Microbiological Oxidation of Long-chain Aliphatic Compounds. Part V.[1] Mechanism of Hydroxylation

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Methyl [(17L)-17-$^{3}H_1$]stearate, methyl [17D)-17-$^{3}H_1$]stearate, and methyl [(17DL)-17-$^{3}H_1$]stearate have been prepared. Each of these compounds, mixed with methyl [U-^{14}C]stearate, has been incubated with *Torulopsis gropengiesseri*, to give, after work-up, methyl 17-L-hydroxystearate and dimethyl octadecane-1,18-dioate. Determination of the ^{3}H : ^{14}C ratios of the latter compounds has established (a) that ω-hydroxylation and ω-1-hydroxylation of stearic acid are independent reactions which involve overall a direct substitution of a hydrogen atom by a hydroxy-group, (b) that the ω-1-hydroxylation of stearic acid is a stereospecific process which takes place with retention of configuration, and (c) that a kinetic isotope effect probably operates during the hydroxylation of [(17L)-17-$^{3}H_1$]stearic acid. A mixture of 1-bromo[(16DL)-16-$^{3}H_1$]heptadecane and 1-bromo[U-^{14}C]heptadecane has been oxidised with *T. gropengiesseri*, to give, after work-up, dimethyl heptadecane-1,17-dioate. No change in the ^{3}H : ^{14}C ratio was observed during this transformation. This result has been interpreted as evidence that alk-1-enes are not intermediates in the terminal oxidation of alkanes by the yeast.

THE concurrent production of ω-hydroxy-acids and ω-1-hydroxy-acids from fatty acids has been observed with rat liver preparations [2] and with yeasts of the genus *Torulopsis* [3,4] which gave ω-1-hydroxy-acids having the L-configuration at the asymmetric centre. By analogy with the reported [5] transformation of octanoic acid to 8-hydroxyoctanoic acid, the oxygen atom introduced in the ω- and ω-1-hydroxy-acids formed by *T. gropengiesseri* is probably derived from molecular oxygen.

Previous work [4] has shown that the ratio of ω-hydroxy-acid to ω-1-hydroxy-acid formed from a fatty acid by *T. gropengiesseri* is critically dependent upon the fatty acid chain-length; both ω- and ω-1-hydroxylations take place most efficiently when the site of hydroxylation is separated from the terminal carboxy-group by a chain of fourteen methylene groups. It has been proposed [4] that both ω- and ω-1-hydroxylations of fatty acids by *T. gropengiesseri* are catalysed by a single enzyme on which a fatty acid can adopt two alignments, one alignment facilitating ω-hydroxylation and the other alignment facilitating ω-1-hydroxylation. The alignment adopted by a particular fatty acid will be largely determined by the tendency to achieve the optimum separation of the site of hydroxylation and the carboxy-group. This hypothesis suggests that fatty acid hydroxylation involves direct displacement of a hydrogen atom by an oxygen species which is presumably derived from molecular oxygen.

Alternative explanations of the concurrent formation of terminally oxidised and sub-terminally oxidised products during microbiological oxidation of aliphatic compounds have been advanced. Leadbetter and Foster [6] accounted for co-production of methyl ketones and fatty acids during the microbiological oxidation of short-chain alkanes by postulating an intermediate

[1] Part IV, D. F. Jones and R. Howe, *J. Chem. Soc.* (*C*), preceding paper.
[2] B. Preiss and K. Bloch, *J. Biol. Chem.*, 1964, **239**, 85; H. Den, *Biochim. Biophys. Acta*, 1965, **98**, 462.
[3] A. P. Tulloch, J. F. T. Spencer, and P. A. J. Gorin, *Canad. J. Chem.*, 1962, **40**, 1326.
[4] D. F. Jones and R. Howe, *J. Chem. Soc.* (*C*), 1968, 2801.
[5] M. Kusunose, E. Kusunose, and M. J. Coon, *J. Biol. Chem.*, 1964, **239**, 1374.
[6] E. R. Leadbetter and J. W. Foster, *Arch. Mikrobiol.*, 1960, **35**, 92.

primary free-radical. This could undergo direct reaction with a hydroxyl-generating species to give a terminally oxygenated product or undergo rearrangement to the more stable secondary free-radical which on reaction with the hydroxyl-generating species would yield a subterminally oxygenated product. This type of mechanism could readily be extended to account for the co-production of ω- and ω-1-hydroxy-acids from fatty acids. McKenna and Kallio,[7] taking account of a hypothesis that alk-1-enes are intermediates in alkane hydroxylation, have suggested that ω- and ω-1-hydroxy-acids could arise from fatty acids as a result of the alternative ring-openings of an intermediate terminally epoxidised fatty acid.

