APPENDIX

Fermentation of Limonene by Enterobacterium

Under exactly identical conditions as described for the fermentation of longifolene, the fermentation of limonene, $\text{C}_{10}\text{H}_{16}$, a monoterpane was carried out by the bacterium isolated from sewage water by enrichment culture techniques identified as "Enterobacterium" by collaboration with the Microbiology Section.

After fermentation for 96 hours, on the rotary shaker, the pooled broth was extracted in the same way as described under longifolene fermentation (p. 46) and the total converted fraction was separated into the neutral and acid transformation products.

The neutral fraction on subjecting to TLC, showed only a trailing and was found to consist of mainly unreacted limonene and some colouring matter. So no further work was carried out on neutral fractions.

The total acid fraction after subjecting to thin layer chromatography was found to consist of two main fractions in the proportion 50:50. The mixed acid fractions were converted to their methyl esters for further separation.
The separation of the methyl esters was brought about by means of column chromatography. The first fraction was eluted in ether : petroleum ether 5:95, showing a single spot on TLC. This was designated as ME$_1$.

The second fraction was eluted in 50:50 ether : petroleum ether showing a single spot on TLC. This fraction was designated as ME$_2$.

**Identification of ME$_2$**

The fraction eluted in 50:50 ether : petroleum ether gave the yield equal to 50 mg and showed a positive TNV test. This fraction showed analysis corresponding to molecular formula C$_{11}$H$_{16}$O$_2$, in accordance with a methyl ester of a monocarboxylic acid.

After saponification of the ester the corresponding acid A$_2$ obtained was a colourless crystalline compound having m.p. 127-128$^\circ$C and showed neutral equivalent = 168 corresponding to molecular formula C$_{10}$H$_{14}$O$_2$.

The infra-red spectrum of A$_2$ (Fig.14) showed a sharp acid band at 1700 cm$^{-1}$, an exocyclic methylene double bond at 1645 and 890 cm$^{-1}$ and a trisubstituted double bond.
FIG. 14.

INFRA RED ABSORPTION SPECTRUM OF THE ACIDIC COMPOUND $A_2$. 
at 780 cm\(^{-1}\). The shoulder at 1615 cm\(^{-1}\) is suggestive of \(\pi - \pi\) unsaturation in the molecule.

From the spectral data it was indicated that the acid may be perillic acid. A spectral comparison of perillic acid revealed that the compounds were identical. Also there was no depression in the mixed melting point. So it was confirmed that the fraction was perillic acid.

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\text{COOH}
\]

Identification of ME

This fraction eluted in 5:95 ether : petroleum ether gave yield = 50 mg showing a positive TNM test. The elemental analysis was C 71.94, H 40.60, O 18.06, corresponding to the molecular formula \(C_{11}H_{18}O_{2}\). Thus this compound was to consist of only two additional hydrogen atoms than perillic acid, methyl ester the rest molecular formula being the same. The possibility of addition of these two hydrogen atoms lies at one of these double bonds.
FIG. 15.

INFRA RED ABSORPTION SPECTRUM OF THE ACIDIC COMPOUND A₁
Thn methyl ester was saponified to the corresponding acid \( A_1 \), which showed neutral equivalent 169 in accordance with the molecular formula \( C_{10}H_{16}O_2 \) for the acid.

The infra red spectrum of \( A_1 \) showed (Fig. 15) a sharp acid band at 1700 cm\(^{-1}\), peaks at 1645 and 890 cm\(^{-1}\) indicating that the exocyclic double bond is intact, which was corroborated by a positive TN\( \rightarrow \)T test. While the peak at 780 cm\(^{-1}\), present in the perillic acid corresponding to a trisubstituted double bond had disappeared, indicating that the addition of the hydrogen atoms must have taken at that position.

From all the above data this acid may be a dihydroperillic acid having the following structure: