Microbiological Oxidation of Steroid Hydrocarbons

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Fungal and bacterial oxidation of steroid hydrocarbons has been examined. A rapid assay method for detection of metabolites derived from 5 α -[4-¹⁴C]cholestane is described. Out of 87 organisms tested, 16 gave isolable oxidation products, while others degraded the hydrocarbon without producing stable intermediates. Preliminary characterization of these compounds is based on chromatographic properties.

INTRODUCTION

Steroid hydrocarbons (steranes) form a significant proportion of the saturated alicyclic fraction of some crude oils and shales. The mixtures often contain 5 α -cholestane, 5 α ,24 ξ -methylcholestane and 5 α ,24 ξ -ethylcholestane, though in some instances a range of isomeric hydrocarbons has been found (Mulheirn & Ryback, 1975). Steranes can be concentrated by distillation and can be isolated by chromatography and clathrate formation (Ryback, 1976). However, their potential as a plentiful source of steroids for conversion to pharmacologically active compounds such as progesterone and cortisone has not been exploited, largely due to the absence of methods of initial introduction of oxygen into the skeleton.

The early studies on hydroxylation and oxidation of steroidal substrates, together with recent detailed investigations by Jones and his co-workers (Bell *et al.*, 1972*a*), have proved the versatility of micro-organisms, particularly fungi, in functionalizing many positions within the steroid nucleus. However, most of these processes involve molecules of the androstane or pregnane series and they are generally ineffective in steroids possessing a large C-17 side-chain (Bell *et al.*, 1972*b*).

Sterols carrying a hydroxyl group at C-3 can serve as the sole carbon source for the growth of many bacteria (Brown & Peterson, 1966), indicating that efficient catabolic processes are indeed available in lower organisms. However, the two main pathways involve, initially, either oxidation and cleavage in ring A or hydroxylation of the C-19 methyl group, followed by aromatization of ring A. Neither pathway is likely to be applicable to steranes, which lack any oxygen atoms by which direct binding of ring A to an enzyme might be achieved. A third and potentially more promising pathway of sterol catabolism in some bacteria is side-chain oxidative degradation. Oxidation of a terminal methyl group of cholesterol has been reported using *Mycobacterium* (Zaretskaya *et al.*, 1968; Schubert *et al.*, 1969; Galli-Keinle *et al.*, 1973) and *Nocardia* species (Sih *et al.*, 1968*a*). Subsequent side-chain breakdown appears to proceed via β -oxidation or a similar process to give C₂₇, C₂₄ and C₂₂ carboxylic acids such as compound 1 (Fig. 1), with the ultimate product being a 17-oxosteroid (compound 2: Fig. 1) (Sih *et al.*, 1968*b*; Marsheck & Karim, 1973). Isolation of the unsaturated acid, compound 3 (Fig. 1) from a *Nocardia* species in a related degradation sequence is consistent with the involvement of a β -oxidation pathway in this process (Sih *et al.*, 1968*b*).

Fig. 1. Steroid structures referred to in the text: (1) 3-oxocholest-4-en-26-oic acid; (2) androst-4-en-3,17-dione; (3) 3-hydroxy-19-norbisnorchola-1,3,5(10)-trien-22-oic acid; (4) 5 α -cholestane; (5) antheridiol.

This paper reports an investigation of the potential of microbial oxidation of the sterane 5 α -cholestane (compound 4: Fig. 1) by a range of bacteria and fungi. A rapid assay method for detecting the production of more polar metabolites using [4- 14 C]cholestane is described. This procedure has been applied to the screening of 87 organisms under several culture conditions; 16 bacterial strains were observed to produce more polar products. Preliminary characterization of these compounds is reported.

METHODS

Sources of organisms and compounds. Most of the organisms used in this study were purchased from the National Collection of Industrial Bacteria, Aberdeen, U.K. Additional strains were obtained from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, or from an internal reference collection of isolates. [4- 14 C]Cholesterol was purchased from Amersham.