To distinguish between the three possible mechanisms for fatty acid hydroxylation, fermentations by *T. gropengiesseri* of stearic acids stereospecifically labelled with tritium at C-17 have been carried out. Hydroxylations of the labelled stearic acid by way of the mechanism involving primary and secondary free-radicals would require that both 18-hydroxystearic acid and 17-hydroxystearic acid should have the same tritium content as the substrate. Hydroxylations of the labelled stearic acid by way of an intermediate epoxy-acid would require that the two hydroxystearic acids should have the same tritium content. Hydroxylations of the labelled stearic acid by direct displacement reactions would require that the tritium content of 18-hydroxystearic should differ from that of 17-hydroxystearic acid. The results reported below confirm that ω- and ω-1-hydroxylations of stearic acid by *T. gropengiesseri* are direct displacement reactions, and establish that ω-1-hydroxylation takes place with retention of configuration.

Methyl stearates stereospecifically labelled with tritium at C-17 were prepared from the enantiomorphs of methyl 17-hydroxystearate by the procedure used by Schroepfer and Bloch [8] for the preparation of [9-$^3H_1$]-stearic acids. Methyl 17-L-hydroxystearate, which was available from the fermentation of octadecane,[4] was converted into methyl 17-D-hydroxystearate by alkaline hydrolysis of the tosylate derivative followed by methylation with diazomethane. Tosylate derivatives of the hydroxystearates were prepared and the tosyloxy-group was replaced by tritium by hydrogenolysis with isotopically labelled lithium aluminium hydride. This type of reaction has been shown to take place largely with an inversion of configuration.[9] The labelled stearyl alcohols formed by the lithium aluminium hydride reduction were oxidised to give stearic acids which were methylated with diazomethane. Methyl [(17DL)-17-$^3H_1$]-stearate was prepared using methyl 17-DL-hydroxystearate which was prepared from methyl 17-oxostearic acid by sodium borohydride reduction.

Fermentations were carried out with *T. gropengiesseri* in a medium containing glucose. Methyl stearates were used for fermentation in preference to the corresponding acids to lessen the risk of the substrate precipitating from the culture medium; aqueous emulsions of the methyl esters were found to be more stable than those of the corresponding acids. Previous work [4] has shown that *T. gropengiesseri* can effect ester hydrolysis. To make the calculation of tritium contents of the hydroxylation products independent of the degree of dilution of the substrates and products by endogenous stearic and hydroxystearic acids, the tritiated methyl stearates were mixed with methyl stearate uniformly labelled with carbon-14 and the ^3H : ^{14}C ratios of the substrates and products were determined.

The products of each of the methyl stearate fermentations were glycolipids which precipitated from the culture medium. These glycolipids were isolated in ethyl acetate and subjected to acid-catalysed methanolysis to release the lipid components which were obtained as methyl esters. Unsaturated lipids were removed as mercuric acetate complexes, and the saturated lipids were chromatographed on a column of aluminium oxide to give methyl 17-hydroxystearate and methyl 18-hydroxystearate which, because it was formed in relatively low yield, could not be freed from contamination by methyl 17-hydroxystearate and by methyl 16-hydroxypalmitate (derived from the endogenous glycolipids of the organism). To provide a measure of the tritium content of the 18-hydroxystearate the mixture of hydroxy-esters was oxidised, and the acidic products were separated from methyl 17-oxostearate by column chromatography and methylated with diazomethane to give a mixture of dimethyl octadecane-1,18-dioate and dimethyl hexadecane-1,16-dioate. This mixture was separated by gas–liquid chromatography. Assuming that oxidation of the primary alcohol group does not affect the tritium substituent at the adjacent carbon atom, the tritium content of dimethyl octadecane-1,18-dioate will be the same as that of methyl 18-hydroxystearate.

It was expected that a proportion of the labelled stearic acids generated by hydrolysis of the methyl stearates would be metabolised by β-oxidation, and that a proportion of the stearic acids might undergo desaturation to give labelled oleic acids which could undergo ω- and ω-1-hydroxylation.[4] Accordingly, the unsaturated lipids obtained from the fermentation products were processed to give methyl 17-L-hydroxyoleate and dimethyl octadec-9-ene-1,18-dioate which were assayed for radio-activity.

The amounts of tritrium relative to carbon-14 in the methyl stearates fermented and in the methyl 17-hydroxystearates and dimethyl octadecane-1,18-dioates obtained from the fermentation products are listed in Table 1. Table 2 gives the amounts of tritium relative

[7] E. J. McKenna and R. E. Kallio, *Ann. Rev. Microbiol.*, 1965, **19**, 183.

[8] G. J. Schroepfer and K. Bloch, *J. Biol. Chem.*, 1965, **240**, 54.

[9] E. R. Alexander, *J. Amer. Chem. Soc.*, 1950, **72**, 3796; G. K. Helmkamp and B. F. Rickborn, *J. Org. Chem.*, 1957, **22**, 479; A. Nickon, J. H. Hammons, J. L. Lambert, and S. J. R. C. Williams, *J. Amer. Chem. Soc.*, 1963, **85**, 3713.

to carbon-14 in the methyl 17-hydroxyoleates and dimethyl octadec-9-ene-1,18-dioates. The latter compounds were not freed from contamination by dimethyl hexadec-7-ene-1,16-dioates.

