Pseudomonas aeruginosa produces, when grown on a glycerol or fructose medium, a unique glycolipide composed of 2 moles each of L-rhamnose and β-hydroxydecanoic acid. The formula of the isolated compound as determined by Jarvis and Johnson (3) is shown in Fig. 1. Various aspects of the conditions under which this rhamnolipide is produced have been investigated previously. Further, a synthetic medium for its production and a suitable assay have been developed (4). In addition to the interest concerning the whole molecule as an example of a simple crystalline glycolipide, both constituent moieties are of considerable significance in their own right.

Rhamnose is the most widely occurring and best known methylpentose, yet no information is available about its origin in nature. The most common L-rhamnose-containing natural products are quercitrin and naringin (5), but the sugar has also been identified in certain cardiac glycosides and saponins (6), in some plant pigments (5), in bacterial polysaccharides (7) and lipopolysaccharides (8), and in plant gums and mucilages (9). Since P. aeruginosa cultures produce relatively large quantities of rhamnolipide (4), they offered a suitable system for the study of rhamnose biosynthesis.

β-Hydroxydecanoic acid is one of a limited number of hydroxylated fatty acids in nature. However, evidence as to the essential role of β-hydroxy fatty acids in the synthesis and breakdown of fatty acids has accumulated in recent years (10) and in this connection the lipide component of the rhamnolipide assumes importance.

In the present paper, data on the incorporation of radioactivity from glycerol-α-C¹⁴, glycerol-β-C¹⁴, and acetate-1-C¹⁴ into rhamnolipide have...
been obtained. From these data, information on possible modes of biosynthesis of both moieties was derived.

EXPERIMENTAL

Production and Isolation of Samples of Rhamnolipide

*P. aeruginosa* was grown on Bacto-peptone (Difco) and synthetic media and rhamnolipide was isolated and assayed as previously described (4).

![Structure of the rhamnolipide of *P. aeruginosa* according to Jarvis and Johnson (3).](image)

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Cultures were shaken in a constant temperature water bath in the hood. Sufficient carrier rhamnolipide was added so that about 1 gm. of the pure substance could be obtained after isolation and recrystallization.

For C\(^{14}\)O\(_2\) incorporation experiments, a special 2 liter flask was designed (Fig. 2) in which 25 ml. of synthetic medium containing 1 per cent glycerol were shaken in contact with a gas phase containing 2 per cent C\(^{14}\)O\(_2\). The CO\(_2\) was liberated by rotation of the two compartment attachment and sampled through the vaccine port.

Glycerol-\(\alpha\)-C\(^{14}\) and glycerol-\(\beta\)-C\(^{14}\) were synthesized by the method of
Gidez and Karnovsky (11) and acetate-1-C\(^{14}\) by that of Sakami et al. (12). When necessary, glycerol was assayed as described by Karnovsky and Brumm (13).

A total of thirteen samples of rhamnolipide for assay of radioactivity and subsequent hydrolysis and degradation was prepared. The following media and substrates were used: Bacto-peptone medium, 3 per cent glycerol-\(\alpha\)-C\(^{14}\) (two experiments, designated Ba-\(\alpha\)); Bacto-peptone medium, 3 per cent glycerol-\(\beta\)-C\(^{14}\) (two experiments, designated Ba-\(\beta\)); synthetic medium, 1 per cent glycerol-\(\alpha\)-C\(^{14}\) (two experiments, designated Sy-\(\alpha\)); synthetic medium, 1 per cent glycerol-\(\beta\)-C\(^{14}\) (three experiments, designated Sy-\(\beta\)); synthetic medium, 1 per cent glycerol (10.83 mmoles per 100 ml.), 0.133 per cent sodium acetate-1-C\(^{14}\) (1.63 mmoles per 100 ml.)\(^2\) (two experiments, designated Sy-Ac); synthetic medium, 1 per cent glycerol, C\(^{14}\)O\(_{2}\)-containing atmosphere (two experiments, designated Sy-CO\(_{2}\)).

### Isolation of Rhamnose and \(\beta\)-Hydroxydecanoic Acid

For the isolation of the constituent moieties the rhamnolipide was hydrolyzed (3) and the hydrolysate was extracted with ether. The aqueous phase was neutralized, taken to dryness under a vacuum, and extracted several times with ethanol. The ethanol extract was decolorized with charcoal and evaporated under nitrogen to yield rhamnose. The ether fraction was taken to dryness under nitrogen and the remaining \(\beta\)-hydroxydecanoic acid was purified by converting it to the sodium salt which was washed with ether. This salt was then converted back to the free fatty acid, the acid was extracted into ether, and the ethereal solution was washed. When the ether was evaporated, the fatty acid was recovered. Its degree of unsaturation was low (iodine numbers by the method of Yasuda (14) ranged from 3 to 6), indicating that virtually no dehydration had occurred.