Chromatographic methods. Thin-layer chromatography (t.l.c.) analytical chromatograms were run on 0.2 mm Merck Kieselgel HF₂₅₄ plates. The chromatograms were initially developed in an unlined tank, using hexane as eluant. This resulted in elution of unchanged hydrocarbon to the solvent front while all observed metabolites were retained on the baseline. Further elution of the plates with ethyl acetate/hexane (15:85, v/v) gave migration of metabolites other than acids to various characteristic R_F values. Standards used included 5 α -cholestane (R_F 0.95), androstan-17-one (0.37), cholestanol (0.16), cholesterol (0.16), methyl cholanoate (0.5) and 5 α -cholanolic acid (0.05).

Radioactive chromatograms were scanned on a Desaga 12-2 plate scanner. Radioactivity of bands eluted from t.l.c. plates with ethyl acetate was measured using a Packard 3375 Tricarb liquid scintillation spectrometer.

Samples were measured in solutions of 2,5-diphenyloxazole (PPO) plus 1,4-di-2(5-phenyloxazolyl)benzene (POPOP) in toluene or 2,5-di[5'-*tert*-butylbenzoxazolyl-(2')l]thiophen (BBOT) in toluene/methanol.

Growth of organisms for sterane metabolism assay. Three basic media were employed for growth of the organisms listed in Table 1. Their compositions (g l^{-1}) were as follows.

Medium A: glucose 20, KH_2PO_4 1.5, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.5, NH_4NO_3 1.0, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.01, L-glutamic acid 0.5, proteose peptone 1.0, yeast extract 0.5, corn steep liquor 1.0.

Medium B: glucose 2.5 or 10 (see Table 1), KH_2PO_4 2.5, yeast extract 2.5, Lab-lemco broth 2.5.

Medium C: KH_2PO_4 4.5, K_2HPO_4 10.5, $(\text{NH}_4)_2\text{SO}_4$ 2.0, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1, and trace elements solution 5 ml; pH 7.1. The trace elements solution contained (g l^{-1}): $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 10.0, $\text{MnCl}_2 \cdot 3\text{H}_2\text{O}$ 0.1, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.4, CaCl_2 0.1.

Medium D, for growth of *Tetrahymena pyriformis*, contained (g l^{-1}): Bactotryptone 5.0, proteose peptone 5.0, sodium acetate 5.0, and KH_2PO_4 1.0 at pH 7.1, supplemented with a solution of 20% (w/v) glucose (50 ml).

Cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml medium and an inoculum of 5 ml, and were shaken at 30 °C for the times shown in Table 1. In some cases dodecane or hexadecane was also added at the concentration indicated. 5 α -[4- ^{14}C]cholestane (1 mg; 2×10^5 c.p.m.) was added in 2,3,4-trimethylpentane (0.1 ml) before sterilization. For testing of cholestane metabolism by *T. pyriformis*, dioxan was used as solvent.

Yields are quoted both on the basis of recovered material and as a percentage of added cholestane.

Assay of radioactive products from microbial incubations. Each flask was acidified to pH 1 with concentrated HCl and left to stand for 30 min. The mixture was then diluted with water and extracted four times with chloroform. The solvent was evaporated and 1% of the residue was removed for scintillation counting. The remainder of the material was analysed by t.l.c. on a 5 $\times$ 20 cm silica plate. After elution in hexane the plate was dried and analysed on the scanning autoradiograph. Unchanged 5 α -cholestane appeared close to the solvent front with the metabolites at the baseline (the intensity of the metabolite radiation was often considerably reduced by absorption effects of residues on the baseline). The plate was then eluted with ethyl acetate/hexane and re-scanned for analysis of the proportions of radioactive products present. For accurate determination of the quantities of various metabolites, the active bands were scraped off and eluted with ethyl acetate, and the products were assayed by scintillation counting.

Synthesis of 5 α -[4- ^{14}C]cholestane. [4- ^{14}C]cholesterol (100 μCi ; 3.7 MBq) was diluted with purified cholesterol (200 mg) and hydrogenated over PtO_2 (40 mg) in ethyl acetate (20 ml) containing perchloric acid (20 μl) for 1.5 h. The catalyst was removed and the solution was washed with 1 M- NaHCO_3 . The product was crystallized from diethyl ether/methanol to give 5 α -cholestanol (138 mg). The liquor was purified by t.l.c. with ethyl acetate/hexane (3:7, v/v), giving coprostanol (8 mg) and 5 α -cholestanol (40 mg).