Each of the methyl stearate fermentations gave approximately a 30% yield of methyl 17-hydroxystearate and a 3% yield of methyl 18-hydroxystearate. About 85% of the methyl hydroxystearates were derived from the labelled methyl stearates, and about 4% of the labelled methyl stearates were converted into methyl 17-hydroxyoleates which contained an equal amount of unlabelled hydroxyoleates. The location of tritium at C-17 in the tritiated ω-1-hydroxystearates and ω-1-hydroxyoleates was confirmed by the loss of tritium which occurred on oxidation of the hydroxy-esters to the corresponding keto-esters. The dimethyl hexadecane-1,16-dioates obtained during the isolation of the C_{18} diesters contained both tritium and carbon-14, but the radioactivity was too weak for accurate measurement of the $^3H : ^{14}C$ ratio.

Table 1 shows that an extensive loss of tritium occurred in the conversion of methyl [(17L)-17-$^3H_1$]-stearate and methyl [(17DL)-17-$^3H_1$]stearate into methyl 17-L-hydroxystearate, whereas relatively little tritium was lost on conversion of methyl [(17D)-17-$^3H_1$]stearate into methyl 17-L-hydroxystearate. Table 1 also shows that the $^3H : ^{14}C$ ratios of the dimethyl octadecane-1,18-dioates were higher than those of the methyl stearates from which the diesters originated. These enrichments in tritium were not produced during purification of the diesters by g.l.c. (compare Sgoutas [10]) because the $^3H : ^{14}C$ ratios of the mixture of C_{16} and C_{18} diesters, prior to g.l.c., were found to be closely similar to those of the purified C_{18} diesters. Assuming that the $^3H : ^{14}C$ ratios of the dimethyl octadecane-1,18-dioates

[10] D. S. Sgoutas, *Nature*, 1966, **211**, 296.

TABLE 1

$^3H : ^{14}C$ ratios of methyl 17-hydroxystearates and dimethyl octadecane-1,18-dioates obtained by microbiological oxidation of methyl stearates stereospecifically labelled with tritium at C-17 and uniformly labelled with carbon-14

Substrate	Products	^3H d.p.m.	^{14}C d.p.m.	$^3H : ^{14}C$	$^3H : ^{14}C$ *
Methyl [U-^{14}C-(17L)-17-$^3H_1$]stearate					
experiment 1		21,250	563	37·7	1·0
experiment 2		13,820	372	38·2	1·0
	Methyl 17-L-hydroxystearate				
	experiment 1	3832	776	4·9	0·13
	experiment 2	1990	433	4·6	0·12
	Dimethyl octadecane-1,18-dioate				
	experiment 1	23,700	161	147	3·9
	experiment 2	6050	44	137	3·6
Methyl [U-^{14}C-(17D)-17-$^3H_1$]stearate					
experiment 1		8511	696	12·2	1·0
experiment 2		5408	451	12·0	1·0
	Methyl 17-L-hydroxystearate				
	experiment 1	11,320	1040	10·9	0·89
	experiment 2	5869	571	10·3	0·86
	Dimethyl octadecane-1,18-dioate				
	experiment 1	1462	60	24·4	2·0
	experiment 2	612	41	14·9	1·24
Methyl [U-^{14}C-(17DL)-17-$^3H_1$]stearate					
experiment 1		14,700	676	21·8	1·0
experiment 2		11,400	481	23·0	1·0
	Methyl 17-L-hydroxystearate				
	experiment 1	13,580	1222	11·1	0·51
	experiment 2	7058	632	11·2	0·49
	Dimethyl octadecane-1,18-dioate				
	experiment 1	3047	52	58·6	2·7

* For ease of comparison, this column lists the $^3H : ^{14}C$ ratios relative to an assigned value of unity for the substrate.

TABLE 2

$^3H : ^{14}C$ ratios of methyl 17-hydroxyoleates and dimethyl octadec-9-ene-1,18-dioates obtained from the fermentation products of methyl stearates stereospecifically labelled with tritium at C-17 and uniformly labelled with carbon-14

Substrates	Products	^3H	^{14}C	$^3H : ^{14}C$	$^3H : ^{14}C$ *
Methyl [U-^{14}C-(17L)-17-$^3H_1$]stearate		21,250	563	37·7	1·0
	Methyl 17-L-hydroxyoleate	8496	939	9·0	0·24
	Dimethyl octadec-9-ene-1,18-dioate †	9479	96	99·0	2·5
Methyl [U-^{14}C-(17D)-17-$^3H_1$]stearate		8511	696	12·2	1·0
	Methyl 17-L-hydroxyoleate	13,940	1327	10·5	0·86
	Dimethyl octadec-9-ene-1,18-dioate †	970	61	15·9	1·3
Methyl [U-^{14}C-(17DL)-17-$^3H_1$]stearate		14,700	676	21·8	1·0
	Methyl 17-L-hydroxyoleate	35,180	2800	12·6	0·58
	Dimethyl octadec-9-ene-1,18-dioate †	3436	78	44	2·0