### Degradation of Rhamnose

In order to locate C\(^{14}\) in the individual carbon atoms of rhamnose, three series of reactions were performed.

#### Isolation of C-1+2+8, C-4, and C-5+6

Rhamnose phenylosazone, prepared by the method of Garard and Sherman (15) for glucose phenylosazone, was oxidized with periodic acid

1 The letter symbols will be used throughout this paper to designate the experiments from which the results under "Results and discussion" were obtained; i.e., Ba refers to Bacto-peptone and Sy to synthetic media and \(\alpha\) or \(\beta\) indicates the position of the label in the glycerol molecule when glycerol-C\(^{14}\) was used. Ac and CO\(_{2}\) refer to experiments in which the radioactivity was present as acetate or carbon dioxide.

2 This ratio of acetate carbon to glycerol carbon had previously been shown to be optimal for rhamnolipide production (4).
and the mesoxaldehyde-1,2-bisphenylhydrazone (C-1+2+3) was collected (16). Acetaldehyde (C-5+6) was aerated into a trap and precipitated as the dimedon complex (17). The formic acid formed (C-4) was oxidized to CO₂ (18) which was trapped as barium carbonate.

**Isolation of C-1, C-5, and C-6**

Rhamnose was oxidized to barium rhamnonate (19) which was converted to the potassium salt by agitating an aqueous suspension with a slight excess of potassium sulfate and removing the resulting barium sulfate by centrifugation. The potassium rhamnonate was oxidized with periodate, essentially as described by Bernstein (20) for potassium ribonate, except that the oxidation was carried out at 0°. Acetaldehyde (C-5+6) and CO₂ (C-1) were simultaneously aerated from the reaction mixture and collected, respectively, in a 2 per cent bisulfite solution and a BaOH-BaCl₂ trap. A flask containing KMnO₄ solution was interposed between the two traps in order to prevent SO₂ from being carried into the CO₂ trap. From the latter, BaCO₃ (C-1) was isolated by centrifugation, washed, and plated. The aeration of acetaldehyde was completed at 100° after removal of the CO₂ trap and neutralization of excess periodate. An aliquot of the acetaldehyde-bisulfite complex was assayed by titration with iodine after destruction of excess bisulfite with iodine and decomposition of the complex with sodium bicarbonate. The remainder of the complex was decomposed with dipotassium hydrogen phosphate and the acetaldehyde was distilled. The iodoform reaction (21) yielded iodoform (C-6) and formic acid (C-5) which was steam-distilled, oxidized to CO₂ (18), and isolated as BaCO₃.

In a control experiment, nitrogen was swept through a solution of glucose-1-C¹⁴ into a solution of formic acid while the sugar was undergoing periodate oxidation.³ No radioactivity, representing formic acid (C-1 of glucose), appeared in the trap. Thus, under the conditions described above for rhammonic acid, the acetaldehyde removed in a stream of nitrogen would not be contaminated with carbons 2, 3, or 4 which appear as formic acid.

**Isolation of C-1+2, C-3+4, and C-5+6**

Another aliquot of rhamnose was converted to the dichlorophenylhydrazone (22) and the derivative was oxidized as described by Topper and Hastings for glucose phenylosazone (16), except that the reaction period was reduced to 5 minutes. The precipitated glyoxal dichlorophenylhydrazone (C-1+2) was centrifuged and recrystallized from 40 per cent ethanol. A similar reaction carried out on glucose has recently been

³ Glucose-1-C¹⁴ and -2-C¹⁴ were obtained from Dr. H. S. Isbell of the National Bureau of Standards, Washington, D. C.
reported (23). Control experiments with glucose-1-C\textsuperscript{14} and -2-C\textsuperscript{14} gave glyoxal dichlorophenylhydrazone with a specific activity greater than 95 per cent of that of the starting material.\textsuperscript{3}

The formic acid in the supernatant solution (C-3+4) was treated as described above. Acetaldehyde (C-5+6) may also be obtained and treated as described above.

Degradation of \(\beta\)-Hydroxydecanoic Acid

Carbons 1+2 of the \(\beta\)-hydroxydecanoic acid were obtained as CO\textsubscript{2} by the method of Bergström et al. (24) adapted for the collection of CO\textsubscript{2} in the Van Slyke apparatus (25).