5 α -Cholestanol (178 mg) in acetone (30 ml) was oxidized with Jones reagent and the isolated product was dissolved in toluene (8 ml). After addition of water (6 ml), acetyl chloride (12 ml) and amalgamated zinc, the mixture was refluxed for 18 h (Enzell, 1966). The isolated product was purified by successive t.l.c. (hexane) on silica and AgNO_3 /silica (10:90), which removed 2–3% of olefinic material. The hydrocarbon was finally treated in dichloromethane (10 ml) with *m*-chloroperbenzoic acid (100 mg) at 20 °C for 1 h. The isolated product was purified by t.l.c. (hexane) to give 5 α -cholestane (155 mg; 2.13×10^8 d.p.m.; 1.37×10^6 d.p.m. mg^{-1}). The product was stored in 2,3,4-trimethylpentane at 3.1 mg ml^{-1} and diluted for use in metabolic studies.

RESULTS AND DISCUSSION

To explore the potential of microbial functionalization and metabolism of steranes, it was essential to employ a rapid screening technique. The method devised for this study involved addition of 5 α -[4- ^{14}C]cholestane to cultures, followed by t.l.c. separation and autoradiography of the recovered lipids. The method can (a) test the overall facility of the organism for cholestane metabolism under a given set of growth conditions, (b) establish the degree of conversion to more polar metabolites, and (c) provide some information on the types of metabolites produced.

Table 1 gives details of growth conditions and radioactivity recovery for 87 fungal and bacterial strains. In the *Candida* strains the radioactivity was located wholly in the culture fluid, while *Cladosporium* showed an even distribution between mycelium and the medium. In all the other fungi tested, radioactivity was located almost exclusively in the disrupted mycelium. Bacteria were not separated from the culture fluid. Acidification and chloroform extraction gave good recovery of lipids. Subsequent saponification may be valuable in some cases, since in one *Corynebacterium* species an increased recovery was observed with this treatment. However, in mycobacteria and other species, solvent extraction was adequate for recovery of lipids.

Table 1. *Screening of fungi, bacteria and a protozoon for metabolism of 5 α -[4-¹⁴C]cholestane*