* For ease of comparison, this column lists the $^3H : ^{14}C$ ratios relative to an assigned value of unity for the substrate. † Contaminated by 10—20% of dimethyl hexadec-7-ene-1,16-dioate.

accurately reflect those of the methyl 18-hydroxystearates from which the diesters were derived, the results show that ω-hydroxylation of the labelled methyl stearates was accompanied by an enrichment of tritium relative to carbon-14.

The results in Table 1 are consistent only with ω- and ω-1-hydroxylation by a mechanism involving direct displacement of a hydrogen atom by an oxygen species which can be readily converted into a hydroxy-group. The diminution of the ^3H : ^{14}C ratio on conversion of the mixture of methyl [(17L)-17-$^3H_1$]stearate and methyl [U-^{14}C]stearate into methyl 17-L-hydroxystearate together with the relatively slight change in ^3H : ^{14}C ratio on conversion of the mixture of methyl [(17D)-17-$^3H_1$]stearate and methyl [U-^{14}C]stearate into methyl 17-L-hydroxystearate indicates that ω-1-hydroxylation of stearic acid takes place stereospecifically with retention of configuration.

Although complete removal or retention of tritium on ω-1-hydroxylation of the tritiated methyl stearates was not observed, the extent of the difference between the experimental results and those expected for a fully stereospecific process is in accord with the amounts of racemisation expected in the reactions used for the preparation of the labelled stearates. The 49—51% decrease in the ^3H : ^{14}C ratio on conversion of the mixture of methyl [(17DL)-17-$^3H_1$]stearate and methyl [U-^{14}C]stearate into methyl 17-hydroxystearate confirms that ω-1-hydroxylation is fully stereospecific.

The diminution in the ^3H : ^{14}C ratio which accompanied the formation of methyl 17-L-hydroxystearate from methyl [(17L)-17-$^3H_1$]stearate and methyl [(17DL)-17-$^3H_1$]stearate is presumably due partly to displacement of the tritium atom by a hydroxy-group and partly to a primary isotope effect.[11] Because of this effect, the rate of ω-1-hydroxylation of [(17L)-17-$^3H_1$]stearic acid would be expected to be lower than that of unlabelled stearic acid or [U-^{14}C]stearic acid. Consequently, the stearic acid which undergoes ω-1-hydroxylation would contain a lower proportion of tritiated molecules than was present in the methyl stearate added to the incubation medium. Conversely, there will be a relative enrichment in tritiated molecules in the stearic acid which is utilised in reactions not involving the tritiated position. One such reaction is ω-hydroxylation. Thus, the kinetic isotope effect will explain the high ^3H : ^{14}C ratios observed in the dimethyl octadecane-1,18-dioates derived from methyl [(17L)-17-$^3H_1$]stearate and [(17DL)-17-$^3H_1$]stearate. The relatively small enrichment in tritium which occurred on ω-hydroxylation of methyl [(17D)-17-$^3H_1$]stearate may have been due to the small amount of methyl [(17L)-17-$^3H_1$]stearate which was present in this material.

Uncertainty of the precise mechanism by which the ^3H : ^{14}C ratio is diminished during ω-1-hydroxylation of [(17L)-17-$^3H_1$]stearic acid does not invalidate the stereochemical conclusions presented earlier, because a kinetic isotope of the type described above can only occur if the tritium atom occupies a position normally occupied by hydrogen atom which is involved in the rate-determining step of the hydroxylation.

The results in Table 2 show that a small proportion of the radioactivity added to *T. gropengiesseri* as labelled methyl stearates appeared in methyl 17-L-hydroxyoleate and methyl 18-hydroxyoleate. The ^3H : ^{14}C ratios of the hydroxyoleates closely parallel those of the corresponding hydroxystearates (Table 1), although the results obtained for the two series of compounds differ sufficiently to rule out the possibility that the hydroxyoleates were formed by desaturation of the hydroxystearates. The labelled hydroxyoleates were presumably formed by ω- and ω-1-hydroxylations of oleic acids which were derived by desaturation of the labelled stearic acids.

Alkane Hydroxylation.—In addition to effecting hydroxylation of fatty acids, *T. gropengiesseri* converts straight-chain alkanes and branched-chain alkanes into alkan-1-ols which are subsequently converted into hydroxy-acids [4] or alkanediols.[12] In recent review articles,[7,13] it has been suggested that terminal oxidation of alkanes by micro-organisms probably involves a direct hydroxylation. However, Azoulay, Chouteau, and Davidovics [14] have claimed that heptane oxidation by a strain of *Pseudomonas aeruginosa* involves the intermediate formation of hept-1-ene, and recently Wagner, Zahn, and Bühring [15] have claimed that the isolation of small amounts of hexadec-1-ene from cultures of a variety of micro-organisms grown on hexadecane is evidence for the involvement of hexadec-1-ene in the microbiological oxidation of hexadecane.