Determination of Radioactivity

Conversion of bacteria, rhamnolipide, and fatty acids to CO\textsubscript{2} for counting was by the Van Slyke-Folch wet combustion technique (26) and C\textsuperscript{14}O\textsubscript{2} was collected as described by Van Slyke et al. (25). Media were counted directly after evaporation. Dimedon complexes, iodoform, osazones, and hydrazones were counted as such (27). All counting was done on stainless steel planchets with a plating area of 1.60 sq. cm. in a proportional gas flow counter (28).

RESULTS AND DISCUSSION

Comparatively little is known about the metabolism of \(P.\ aeruginosa\). Of late, however, interest has been directed toward this and related organisms, and present knowledge has recently been reviewed (29). Most of the literature is, however, concerned with the breakdown of glucose, and no information is as yet available about the pathways of glycerol or acetate in this organism. This would, of course, be the information needed to formulate a metabolic scheme to account for the formation of rhamnolipide from these substrates.

While details about the stepwise transformation of glycerol and acetate by \(P.\ aeruginosa\) are lacking, an over-all hypothesis might be advanced to account for the production of the two components of rhamnolipide. The results detailed in this paper, particularly those obtained on synthetic media, for which the carbon sources were clearly defined, are thus compared with the hypothesis which is outlined below.

Hypothesis for Origin of Carbon of Rhamnolipide Moieties

Rhamnose can be imagined to have been formed from two 3-carbon units arising from glycerol, while each fatty acid might have been synthesized from five 2-carbon units, each being formed from the 3-carbon chain of glycerol by the loss of 1 carbon atom. A total of 14 glycerol
molecules is postulated to have contributed the carbon of 1 rhamnolipide molecule, 4 molecules of glycerol yielding the rhamnose and 10 forming the fatty acid moieties.

Such a hypothesis, represented diagrammatically in Fig. 3, allows a postulation of the following: (1) the relationship between the specific activity of substrate carbon and product carbon in experiments with synthetic media, (2) the proportions of the activity of rhamnolipide to be found in rhamnose carbon or in fatty acid carbon, (3) the disposition of activity within each moiety.

![Diagram](http://www.jbc.org/)

**Fig. 3.** Schematic representation of the hypothesis for rhamnolipide (RL) formation. ○, C\(^{14}\) atoms. The following relationships can be derived: (1) average specific activity of glycerol-\(\alpha\)-C\(^{14}\) carbon = \(\sigma_1/3\); for rhamnolipide components formed from glycerol-\(\alpha\)-C\(^{14}\), average specific activity of rhamnose carbon = \(\sigma_1/3\), average specific activity of \(\beta\)-hydroxydecanoic acid carbon = \(\sigma_1/4\); thus the average specific activity of rhamnose carbon is 100 per cent and that of the \(\beta\) hydroxydecanoic acid 75 per cent of the average specific activity of glycerol-\(\alpha\)-C\(^{14}\) carbon. (2) Average specific activity of glycerol-\(\beta\)-C\(^{14}\) carbon = \(\sigma_1/3\); for rhamnolipide components formed from glycerol-\(\beta\)-C\(^{14}\), average specific activity of rhamnose carbon = \(\sigma_1/3\), average specific activity of \(\beta\)-hydroxydecanoic acid carbon = \(\sigma_1/2\); thus the average specific activity of rhamnose carbon is 100 per cent and that of \(\beta\)-hydroxydecanoic acid 150 per cent of the average specific activity of glycerol-\(\beta\)-C\(^{14}\) carbon.

Thus (1) the rhamnose and fatty acid would have a carbon specific activity of 100 and 75 per cent, respectively, of that of the substrate in rhamnolipide derived from glycerol-\(\alpha\)-C\(^{14}\) and of 100 and 150 per cent, respectively, when glycerol-\(\beta\)-C\(^{14}\) was used (Fig. 3). If, now, each moiety is properly weighted for the number of carbons it contains, rhamnolipide from \(\alpha\)-labeled glycerol could be calculated to have a carbon specific activity of \(((100 \times 12) + (75 \times 20))/32 = 84.4\) per cent of that of the substrate glycerol carbon. The analogous figure in the case of \(\beta\)-labeled glycerol would be 131.2 per cent.

(2) The fraction of the activity of the whole rhamnolipide contained in the sugar moieties would, when \(\alpha\)-labeled glycerol was used, be \(((100 \times 12) \times 100)/((100 \times 12) + (75 \times 20)) = 44.5\) per cent. The 2 moles
of \( \beta \)-hydroxydecanoic acid would be expected to contain 55.5 per cent of the activity of rhamnolipide.