Strain	Medium*	Glucose concn (%)	Growth period (d)	Recovered activity (%)	Conversion (%)†	
					Recovered	Overall
<i>Cladosporium resinae</i>	A	2.0	4	89	—	—
<i>Penicillium lilaceum</i>	A	2.0	4	70	—	—
<i>Aspergillus niger</i>	A	5.0	4	65	—	—
	A	5.0	4	64	—	—
<i>Cunninghamella blakesleeana</i>	A	5.0	4	52	—	—
	A	5.0	4	88	—	—
<i>Candida</i> (ex cetane)	A	2.0	5	92	—	—
	A ^b	2.0	7	33	—	—
<i>Candida intermedia</i>	A	2.0	5	96	—	—
	A ^b	2.0	7	121	—	—
<i>Candida lipolytica</i>	A	2.0	5	101	—	—
	A ^b	2.0	3	96	—	—
<i>Nocardia</i> A23-3‡	B	0.25	3	89	—	—
	B ^d	1.0	6	73	—	—
<i>N. opaca</i>	B	1.0	5	22	5	1
	B ^d	1.0	6	34	3	1
	B ^d	—	7	84	5	4
<i>N. erythropolis</i>	B	1.0	5	58	10	6
	B ^d	1.0	6	61	12	7
	B ^d	—	7	114	<5	<5
	B ^d	—	7	94	5	5
<i>N. calcarea</i> NCIB 8863	B	0.25	7	57	—	—
<i>N. cellulans</i> NCIB 8868	B	0.25	7	111	—	—
<i>N. coeliaca</i> NCIB 8939	B	0.25	7	85	—	—
<i>N. globerula</i> NCIB 9159	B	0.25	7	86	—	—
<i>N. hydrocarbonoxydans</i> NCIB 9436	B	0.25	7	93	—	—
<i>N. opaca</i> NCIB 9409	B	0.25	7	23	70	16
<i>N. petroleophila</i> NCIB 9438	B	0.25	7	100	—	—
<i>N. rugosa</i> NCIB 8926	B	0.25	7	89	—	—
<i>Nocardia</i> NCIB 8862	B	0.25	7	88	—	—
<i>Nocardia</i> NCIB 9160	B	0.25	7	88	40	35
<i>N. apis</i> NCIB 9378	B	0.25	7	101	—	—
<i>N. italica</i> NCIB 9386	B	0.25	7	99	—	—
<i>N. aurantiaca</i> NCIB 9557	B	0.25	7	73	66	48
<i>Corynebacterium</i> JEIC	B ^b	0.25	3	73	5	3
	C ^c	—	6	82	10	8
	B	0.25	6	108	5	5
	B ^a	0.25	6	71	10	7
<i>C. laevaniformans</i>	B	1.0	5	30	5	2
	B ^d	1.0	21	84	—	—
<i>C. aquaticum</i> NCIB 9460	B	0.25	7	143	—	—
<i>C. manihot</i> NCIB 9097	B	0.25	7	102	—	—
<i>C. mediolanum</i> NCIB 7206	B	0.25	7	105	—	—
<i>C. rubrum</i> NCIB 9433	B	0.25	7	83	—	—
<i>C. acetoacidophilum</i> NCIB 9661	B	0.25	7	96	—	—
<i>C. barkeri</i> NCIB 9658	B	0.25	7	93	—	—
<i>C. herculis</i> NCIB 9694	B	0.25	7	115	—	—
<i>Coryneform</i> NCIB 9568	B	0.25	7	95	—	—
<i>Coryneform</i> NCIB 9646	B	0.25	7	66	33	20
<i>Coryneform</i> NCIB 9569	B	0.25	7	77	—	—
<i>Coryneform</i> NCIB 9647	B	0.25	7	94	<5	4
<i>Coryneform</i> NCIB 9648	B	0.25	7	150	—	—
<i>Coryneform</i> NCIB 9649	B	0.25	7	48	33	16
<i>Mycobacterium phlei</i> NCIB 8573	B	1.0	5	60	20	12
	B ^c	1.0	21	99	—	—
<i>M. phlei</i> NCIB 8575	B	1.0	7	64	15	10
	B ^c	1.0	21	69	5	3
<i>M. phlei</i> CBS 49466	B	1.0	7	28	50	14
	B ^c	1.0	21	94	<1	<1
<i>M. smegmatis</i>	B	1.0	5	30	5	2
	B ^d	1.0	21	84	—	—
	B	0.25	7	50	75	40

