STUDIES ON THE PRODUCTION OF GLYCOLIPIDE BY
PSEUDOMONAS AERUGINOSA

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A crystalline acidic glycolipide has recently been isolated from cultures of Pseudomonas aeruginosa by Jarvis and Johnson (1949). These authors investigated its chemical structure and found it to be composed of two moles each of L-rhamnose and 1-β-hydroxydecanoic acid linked as follows:

\[
\text{rhamnose}^{1,3\text{glycoside}} \text{link} \text{rhamnose} \text{glycoside link} \beta-\text{OH-decanoic acid}^\text{ester} \beta-\text{OH-decanoic acid}
\]

The component units are the same as those of the substance obtained from P. aeruginosa by Bergström et al. (1947) and referred to by them as pyolic acid.

The compound reported by Jarvis and Johnson (1949) seemed worthy of extended study since it represents not only a unique example of a crystalline glycolipide, but might also be of interest in a consideration of the origins of its components. Very little is known of the biosynthesis of L-rhamnose, and β-hydroxydecanoic acid is important as an example of an intermediate in the general mechanism of fatty acid metabolism (Lynen, 1953).

The original investigators (Jarvis and Johnson, 1949) isolated the glycolipide from cultures grown on a peptone-glycerol broth, both constituents of which seemed to be essential for its production. The present communication deals with the development of a synthetic medium containing glycerol as the sole carbon source, the selection and application of an assay procedure for the glycolipide, and some characteristics of glycolipide production by P. aeruginosa. Studies demonstrating the incorporation of α- and β-C\(^{14}\)-labeled glycerol into both the sugar and lipide moieties, as well as the distribution of radioactivity in the glycolipide carbons, will be reported later. A preliminary report of portions of this work has been published (Hauser and Karnovsky, 1953).

MATERIALS AND METHODS

Bacterial strain. The strain of P. aeruginosa used in these experiments was obtained from the Department of Bacteriology, Harvard Medical School. It was maintained by frequent transfers on tryptic digest nutrient agar slants, a fresh slant being prepared 18 to 24 hours before the inoculation of cultures.

Media. The 4 per cent lactopeptone-3 per cent glycerol broth used successfully by Jarvis and Johnson (1949) was employed for glycolipide production on a nonsynthetic medium. Several synthetic media with glycerol as the sole carbon source were tried, among them those of Birkofer and Birkofe (1948), Norris et al. (1949), and Cutchins et al. (1952); but best results were obtained with a modification of the minimal medium for Escherichia coli of Davis and Mingioli (1950). It contained K\(_2\)HPO\(_4\), 7.0 g/L; KH\(_2\)PO\(_4\), 3.0 g/L; MgSO\(_4\)·7H\(_2\)O, 0.1 g/L; (NH\(_4\))\(_2\)SO\(_4\), 1.0 g/L; pH 7.0. A stock solution was prepared in tenfold strength with chloroform as preservative. It was diluted as needed, usually 1:10, and suitable amounts of glycerol (Mallinkrodt—94 per cent pure) were added before autoclaving. Where necessary, other energy sources were autoclaved in concentrated solution or sterilized by filtration before addition to the medium. The radioglycerol used for radioglycolipide preparation was that made by Gidez and Karnovsky (1952). Culture media were autoclaved at 15 lb pressure for 15 min.

Conditions of incubation. Erlenmeyer flasks were used throughout. In no case did the medium occupy more than one-quarter of the flask.
volume since greater amounts sharply decreased glycolipide production.

Except where stated otherwise in the text, cultures were allowed to grow for approximately five days (120 ± 12 hours) with shaking. Two types of shakers were used: a reciprocal type with a 2.5 inch stroke, set at 70 to 100 excursions per minute, and a rotary type (New Brunswick Scientific Co., New Brunswick, N. J.) adjusted for 160 rotations per minute. Both served equally well.

Early experiments were carried out in the warm room at 36 C to 38 C, but glycolipide production improved upon lowering the temperature and most runs were done at 31 C to 34 C.

**Isolation of glycolipide.** The isolation of the crystalline glycolipide was accomplished in essentially the manner described by Jarvis and Johnson (1949), with modifications. The organisms were centrifuged off in a Servall SS centrifuge (10 min at about 11,000 X G) and discarded after it had been shown that they contained only minimal amounts of glycolipide. The supernatant was acidified with 10 n sulfuric acid to pH 2. Refrigeration for at least three days yielded crystals which were harvested by centrifugation. After two water washes, an aqueous suspension was filtered on a Büchner funnel, with suction, through Whatman no. 1 filter paper. In this manner clogging of the filter could be controlled. Frequently a small second crop could be isolated from the medium upon further standing in the cold room.

Several recrystallizations from acetone and water, with the addition of decolorizing charcoal (norite) where necessary, were carried out in order to obtain a pure product. About 75 ± 10 per cent of the glycolipide produced (as shown by the assay described below) could be isolated, but further losses were incurred during the recrystallization process.