In experiments with \( \beta \) labeled glycerol the sugar moieties of rhamnolipide would analogously carry 28.6 per cent, and the fatty acids 71.4 per cent of the activity of the whole rhamnolipide molecule.

(3) Experiments with glycerol-\( \alpha \)-C\(^{14} \) would yield rhamnose with 25 per cent of its specific activity in each of carbon atoms 1, 3, 4, and 6. In the case of experiments with glycerol-\( \beta \)-C\(^{14} \), 50 per cent of the activity of the rhamnose might be expected in each of carbon atoms 2 and 5. In the \( \beta \)-hydroxydecanoic acid tracer might be found in carbon atoms 2, 4, 6, 8, and 10 to an equal extent after growth on glycerol-\( \alpha \)-C\(^{14} \). Similarly, carbon atoms 1, 3, 5, 7, and 9 would be labeled equally in experiments with glycerol-\( \beta \)-C\(^{14} \). This follows from the fact that the labeled 2-carbon units formed from glycerol-\( \alpha \)-C\(^{14} \) would be methyl-labeled, while those from glycerol-\( \beta \)-C\(^{14} \) would be carboxyl-labeled. The terminal 2-carbon fragment would, in either case, contain 20 per cent of the activity of the whole fatty acid.

The figures in columns labeled “Hypothetical” in Tables I to IV are derived as described above, and, as may be seen, the agreement between these figures and those found is good.

**Relationship of Rhamnolipide Carbon to Substrate Glycerol Carbon**

On Bacto-peptone media about 75 per cent of rhamnolipide carbon appeared to have come from glycerol when glycerol-\( \alpha \)-C\(^{14} \) was the substrate, while about 55 per cent was contributed by glycerol in the experiments with the \( \beta \)-C\(^{14} \) compound (Table I). These calculations are based on the sum of the activities of two rhamnose and of two fatty acid fragments. In early experiments rhamnolipide was contaminated by small amounts of bacterial protein, much more active than rhamnolipide, since it had not been diluted by carrier. This contaminant was not present in the isolated rhamnose and fatty acid, nor in rhamnolipide derived from synthetic media.

In experiments with either glycerol-\( \alpha \)-C\(^{14} \) or \( \beta \)-C\(^{14} \) on synthetic media about 90 per cent of rhamnolipide carbon appeared to have been derived from glycerol. The 10 per cent short fall is believed to have been due to experimental errors. Recovery of rhamnolipide activity in its component moieties was good (99 ± 2 per cent).

**Comparison of Specific Activities of Rhamnose and \( \beta \)-Hydroxydecanoic Acid with That of Rhamnolipide**

Examination of the isolated sugar and lipide moieties revealed that they contained 46.2 and 53.8 per cent of the rhamnolipide activity, respectively, in experiments with glycerol-\( \alpha \)-C\(^{14} \) on Bacto-peptone media and 45.5 and
54.9 per cent, respectively, in similar experiments on synthetic media (Table II). When glycerol-β-C\textsuperscript{14} was used, the average percentages were 32.5 and 67.5 for Bacto-peptone and 31.1 and 65.8 for synthetic media. Thus there was no essential difference in the disposition of radioactivity

<table>
<thead>
<tr>
<th></th>
<th>Rhamnolipide carbon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Found (a)</td>
</tr>
<tr>
<td></td>
<td>c.p.m. per mmole</td>
</tr>
<tr>
<td>Ba-α*</td>
<td>6,450 ± 390</td>
</tr>
<tr>
<td>Ba-β</td>
<td>7,170 ± 400</td>
</tr>
<tr>
<td>Sy-α</td>
<td>7,420 ± 190</td>
</tr>
<tr>
<td>Sy-β</td>
<td>12,040 ± 240</td>
</tr>
</tbody>
</table>

The mean values are given, with the average deviation from the mean. The specific activity of substrate glycerol carbon was normalized to 10,000 c.p.m. per mmole.

* For explanation of the symbols see footnote 1.

<table>
<thead>
<tr>
<th>α*†</th>
<th>β*†</th>
<th>Sy-Ac*§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypothetical</td>
<td>Found</td>
<td>Hypothetical</td>
</tr>
<tr>
<td>Sugar moiety§</td>
<td>45.8 ± 1.6</td>
<td>28.6</td>
</tr>
<tr>
<td>Lipide</td>
<td>54.5 ± 1.4</td>
<td>71.4</td>
</tr>
</tbody>
</table>

The values are given as percentages of radioactivity in the rhamnolipide molecule, with the average deviation from the mean.

* For explanation of the symbols see footnote 1.
† The results obtained on Bacto-peptone and synthetic media were combined.
‡ No hypothetical figures could be derived for these experiments.
§ The sugar moiety consists of 2 moles of rhamnose; the lipide moiety of 2 moles of β-hydroxydecanoic acid.

in the moieties of the rhamnolipide molecule whether it had been formed on Bacto-peptone or synthetic medium. Depending, however, on the position of the label in the substrate glycerol, marked differences in the disposition of rhamnolipide activity in its components were found.

*Patterns of Isotope Distribution in Rhamnose*

Degradation of the isolated rhamnose samples by a combination of some or all of the degradation methods described above yielded the results
summarized in Table III. Despite occasional difficulties experienced in obtaining acceptable replication of results because of low activity, the data are considered to be accurate within 10 per cent. No radioactivity or very small amounts of radioactivity were found in rhamnose carbon 5 in experiments with glycerol-α-C¹⁴ and in rhamnose carbons 1, 3+4, 4, and 6 in experiments with glycerol-β-C¹⁴.

In the Bacto-peptone experiments only the periodate cleavage of rhamnose phenylosazone and the iodoform reaction on acetaldehyde were carried out to obtain the general distribution of radioactivity within the molecule. In these experiments it appears that there may be a significantly greater amount of activity in the top half of the molecule than in the bottom half.

When glycerol constituted the sole carbon source, i.e. in synthetic media, it became profitable to carry the degradation of the rhamnose molecule sufficiently far to permit the calculation of the relationship of each individual carbon atom to the entire molecule (Table IV).

The following points might be made with respect to the data in Table IV: (1) The values for C-1, C-4, and C-6 were individually determined in all cases. (2) In the experiments designated Sy-α, the figure given for C-5 was obtained by subtraction of the value for C-6 from that for C-5+6; in the experiments designated Sy-β, the results for both C-5 and C-6 were individually obtained. (3) The difference between the values for C-1+2 and C-1 was taken as that for C-2. (4) The value for C-3 was calculated both as the difference between the values for C-3+4 and C-4 and as the difference between those for C-1+2+3 and C-1+2. The result given is

| Rhamnose carbons | Glycerol-α-C¹⁴ | | | Glycerol-β-C¹⁴ |
|------------------|----------------|----------------|----------------|
|                  | Hypothetical   | Ba-α*          | Sy-α*          | Hypothetical   | Ba-β*          | Sy-β*          |
| C-1              | 25             | 25 ± 4         | 0              | 4 ± 1          |
| C-1+2            | 25             | 29 ± 2         | 50             | 51 ± 2         |
| C-1+2+3          | 50             | 56 ± 1         | 50 ± 1         | 57 ± 1         | 50 ± 1         |
| C-3+4            | 50             | 52 ± 3         | 0              | 8 ± 2          |
| C-4              | 25             | 26 ± 1         | 24 ± 1         | 4 ± 3          | 2 ± 1          |
| C-5+6            | 25             | 25 ± 1         | 50             |               |
| C-5              | 0              | 0†             | 50             | 43†            | 48 ± 1         |
| C-6              | 25             | 21†            | 21 ± 1         | 0              | 0†             |

The values are given as percentages of radioactivity in the rhamnose molecule, with the average deviation from the mean.

* For explanation of the symbols see footnote 1.
† Single experiment only.
the average of all calculations. Despite small variations from one experi-
ment to the next it is clear that the $\alpha$-carbons of glycerol primarily furnished
carbons 1, 3, 4, and 6 of rhamnose, while $\beta$-carbons were responsible for
carbons 2 and 5.

This pattern of labeling of rhamnose points clearly toward the suggested
combination of two 3-carbon units directly derived from the glycerol
carbon skeleton. Virtually no randomization of the label has occurred in
the synthesis of rhamnose by \textit{P. aeruginosa}. By analogy with the origin
of glucose carbon an aldol condensation of \textit{L}-lactaldehyde and dihydroxy-
acetone phosphate might be postulated. Hough and Jones (30) found
that the condensation of these two compounds in the presence of pea
aldolase \textit{in vitro} yielded 6-deoxy-\textit{L}-sorbose-1-phosphate and 6-deoxy-\textit{D}-fruc-
tose-1-phosphate. Both these sugars have the \textit{D}-threo configuration at
carbons 3 and 4 (\textit{i.e.} carbon 3 is \textit{L} and carbon 4 is \textit{D}), whereas \textit{L}-rhamnose
has the \textit{L}-threo configuration.