Alk-1-ene oxidation by *T. gropengiesseri* [4] gave products which differed from those obtained from the corresponding alkanes. This suggested that alk-1-enes are not intermediates in alkane oxidation by *T. gropengiesseri* but did not rule out the possibility of an enzyme-bound alk-1-ene intermediate. To provide further evidence on this point, [(17DL)-17-$^3H_1$]stearic acid was converted by the Hunsdiecker reaction into 1-bromo[(16DL)-16-$^3H_1$]heptadecane which was mixed with 1-bromo[U-^{14}C]heptadecane and fermented by *T. gropengiesseri*, to give dimethyl heptadecane-1,17-dioate. Previous work [16] has established that αω-dicarboxylic acids are formed from 1-bromoalkanes by the reaction sequence 1-bromoalkane ⟶ ω-bromoalkanol ⟶ ω-bromoalkanoic acid ⟶ ω-bromo-ω-hydroxyalkanoic acid ⟶ ω-formylalkanoic acid ⟶ αω-alkanedioic acid. Table 3 shows that no change in ^3H : ^{14}C ratio occurred during the transformation of the labelled bromoheptadecane to heptadecanedioic acid. This result rules out the possibility that an ω-bromoalk-1-ene is an intermediate in the formation of an

[11] E. A. Evans, ' Tritium and Its Compounds,' Butterworths, London, 1966, p. 311.
[12] D. F. Jones, *J. Chem. Soc.* (*C*), 1968, 2809.
[13] A. C. van der Linden and G. J. E. Thijsse, *Adv. Enzymol.*, 1965, **27**, 469.
[14] E. Azoulay, J. Chouteau, and G. Davidovics, *Biochim. Biophys. Acta*, 1963, **77**, 554.
[15] F. Wagner, W. Zahn, and U. Büring, *Angew. Chem. Internat Edn.*, 1967, **6**, 359.
[16] D. F. Jones and R. Howe, *J. Chem. Soc.* (*C*), 1968, 2816.

TABLE 3

^3H : ^{14}C ratio of dimethyl heptadecane-1,17-dioate obtained from fermentation products of 1-bromo[U-^{14}C-(16DL)-16-$^3H_1$]heptadecane

Compound	^3H	^{14}C	^3H : ^{14}C	^3H : ^{14}C *
1-Bromo[U-^{14}C-(16DL)-16-$^3H_1$]heptadecane				
experiment 1	14,940	1710	8·6	1·0
experiment 2	14,670	1680	8·73	1·0
Dimethyl heptadecane-1,17-dioate				
experiment 1	9166	1062	8·6	1·0
experiment 2	16,670	1955	8·5	0·97

* For ease of comparison, this column lists the ^3H : ^{14}C ratios relative to an assigned value of unity for the substrate.

ω-bromoalkanol provided that dehydrogenation is not a non-stereospecific reaction which discriminates between hydrogen and tritium in such a way that only hydrogen atoms are eliminated. 1-Bromoalkanes and alkanes are metabolised by *T. gropengiesseri* by essentially the same reaction sequence,[16] so it is probable that alkane hydroxylation by the yeast does not involve an alk-1-ene intermediate but involves direct displacement of hydrogen by an oxygen species.

The biological hydroxylation of the steroid ring system requires molecular oxygen and NADPH, and involves a stereospecific displacement reaction which takes place with retention of configuration.[17] The biological hydroxylation of alkanes and fatty acids also requires molecular oxygen and reduced pyridine nucleotide,[5,18,19] and, as the present work shows, involves a direct displacement reaction which, when the site of attack is a secondary carbon atom, takes place with retention of configuration. Thus, it seems likely that a single general mechanism is involved in the enzymatic hydroxylation of aliphatic and alicyclic systems, although the enzymes which orientate the substrates for hydroxylation presumably differ with different types of substrate. Some features of the enzyme which catalyses fatty acid hydroxylation in *T. gropengiesseri* have been summarised in a previous paper.[1]

Retention of configuration, which is a feature of the enzymic hydroxylation of steroids and fatty acids, may be due to the fact that the substrates are so orientated on an enzyme that only one of the two methylene hydrogens is exposed to attack. Consequently C–H bond fission and C–O bond formation may have to take place on the same side of the carbon atom, and hence the stereochemistry of the hydroxylation does not necessarily provide a distinction between reaction mechanisms involving nucleophilic substitution, electrophilic substitution, or a radical process.

EXPERIMENTAL

Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer which was adjusted to give less than 0·01% overlap of ^3H into the ^{14}C channel and 15% overlap of ^{14}C into the ^3H channel. Butyl PBD in toluene was used as the scintillation medium. Counting efficiencies were approx. 30% for ^3H and approx. 50% for ^{14}C. Background counts were approx. 14 counts/min. in the ^3H channel and 10 in the ^{14}C channel. Optical rotations were measured on a Bendix automatic polarimeter, and n.m.r. spectra were measured on a Varian A60 spectrometer with tetramethylsilane (τ 10) as internal standard. [U-^{14}C]Stearic acid, 98% radiochemical purity, was purchased from the Radiochemical Centre, Amersham, Bucks. Tritium-labelled lithium aluminium hydride was purchased from New England Nuclear Corp., Boston, U.S.A. Methyl stearate and stearyl alcohol were prepared from stearic acid of 'specially purified' grade obtained from British Drug Houses, Poole, Dorset. Aluminium oxide (neutral, grade III) for chromatography was obtained from Woelm, Eschwege, Germany. Light petroleum refers to the fraction b.p. 60—80°.

*Preparation of Methyl Stearates Labelled Stereospecifically with Tritium.—Methyl 17-toluene-*p*-sulphonyloxystearates.* Methyl 17-L-hydroxystearate, m.p. 52—53°, $[\alpha]_D^{22}$ +3·5° (*c* 4·5 in $CCCl_3$) (Found: C, 72·6; H, 12·0. Calc. for $C_{19}H_{38}O_3$: C, 72·6; H, 12·2%), was obtained from the products of fermentation of octadecane by *T. gropengiesseri*.[4] Treatment of the hydroxy-ester (1 g.) with toluene-*p*-sulphonyl chloride (6 g.) in pyridine (70 ml.) at 0° for 7 days gave *methyl* 17-L-*toluene*-p-*sulphonyloxystearate*, m.p. 68—69°, $[\alpha]_D^{23}$ +1·3° (*c* 5·0 in $CHCl_3$) (Found: C, 66·7; H, 9·3. $C_{26}H_{44}O_5S$ requires C, 66·4; H, 9·5%). This tosylate (1 g.) in dioxan (20 ml.) and 4N-sodium hydroxide (100 ml.) was heated under reflux with stirring for 12 hr., to give a hydroxy-acid. Methylation with diazomethane and purification by column chromatography on aluminium oxide gave *methyl* 17-D-*hydroxystearate*, m.p. 56°, $[\alpha]_D^{22}$ −3·3° (*c* 4·0 in $CHCl_3$) (Found: C, 72·7; H, 12·2. $C_{19}H_{38}O_3$ requires C, 72·6; H, 12·2%). Treatment of the hydroxy-ester with toluene-*p*-sulphonyl chloride in pyridine gave *methyl* 17-D-*toluene*-p-*sulphonyloxystearate*, m.p. 69°, $[\alpha]_D^{22}$ −1·0 (*c* 3·0 in $CHCl_3$) (Found: C, 66·4; H, 9·3. $C_{26}H_{44}O_5S$ requires C, 66·4; H, 9·5%). Methyl 17-DL-hydroxystearate, m.p. 62°, $[\alpha]_D^{23}$ 0·0° (*c* 3·0 in $CHCl_3$) (Found: C, 72·8; H, 12·2%) was prepared from methyl 17-L-hydroxystearate by oxidation to give methyl 17-oxostearate [20] and reduction of the keto-ester with sodium borohydride in methanol. Treatment of the hydroxy-ester with toluene-*p*-sulphonyl chloride in pyridine gave methyl 17-DL-toleuene-*p*-sulphonyloxystearate, m.p. 71—72° (Found: C, 66·6; H, 9·2%).

The identities of the above compounds were confirmed by n.m.r. spectroscopy, the hydroxy-esters showing τ ($CDCl_3$) 6·25m, 7·92s, and 8·83d [$CH_2CH(OH)CH_3$] and 7·74t and 6·32s ($CH_2CH_2CO_2CH_3$), and the tosylates showing τ (CCl_4) 2·15—2·8m, 5·5m, and 7·58s [$CH_2CH(CH_3)OSO_2C_6H_4CH_3$]. Analysis by t.l.c. and g.l.c. showed that the purities of the compounds were in excess of 98%.