Table 1—continued

Strain	Medium*	Glucose concn (%)	Growth period (d)	Recovered activity (%)	Conversion (%)†	
					Recovered	Overall
<i>M. rhodochrous</i>	B	1.0	3	70	—	—
	B ^d	1.0	6	62	—	—
<i>Arthrobacter</i> PG§	B	1.0	6	31	1	<1
	B ^b	0.25	6	12	<1	<1
	B ^d	1.0	6	34	<1	<1
<i>A. globiformis</i> NCIB 8602	B	0.25	6	76	—	—
<i>A. globiformis</i> NCIB 8605	B	0.25	6	83	—	—
<i>A. tumescens</i> NCIB 8914	B	0.25	6	80	—	—
<i>A. aurescens</i> NCIB 8912	B	0.25	6	72	—	—
<i>A. citreus</i> NCIB 8915	B	0.25	6	78	—	—
<i>A. histidinolovorans</i> NCIB 9541	B	0.25	6	67	—	—
<i>A. pascens</i> NCIB 8910	B	0.25	6	44	—	—
<i>A. globiformis</i> NCIB 8717	B	0.25	6	74	—	—
<i>A. atrocyaneus</i> NCIB 9220	B	0.25	7	70	—	—
<i>A. aurescens</i> NCIB 8912	B	0.25	7	72	—	—
<i>A. citreus</i> NCIB 8915	B	0.25	7	75	—	—
<i>A. crystallopoietes</i> NCIB 9499	B	0.25	7	104	—	—
<i>A. duodecadis</i> NCIB 9222	B	0.25	7	85	—	—
<i>A. flavescens</i> NCIB 9221	B	0.25	7	102	—	—
<i>A. globiformis</i> NCIB 8907	B	0.25	7	83	—	—
	B	0.25	7	76	—	—
	B	0.25	7	89	—	—
<i>A. nicotianae</i> NCIB 9458	B	0.25	7	76	—	—
<i>A. oxydans</i> NCIB 9333	B	0.25	7	86	—	—
	B	0.25	7	98	—	—
<i>A. pascens</i> NCIB 8910	B	0.25	7	44	—	—
<i>A. ramosus</i> NCIB 9066	B	0.25	7	146	—	—
<i>A. simplex</i> NCIB 8929	B	0.25	7	<30	—	—
<i>A. tumescens</i> NCIB 8914	B	0.25	7	80	—	—
<i>A. ureafaciens</i> NCIB 7811	B	0.25	7	>80	—	—
<i>A. variabilis</i> NCIB 9455	B	0.25	7	95	—	—
<i>Arthrobacter</i> NCIB 9423	B	0.25	7	>80	—	—
<i>Arthrobacter</i> NCIB 9459	B	0.25	7	107	—	—
<i>A. histidinolovorans</i> NCIB 9541	B	0.25	7	67	—	—
<i>Arthrobacter</i> NCIB 9666	B	0.25	7	90	—	—
Strain K ₂ §	B	0.25	7	78	—	—
Strain K ₃ §	B	0.25	7	79	—	—
Strain K ₄ §	B	0.25	7	92	—	—
Strain K ₅ §	B	0.25	7	13	—	—
Strain K ₁ §	B	0.25	7	13	<1	<1
Strain K _p §	B	0.25	7	48	—	—
<i>Pseudomonas</i> 473	B	0.25	7	85	—	—
Strain 13(7)‖	B ^b	0.25	6	84	—	—
Strain 9(7)‖	B ^b	0.25	6	105	—	—
Strain 14(7)‖	B ^b	0.25	6	66	—	—
Strain 10(7)‖	B ^b	0.25	12	38	—	—
Strain 1(7)‖	B ^b	0.25	19	34	trace	trace
Strain decalin GR	B ^b	0.25	19	46	—	—
Strain 18(7)‖	B ^b	0.25	19	44	—	—
Strain cholestane	B ^b	0.25	19	29	—	—
Strain perhydrophenanthrene	B ^b	0.25	19	53	—	—
<i>Tetrahymena pyriformis</i>	D	1.0	5	90	—	—

* Details of the culture media are given in Methods. In some experiments dodecane or hexadecane was added to the media, as indicated by the superscripts: *a*, 0.2% dodecane; *b*, 0.4% dodecane; *c*, 0.6% hexadecane (final concentrations, v/v).

† The recovered conversion is based on total recovered radioactivity and the overall conversion on total added sterane.

‡ Isolated on butane.

§ Isolated on benzene.

‖ Isolated on pristane.

Table 2. *Cholestane metabolism by selected bacteria*

Organism	Conversion (%)		Metabolite groups*			
	Recovered	Overall	Esters	Ketone	Mono-alcohol	Acid, etc.
<i>Nocardia opaca</i>	3-5	1-4	?†	—	—	++
<i>N. erythropolis</i>	10-12	5-7	?†	—	+	++
<i>N. opaca</i> NCIB 9409	70	16	—	+	+	+++
<i>Nocardia</i> NCIB 9160	40	35	—	++	+	—
<i>N. aurantiaca</i> NCIB 9557	66	48	+++	—	—	—
<i>Corynebacterium</i> JEIC	5-10	3-8	—	?‡	+‡	++
<i>C. laevaniformans</i>	5	2	—	—	—	—§
Coryneform NCIB 9646	33	20	—	—	—	+++
Coryneform NCIB 9647	<5	4	—	—	—	+
Coryneform NCIB 9649	33	16	—	?	—	+++
<i>Mycobacterium phlei</i> NCIB 8573	20	12†	+++	++	+	+
<i>M. phlei</i> NCIB 8575	5-15	3-10	+++	++	+	+‡
<i>M. phlei</i> CBS 49466	50	14†	+++	+	—	+++†
<i>M. smegmatis</i>	5-75	2-40	++	+	+	++
<i>Arthrobacter</i> PG	1	<1	?	—	—	+
Strain K ₁	<1	<1†	—	—	—	—§

* Identified by R_F values on t.l.c.: +++, major metabolites; ++, significant metabolites; +, trace metabolites.