However, this does not preclude the existence of an enzyme in \textit{P. aeru-
ginosa} which could effect the condensation of fragments similar to those
above to yield \textit{L}-rhamnulose-1-phosphate which, after dephosphorylation,
could isomerize to the aldose form. \textit{L}-Rhamnulose and its 1-phosphate
ester have recently been found to be implicated in the metabolism of
\textit{L}-rhamnose by \textit{Escherichia coli} (31) and by a mutant of \textit{Pasteurella pestis}
(32).

\begin{table}[ht]
\centering
\caption{Disposition of Radioactivity in Individual Carbon Atoms of Rhamnose}
\begin{tabular}{l|c|c|c|c}
\hline
Rhamnose carbon No. & \multicolumn{2}{c|}{Sy-$\alpha^*$} & \multicolumn{2}{c}{Sy-$\beta^*$} \\
\hline
 & Hypothetical & Found & Hypothetical & Found \\
\hline
CHO 1 & 25 & 25 $\pm$ 4 & 0 & 4 $\pm$ 1 \\
HCOH 2 & 0 & 4 $\pm$ 2 & 50 & 47 $\pm$ 1 \\
HCOH 3 & 25 & 25 $\pm$ 4 & 0 & 4 $\pm$ 2 \\
HOCH 4 & 25 & 24 $\pm$ 1 & 0 & 2 $\pm$ 1 \\
HOCH 5 & 0 & 4 $\pm$ 1 & 50 & 48 $\pm$ 1 \\
CH$_3$ 6 & 25 & 21 $\pm$ 1 & 0 & 0 \\
\hline
Carbons 1–6\ldots & 100 & 103 $\pm$ 2 & 100 & 105 $\pm$ 1 \\
\hline
\end{tabular}
\end{table}

The values are given as percentages of radioactivity in the rhamnose molecule,
with the average deviation from the mean.

* For explanation of the symbols see footnote 1.
Incorporation of Radioactive Acetate Carbon into Rhamnolipide

Since acetate is an excellent source of the 2-carbon units needed for fatty acid synthesis in other systems, it was of interest to study its incorporation into the lipide moiety of rhamnolipide as well as to determine whether rhamnose carbon can be furnished by acetate. The sodium acetate-1-C\textsuperscript{14} used has a specific activity of 133,700 c.p.m. per mmole and the specific activity of the total carbon in the medium containing an optimal mixture of glycerol and sodium acetate (see above) was 6080 c.p.m. per mmole. Rhamnolipide with a specific activity of 226,900 c.p.m. per mmole was formed, but it is clear that no acetate carbon enters the sugar moiety of rhamnolipide and that all of the activity that is incorporated can be recovered in the 2 fatty acid molecules (Table II).

### Table V

<table>
<thead>
<tr>
<th>Fraction of Radioactivity of (\beta)-Hydroxydecanoic Acid Found in Terminal (\beta)-Carbon Unit (Carbons 1 and 2)</th>
<th>(\alpha^\dagger)</th>
<th>(\beta^\dagger)</th>
<th>Sy-Ac*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Found (a)</td>
<td>17.3 ± 1.1</td>
<td>23.0 ± 0.5</td>
<td>23.2 ± 0.8</td>
</tr>
<tr>
<td>Hypothetical (b)</td>
<td>20.0</td>
<td>20.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Ratio, (a)/(b)</td>
<td>87 ± 4</td>
<td>115 ± 2</td>
<td>116 ± 4</td>
</tr>
</tbody>
</table>

The values are given as percentages, with the average deviation from the mean.

* For explanation of the symbols see footnote 1.

† The results obtained on Bacto-peptone and synthetic media were combined.

Isotope Distribution Patterns of \(\beta\)-Hydroxydecanoic Acid

In order to establish whether the fatty acids which form the lipide portion of rhamnolipide are synthesized by *P. aeruginosa* in accordance with classical pathways, a partial stepwise degradation of the carbon chain would be desirable. In the present work, the activity of the 2 terminal carbon atoms only was measured. This was felt to be a sufficient indication of the probable distribution of radioactivity in the fatty acid, especially in view of the data on the incorporation of glycerol carbon into the lipide moiety (Table II). The preferential incorporation of the \(\beta\)-carbon of glycerol into rhamnolipide over the \(\alpha\)-carbon (Table II) pointed toward the role of 2-carbon units.