Hydrogenolyses with $LiAl^3H_4$.—Each of the methyl 17-toluene-*p*-sulphonyloxystearates (2 mg.) was heated under reflux with stirring for 18—20 hr. with tritium-labelled lithium aluminium hydride (2·1 mg.; 5 mc) in tetrahydrofuran (10 ml.; freshly distilled from lithium aluminium

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[18] K. Wakabayshi and N. Shimazono, *Biochim. Biophys. Acta*, 1963, **70**, 132.
[19] R. K. Gholson, J. N. Baptist, and M. J. Coon, *Biochemistry*, 1963, **2**, 1155.
[20] P. A. J. Gorin, J. F. T. Spencer, and A. P. Tulloch, *Canad. J. Chem.*, 1961, **39**, 846.

hydride in a darkened apparatus). The excess of hydride was decomposed with 3N-hydrochloric acid, and unlabelled stearyl alcohol (49 mg.) was added. The reaction mixture was diluted with water (5 ml.) and extracted with ether (3 × 10 ml.). The ethereal solution, washed with water (5 ml.) and dried ($MgSO_4$), was evaporated, and the residue in acetone (3 ml.) was treated dropwise with a solution of chromium trioxide (2·7 g.) in conc. sulphuric acid (2·3 ml.) and water (7 ml.) until an excess of the reagent was present. The reaction mixture was diluted with water (5 ml.) and extracted with ether (3 × 10 ml.). The ethereal solution was washed with water (3 ml.) and extracted with 3N-sodium hydroxide (2 × 3 ml.). The combined alkali extracts were washed with ether, acidified with 3N-hydrochloric acid, and extracted with ether (3 × 10 ml.). The ethereal solution, washed with water (2 × 3 ml.) and dried ($MgSO_4$), was concentrated to 3 ml. and treated with ethereal diazomethane. The excess of diazomethane and ether were evaporated, and the residue in light petroleum was chromatographed on a column (5 × 1 cm.) of aluminium oxide in light petroleum. Elution of the column with light petroleum (20 ml.) gave methyl stearate which was crystallised twice from aqueous methanol at 0°.

By this procedure, methyl 17-L-toluene-*p*-sulphonyloxystearate was converted into methyl [(17D)-17-$^3H_1$]stearate, m.p. 39°, sp. act. 0·19 μc/mg., methyl 17-D-toluene-*p*-sulphonyloxystearate was converted into methyl [(17-L)-17-$^3H_1$]stearate, m.p. 39°, sp. act. 0·27 μc/mg., and methyl 17-DL-toluene-*p*-sulphonyloxystearate was converted into methyl [(17DL)-17-$^3H_1$]stearate, m.p. 39°, sp. act. 0·3 μc/mg. Analysis by g.l.c. showed that the chemical purity of the labelled stearates was in excess of 98%. The radiopurity of the methyl stearates was determined by the two g.l.c. procedures described by Schroepfer and Bloch,[8] and by t.l.c. on silica gel–anthracene in hexane containing ether (25%). Radioactive components on the thin-layer chromatograms were detected by placing the chromatogram in contact with *X*-ray film (Kodak, Royal Blue) in an aluminium box at −70° for 40 hr. The chromatograms were also assayed by stripping off segments and counting the adsorbent in a liquid scintillation counter. By these three procedures the radiopurity of the tritiated methyl stearates was found to be in excess of 95%.

Preparation of 1-*Bromo*[(16DL)-16-$^3H_1$]*heptadecane and* 1-*Bromo*[U-^{14}C]*heptadecane*.—Methyl [(17DL)-17-$^3H_1$]stearate (7 mg., 2·1 μc) was mixed with unlabelled methyl stearate, and the mixture saponified. The resulting stearic acid was converted into 1-bromoheptadecane (60% yield) by the Hunsdiecker reaction using the reaction conditions employed by Lüttringhaus and Schade [21] for the conversion of lauric acid into 1-bromoundecane. Distillation of the crude bromoheptadecane gave 1-bromo-[(16DL)-16-$^3H_1$]heptadecane, b.p. 144°/0·3 mm. (Found: C, 63·9; H, 10·7. Calc. for $C_{17}H_{35}Br$: C, 63·95; H, 11·0%), τ ($CDCl_3$) 6·6t (CH_2CH_2Br) and 9·1m (CH_2CH_3).

In a similar manner, [U-^{14}C]stearic acid was converted into 1-bromo[U-^{14}C]heptadecane, b.p. 157°/0·8 mm. (Found: C, 63·8; H, 10·7%), τ ($CDCl_3$) 6·6t and 9·1m.

Analysis by t.l.c. [silica gel G, hexane–ether (5 : 1)] and g.l.c. [silicone rubber gum (S.E. 30) and 6% diethylene glycol succinate polyester columns] established that the chemical and radiochemical purities of the labelled 1-bromoheptadecanes were in excess of 96%.

Fermentations of Labelled Methyl Stearates.—Portions (approx. 2 μc) of the tritium-labelled methyl stearates were mixed with small amounts of methyl [U-^{14}C]stearate (prepared from [U-^{14}C]stearic acid and diazomethane), and the mixtures were made up to 0·45 g. with unlabelled methyl stearate. Each of the stearate mixtures was emulsified in water containing Tween 80 and Span 80, and added to a 500 ml. Erlenmeyer flask containing a 24 hr. old culture of *T. gropengiesseri*. The culture medium, conditions of incubation, and procecure used to obtain the lipid constituents of the fermentation products were the same as those previously reported.[4] Two fermentations were carried out with each of the tritiated methyl stearates.