† Formed only in cultures lacking *n*-alkanes.

‡ Major metabolite in presence of *n*-alkanes.

§ Products not detected by autoradiography.

|| Isolated on benzene.

Four fungal species and three yeast strains were screened, with no evidence of metabolite formation. With the exception of *Aspergillus niger* and *Candida* (ex cetane), efficient recovery of the hydrocarbon was obtained. The *in vivo* production of ergosterol and related steroids, which play a vital role in the growth and mitochondrial function of fungi, might be expected to be accompanied by rigid control of associated catabolic processes. Thus, processes of side-chain degradation are unlikely to be efficient in these organisms. Species such as *Achlya*, which do oxidize the sterol side-chain to produce the fungal sex hormone antheridiol (compound 5: Fig. 1) (McMorris & Barksdale, 1967), might offer more potential for sterane metabolism.

Screening of bacteria gave more encouraging results. Of the 16 *Nocardia* strains examined, five gave promising levels of metabolite formation under a variety of growth conditions. Of these five strains, two strains of *N. opaca* gave low recovery of total radioactivity and little useful conversion of cholestane to polar metabolites ($\leq 16\%$). Assay results indicated that the major products were acids (Table 2). The data suggest that the low conversion results from extensive degradation of the initially formed metabolites. *Nocardia erythropolis* was less efficient at degrading cholestane, giving substantially higher recovery of material. Maximum observed conversion of cholestane to useful metabolites by this strain was 7% in these trials. The major products of most strains were acids, though formation of mono-alcohols and esters was also indicated. In several strains the presence of an excess of an *n*-alkane in the medium had no major influence on sterane metabolism. The use of *n*-alkane as the carbon source gave higher recoveries with no decrease in metabolite production. In later experiments, two further strains, *Nocardia* sp. NCIB 9160 and *N. aurantiaca*, also gave high recoveries (70-90%) together with high conversion to useful metabolites (35-48%). Significantly, strain NCIB 9160 appeared to produce compounds having the t.l.c. polarity of androstan-17-one,

Fig. 2. Degradation of 5 α -[4- ^{14}C]cholestane in the presence of [^3H]hexadecane by *Corynebacterium* JEIC grown (a) on the complex medium B, and (b) on the mineral medium C (see Methods). Cultures were grown in 50 ml medium containing 0.3 ml hexadecane and 1 mg 5 α -cholestane. The curves represent the recovery of radioactive 5 α -cholestane (□) and hexadecane (●) at various times during the incubations.

making this strain a suitable candidate for a further study. The products from *N. aurantiaca* appeared to be less polar.

Nine strains of *Corynebacterium* were examined, two of which gave promising results. The presence of *n*-alkanes had no deleterious effects on conversions in *Corynebacterium* strain JEIC, yields of polar metabolites being 3–8%. In one experiment with *C. laevaniformans*, metabolites were not detected when *n*-hexadecane was incorporated, though this result was questionable since growth appeared to be retarded by the hydrocarbon. Six further coryneform bacteria were examined subsequently, three of which gave significant (4–20%) conversion of cholestane to polar acidic metabolites. The structures of these compounds remain to be determined.

Preliminary experiments on metabolism of 5 α -[4- ^{14}C]cholestane by *Corynebacterium* strain JEIC showed that about 5% conversion to more polar compounds could be achieved under a variety of conditions, using the standard assay concentration of 20 mg cholestane l $^{-1}$. On a glucose-containing medium, products were formed without major breakdown of the steroids (Table 1). Addition of *n*-dodecane to the medium appeared to enhance both cholestane conversion to useful metabolites and the further breakdown of these metabolites. In a mineral medium supplemented with *n*-dodecane, significant oxidation was also obtained without a major increase in breakdown of the sterane or its metabolites. These results can be interpreted in terms of either an enhancement of enzyme induction by the alkane or an increased rate of absorption of the sterane by the cells in the presence of the alkane.

