from a number of geological deposits have hitherto posed a considerable problem, since corresponding biochemical precursors could not be postulated (Maxwell *et al.*, 1971).

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