According to Bergström et al. (24) oxidation of \(\beta\)-hydroxydecanoic acid with \(CrO_3\) in acetic acid will convert C-1 and C-2 of the fatty acid directly to CO\textsubscript{2}. CO\textsubscript{2} was easily obtained, but it is evident that fatty acids from experiments with glycerol-\(\alpha\)-C\textsuperscript{14} gave values 10 to 20 per cent lower than anticipated, while those from glycerol-\(\beta\)-C\textsuperscript{14} experiments are higher to a similar degree (Table V). The most plausible explanation of these findings
RHAMNOSE AND RHAMNOLIPIDE BIOSYNTHESIS

is that C-1 and C-2 yield CO₂ to an unequal extent, the conversion of C-2 to CO₂ being only 70 to 80 per cent as efficient as that of C-1. While what evidence is available strongly suggests that such is the case, the possibility is not precluded that relatively small amounts of activity may be present in the carboxyl or the α-carbon of the fatty acid in experiments with glycerol-α-C¹⁴ or -β-C¹⁴, respectively. Chromic acid oxidation of the fatty acids from experiments designated Sy-Ac (in which labeled acetate and unlabeled glycerol were used) yielded results which exhibit the same phenomenon as those from experiments with glycerol-β-C¹⁴ (Table V). They are higher than the hypothetical value by about the same amount, presumably, again, because of unequal oxidation of C-1 and C-2 of the fatty acid to CO₂.

If one assumes that each glycerol and each acetate molecule in the medium could furnish one activated 2-carbon unit and that these units are incorporated into the fatty acids in the same ratio in which the substrates are present in the medium (6.64:1; see above), the contribution of acetate carbon to the fatty acid can be calculated to amount to 13.1 per cent. Since the specific activity of the acetate-1-C¹⁴ was 66,800 c.p.m. per mmole of carbon and that of the β-hydroxydecanoic acid formed was 11,240 ± 260 c.p.m. per mmole of carbon, acetate actually contributed 16.8 ± 0.4 per cent of the carbon of the fatty acid. Thus, while glycerol is successful in competing with acetate to supply 2-carbon units, it contributes 25 to 30 per cent less than its share.

From the radioactivity content of the terminal 2-carbons of the fatty acid in experiments with glycerol C¹⁴ and with acetate-C¹⁴ as well as from the fact that acetate is incorporated readily into the lipide moiety of rhamnolipide, it is tentatively concluded that the fatty acid is synthesized from 2-carbon units. However, the β-hydroxydecanoic acid has been shown to possess the D(-) configuration (33), while β-ketoacyl-coenzyme A reductase (β-hydroxyacetyl-coenzyme A dehydrogenase) has been shown to implicate specifically the L(+) compound (34). It is interesting to note that the dehydrating enzyme (α,β-unsaturated acyl-coenzyme A hydrase), described in the literature (35), is also specific for the L(+) series of β-hydroxy fatty acids. The possibility exists that in the synthesis of the fatty acid of rhamnolipide the keto group of β-ketodecanoic acid is hydrogenated subsequent to the loss of the activating coenzyme A group. By analogy with β-hydroxybutyric acid (36), hydrogenation of the β-keto group could thus yield the D(-) form of the β-hydroxy fatty acid. Alternatively, L(+) β-hydroxydecanoyl-coenzyme A could be the primary hydrogenation product of the keto compound and could then be converted to the D(-) compound by a racemase similar to that found by Stern, del Campillo, and Lehninger (37).
Failure of Cultures to Incorporate C\textsuperscript{14}O\textsubscript{2} into Rhamnolipide

In view of the slight deficit in accounting for all the rhamnolipide carbon in synthetic media with glycerol as the sole carbon source (Table I), and in view of the CO\textsubscript{2} requirement by \textit{P. aeruginosa} (38), the role of CO\textsubscript{2} assimilation in rhamnolipide synthesis was investigated. Bacteria were grown in the presence of C\textsubscript{14}O\textsubscript{2} under conditions favorable for rhamnolipide production. Although minimal quantities of rhamnolipide were made and large amounts of carrier had to be added for isolation, the results indicated that far less than 1 per cent of rhamnolipide carbon was derived from CO\textsubscript{2} (Table VI).