In a more detailed examination of the influence of aliphatic hydrocarbons on the course of cholestane breakdown, *Corynebacterium* strain JEIC was incubated with 5 α -[4- ^{14}C]cholestane (0.67 mg; 4×10^5 c.p.m., in 0.2 ml iso-octane) and *n*-[U- ^3H]hexadecane [0.3 ml; 1.9 $\mu\text{Ci g}^{-1}$ (70 kBq g $^{-1}$); 4.5×10^5 c.p.m.] in both glucose-supplemented and mineral media. Breakdown of the *n*-alkane was virtually complete after 3–4 d in the glucose medium (medium B: Fig. 2a). Cholestane metabolism appeared to go through an initial lag phase, with significant metabolism only commencing as the *n*-hexadecane became depleted. In the mineral medium (medium C) the picture was broadly similar, though the time scale was considerably contracted, *n*-alkane breakdown being complete in 2–3 d (Fig. 2b). Cholestane metabolism also proceeded more rapidly, without a significant lag phase, and appeared to be maintained for a longer period, since the yield of recovered ^{14}C -labelled compounds was only 7.6% after 10 d, considerably lower than in the glucose medium. The lag phase for cholestane breakdown in the glucose-supplemented medium may reflect either preferential

breakdown of the *n*-alkane by a common metabolic pathway such as β -oxidation or the requirement for the organism to induce a distinct set of enzymes for cholestane metabolism.

Quantitative aspects of cholestane oxidation by this organism were not encouraging. While the time-course experiments showed efficient loss of 5 α -cholestane, conversion to useful metabolites in the experiment was usually less than 5% and gave no apparent maximum. In other experiments conversions varied from 2% to 10%. Increasing cholestane concentration from 20 mg l⁻¹ to 200 and 400 mg l⁻¹ gave significantly lower yields. This unreliable production of metabolites prevented detailed work on their structures. T.l.c. analysis of diazomethane-treated products confirmed that the predominant compounds were sterane monocarboxylic acids. Evidence for the formation of a small quantity of mono-alcohols and possibly ketones was obtained for cultures grown in the presence of *n*-alkane.

Four of the five *Mycobacterium* species screened proved efficient at cholestane metabolism, giving high yields of oxidized products (2–40%). However, in contrast to the corynebacteria, the presence of liquid *n*-alkanes had a deleterious effect on metabolism, due, at least in part, to growth retardation. Detailed investigation of growth conditions and the structures of metabolites of representative strains of *M. phlei* will be reported elsewhere.

Rather surprisingly, *Arthrobacter*, *Pseudomonas* and related species, which have exceptional ability in *n*-alkane oxidation, showed little promise as practical sterane oxidizers, even in the presence of an *n*-alkane as an inducer of paraffin oxidation. From 28 *Arthrobacter* strains, a *Pseudomonas* strain and 15 related strains which had been isolated during studies on hydrocarbon oxidation, only two showed slight evidence of the formation of stable intermediates. However, the levels of recovered radioactivity were again low in 10 cases, suggesting that functionalization of the hydrocarbon may be proceeding, but only for the intermediate metabolites to undergo more efficient extended degradation. No investigations of the use of inhibitors or the potential of mutation in limiting degradation have yet been carried out.

The protozoan *Tetrahymena pyriformis* is capable of dehydrogenating cholesterol in both the ring system and side-chain, giving cholesta-5,7,22-trienol (Conner *et al.*, 1969). This product is also obtained from cholest-7-en-3 β -ol and from 5 α -cholestanol (Mulheirn *et al.*, 1971). Incubation with 5 α -cholestane, however, gave no conversion to olefinic material, indicating the necessity for the presence of a 3 β -hydroxyl group for binding to the dehydrogenase enzymes.

This study shows that a large number of bacteria are capable of degrading steroid hydrocarbons such as cholestane, particularly members of the genera *Nocardia*, *Corynebacterium* and *Mycobacterium*. Nine strains gave overall conversions to oxidized products in excess of 10% in sub-optimal conditions. While *Arthrobacter* and *Pseudomonas* strains appear to be unable to oxidize cholestane to useful products, 11 strains gave low recovery of the hydrocarbon and may, therefore, contain active metabolic pathways whose inhibition could lead to accumulation of functionalized steroids.

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