Separation of Saturated and Unsaturated Hydroxy-esters.—Each of the lipid mixtures (approx. 0·25 g.), obtained from the fermentation products of the labelled methyl stearates, was heated under reflux in methanol (50 ml.) containing mercuric acetate (3 g.) for 2 hr. The residue obtained on evaporation of the solvent was chromatographed on a column (25 × 2 cm.) of silica gel M.F.C. (Hopkin and Williams, Chadwell Heath, Essex) in light petroleum. Elution of the column with ether (approx. 250 ml.) removed the saturated lipids, and elution with methanol containing acetic acid (2%) (200 ml.) removed the mercuric acetate complexes of the unsaturated lipids. These complexes were decomposed by the addition of water (5 ml.) and conc. hydrochloric acid (15 ml.), and the unsaturated lipids were isolated in ether, and treated with diazomethane.

Isolation of Methyl 17-*Hydroxystearate*.—The saturated lipids obtained above were chromatographed on a column (5 × 1 cm.) of aluminium oxide in light petroleum. The column was eluted with increasing proportions of acetone (0·1—2·0%) in light petroleum, and fractions (10 ml.) were collected and examined by g.l.c. Fractions containing virtually pure methyl 17-hydroxystearate (approx. 150 mg.) were combined and crystallised from acetone–light petroleum, and samples were assayed for radioactivity.

*Production and Purification of Dimethyl Octadecane-*1,18-*dioate*.—Late fractions from the aluminium oxide column described above contained methyl 17-hydroxystearate and small amounts of methyl 18-hydroxystearate, methyl 15-hydroxypalmitate, and methyl 16-hydroxypalmitate. These fractions (approx. 50 mg.) in acetone (3 ml.) were treated dropwise with a slight excess of a solution of chromium trioxide (2·7 g.) in conc. sulphuric acid (2·3 ml.) and water (7 ml.). The oxidation products were isolated in ether and chromatographed on a column (5 × 1 cm.) of silica gel in light petroleum. The column was eluted with light petroleum containing ether (10%), and fractions (5 ml.) were collected and examined by t.l.c. Early fractions contained methyl 17-oxostearate and methyl 15-oxopalmitate, and late fractions contained a mixture (2—4 mg.) of the monomethyl esters of hexadecane-1,16-dioic acid (2 parts) and octadecane-1,18-dioic acid (1 part). This mixture was methylated with diazomethane, and the component diesters separated by preparative g.l.c. on a 6% diethylene glycol succinate polyester column (Varian Aerograph model 204) and assayed for radioactivity.

Isolation of Methyl Oleate Derivatives.—By procedures analogous to those described above the unsaturated lipids obtained from the methyl stearate fermentations were processed, to give methyl 17-hydroxyoleates and dimethyl octadec-9-ene-1,18-dioates. The latter compounds, which were not purified by g.l.c., were contaminated by 10—20% of dimethyl hexadec-7-ene-1,16-dioates.

Characterisation of the Fermentation Products.—The

[21] A. Lüttringhaus and D. Schade, *Ber.*, 1941, **74**, 1565.

specimens of methyl 17-L-hydroxystearate obtained from methyl stearate fermentations were authenticated by m.p., elemental analysis, optical rotation, g.l.c. retention time, and n.m.r. spectroscopy. Specimens of methyl 17-L-hydroxyoleate were authenticated by g.l.c. retention time, optical rotation, and n.m.r. spectroscopy. Further characterisation of the ω-1-hydroxy-esters was obtained by oxidation to give methyl 17-oxostearate and methyl 17-oxo-oleate. These oxidations were accompanied by the loss of 95—98% of the tritium content of the hydroxy-esters. Dimethyl octadecane-1,18-dioate and dimethyl octadec-9-ene-1,18-dioate were characterised by comparison with authentic specimens on t.l.c. [silica gel, hexane–ether (2 : 1)] and on g.l.c. (silicone rubber gum and diethylene glycol succinate polyester columns).

Fermentation of Labelled 1-*Bromoheptadecane*.—1-Bromo-[(16DL)-16-$^{3}H_{1}$]heptadecane (approx. 1 μc) was mixed with a small amount of 1-bromo[U-^{14}C]heptadecane. The mixture was made up to 0·5 g. with unlabelled 1-bromoheptadecane, and fermented by *T. gropengiesseri*. The lipid constituents (approx. 120 mg.) of the fermentation product were isolated as described previously,[16] and chromatographed on a column (6 × 1 cm.) of aluminium oxide in light petroleum containing acetone (0·2%). Fractions (10 ml.) were collected and examined by g.l.c. and t.l.c. Fractions containing pure dimethyl heptadecane-1,17-dioate were combined, crystallised from methanol, and samples assayed for radioactivity. The specimens of dimethyl heptadecane-1,17-dioate, m.p. 51—52°, were characterised by mass spectrometry and n.m.r. spectroscopy.

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