TABLE VI
Fization of Carbon Dioxide into Rhamnolipide and Bacteria

<table>
<thead>
<tr>
<th>Addition of C\textsubscript{14}O\textsubscript{2} at</th>
<th>0 hr.</th>
<th>39 hrs.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity of C\textsubscript{14}O\textsubscript{2}</td>
<td>(3 \times 10^4)</td>
<td>(3 \times 10^4)</td>
</tr>
<tr>
<td>Total rhamnolipide produced (mg. in 25 ml.)</td>
<td>0.88</td>
<td>0.48</td>
</tr>
<tr>
<td>Maximal specific activity of rhamnolipide</td>
<td>(1.6 \times 10^4)</td>
<td>(2.1 \times 10^4)</td>
</tr>
<tr>
<td>1% incorporation level (specific activity of rhamnolipide C)</td>
<td>(3 \times 10^4)</td>
<td>(3 \times 10^4)</td>
</tr>
<tr>
<td>Specific activity of bacterial C</td>
<td>(8 \times 10^3)</td>
<td>(1.7 \times 10^3)</td>
</tr>
<tr>
<td>% bacterial C derived from C\textsubscript{14}O\textsubscript{2}</td>
<td>27</td>
<td>6</td>
</tr>
</tbody>
</table>

The specific activities are given in counts per minute per millimole.

* Attempt to favor incorporation specifically into rhamnolipide by liberating C\textsubscript{14}O\textsubscript{2} at the end of the period preceding rhamnolipide formation (4).

Incorporation of Activity into Bacteria

Of the radioactivity remaining in the culture at the end of the incubation period, 3 to 5 per cent was found in the organisms. A comparison of bacterial and glycerol carbon in the experiments with synthetic media revealed that, when glycerol-\(\alpha\)-C\textsuperscript{14} was used, the bacterial carbon had a specific activity 81 per cent of that of the substrate. The corresponding figure in experiments with glycerol-\(\beta\)-C\textsuperscript{14} was 125 per cent (Table VII). Similar findings have been reported with tubercle bacilli (39) and can be explained by assuming that bacterial fatty acids, or other compounds, were biologically synthesized from 2-carbon fragments. 2 molecules of glycerol-\(\alpha\)-C\textsuperscript{14} may give rise to two 2-carbon units, only one of which is labeled, whereas every 2-carbon unit formed from glycerol-\(\beta\)-C\textsuperscript{14} would be tagged (Fig. 3). The latter could increase the specific activity of bacterial carbon deriving from glycerol-\(\beta\)-C\textsuperscript{14} above 100 per cent if the bacterial
body were largely composed of substances derived from 2-carbon units. Atmospheric CO₂ was incorporated well into the bacterial cell (Table VII). In one experiment in which 17 per cent of the carbon available to the organism represented CO₂ in the atmosphere in contact with the medium, 27 per cent of the bacterial carbon was supplied by CO₂. The difference between the two experiments designated Sy-CO₂ reflects the time at which the C¹⁴O₂ was made available to the metabolizing organism.

P. aeruginosa also incorporates acetate carbon in preference to glycerol carbon into its structure. In the two experiments designated Sy-AC the bacterial cell carbon had a specific activity of 10,370 c.p.m. per mmole, an average of 70 per cent greater than that of the total carbon of the medium. These findings support the figures obtained in the case of glycerol-α-C¹⁴ and -β-C¹⁴ mentioned above. They are also in accord with the data of Table V,

**Table VII**  
*Relative Specific Activities of Bacterial and Glycerol Carbon*

<table>
<thead>
<tr>
<th></th>
<th>Sy-α*</th>
<th>Sy-β*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity of bacterial C, c.p.m. per mmole</td>
<td>53,800 ± 2300</td>
<td>21,100 ± 100</td>
</tr>
<tr>
<td>Specific activity of glycerol C, c.p.m. per mmole</td>
<td>66,700</td>
<td>16,900</td>
</tr>
<tr>
<td>% bacterial C which appeared to have been derived from glycerol C</td>
<td>80.7 ± 2.8</td>
<td>124.9 ± 0.6</td>
</tr>
</tbody>
</table>

The mean values are given, with the average deviation from the mean.

* For explanation of the symbols see footnote 1.

where the contribution of acetate and glycerol carbon to β-hydroxydecanoic acid is recorded.

**SUMMARY**

1. Glycerol carbon can furnish all of the carbon of the *Pseudomonas aeruginosa* rhamnolipide, whereas acetate carbon can supply only β-hydroxydecanoic acid carbon.

2. The rhamnose moiety of rhamnolipide appears to be derived by condensation of two 3-carbon units formed from glycerol without cleavage of its carbon-carbon bonds.

3. The carbon skeleton of the fatty acid seems to be synthesized by classical pathways from 2-carbon units.

4. CO₂ does not furnish rhamnolipide carbon.

5. Glycerol, acetate, and CO₂ carbon is incorporated into the bacterial cell carbon, much of which apparently represents lipide.
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