**Assay of glycolipide.** The method of Dische and Shettles (1948) for methylpentoses was used for the determination of glycolipide. Samples of the supernatant medium were taken before acidification (see Isolation of glycolipide above), suitably diluted, and directly subjected to the procedure. At least two hours were allowed to elapse after carrying out the CyR10 reaction before reading the solutions in the Beckman Model DU spectrophotometer. The absorption peak of rhamnose was found to be at 4030 A, and since no interfering hexoses were present, readings were taken at this wavelength as well as at 4300 A. Optical densities were of about the same magnitude as those observed by Dische and Shettles (1948) for fucose. Control tubes to which no cysteine was added were run in all cases because considerable extraneous color often developed on heating with sulfuric acid, especially in the case of nonsynthetic media. Suitable standards and blanks were included in each set of determinations, and appropriate corrections made as indicated by Dische and Shettles (1948). Linearity could be obtained from 3 µg to 30 µg of methylpentose. A concentrated rhamnose standard was prepared in saturated benzoic acid (Folin, 1934); it can be kept this way indefinitely in the cold.

**Determination of glycerol.** Glycerol was determined by a method involving oxidation with periodic acid and colorimetric determination of the resulting formaldehyde with chromotropic acid (MacPadyen, 1945; Karnovsky and Brumm, 1954, in press). During early experiments the formaldehyde was precipitated as the dimedone complex and determined gravimetrically (Yoe and Reid, 1941). The two methods are in agreement.

**Determination of growth. (a) Turbidimetric.** Aliquots of the culture were centrifuged, the organisms washed with distilled water, suspended in 1:10 4 (0.001 per cent) “duponol”, and read in the Klett colorimeter with a no. 42 or no. 56 filter. Since the response of the instrument to turbidity is not linear, calibration curves were plotted and necessary corrections made.

(b) Viable counts. Counts of viable organisms were made by streaking serially diluted media on tryptic digest nutrient agar plates and making plate counts after 24 hours of incubation at 37 C.

**Measurement of radioactivity.** For counting as barium carbonate, glycolipide samples were combusted to CO₂ by the micro-wet carbon combustion of Van Slyke and Folch (1940) as adapted for the precipitation of C14O₂ as BaC14O₃ by Van Slyke et al. (1951).

ml of sulfuric acid (6 vol conc H₂SO₄; 1 vol H₂O) and subsequent color development with 3 per cent cysteine hydrochloride solution.

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2 The term “CyR10 reaction” is used by Dische and Shettles (1948) to indicate a 10 minute heating period (100 C) of 1 ml solution under test with 4.5
For counting directly without combustion samples were dissolved in chloroform and plated onto planchets with a disk of lens paper covering the plating area to facilitate even distribution of the material (Calvin et al., 1949).

All samples to be counted were mounted on stainless steel disks with a plating area of 1.60 cm² and their radioactivity determined with a proportional gas flow-counter (Robinson, 1950), after solvent removal to constant planchet weight. Self-absorption factors for BaCO₃ (Robinson, unpublished data) were found to be applicable throughout.

RESULTS AND DISCUSSION

Preliminary experiments. Numerous preliminary experiments were carried out in 2 L flasks containing 500 ml of bactopeptone medium each in order to confirm the findings of Jarvis and Johnson (1949) and to standardize an efficient method for the production and isolation of the glycolipide. Some representative results are shown in table 1. Since these experiments were carried out before the development of a method for the assay of glycolipide, column 4 represents the amounts of substance actually isolated. More than 5 g glycolipide per liter of medium has on occasion been produced; however, the variation from run to run is considerable.

The data indicate that good glycolipide production can be attained even with glycerol concentrations lower than 3 per cent. Apparently only small amounts of glycerol were utilized during the first two days of incubation, while frequently only minimal amounts remained after five. In the absence of CO₂ in the atmosphere in contact with the culture, glycolipide is produced in greatly reduced quantities, despite fair growth. This is in agreement with the concept of CO₂ requirement even for so-called heterotrophic organisms (Werkman, 1949), and with the specific findings of Gladstone et al. (1935) for P. aeruginosa. These authors found complete growth inhibition under conditions of continuous CO₂ removal, but were unable to prevent growth in this manner in the presence of peptone.

The physical properties of the isolated glycolipide were identical with those found by Jarvis and Johnson (1949). Additional evidence for the identity of the sugar moiety was adduced by subjecting the water-soluble fraction obtained by acid hydrolysis to descending paper chromatography. The mobile phase consisted of ethyl acetate:acetic acid:water 3:1:3 v/v (Jermyn and Isherwood, 1949), and the chromatograms were developed with aniline hydrogen oxalate (Partridge, 1949) after 18 hours. The unknown moved the same distance as l-rhamnose, an average of 2.52 times that moved by glucose. No sugar other than rhamnose could be detected.

Assay of glycolipide. Several unsuccessful attempts were made to quantitate glycolipide production before a method of Dische and Shettles (1948) for the determination of methylpentoses was found to give accurate and reproducible results. This method was originally used for the quantitative determination of fucose in hydrolyzed and unhydrolyzed polysaccharides of blood group substances. Its outstanding advantage is the ease with which it is carried out; isolation, extraction, or preliminary hydrolysis of the glycolipide is obviated, the last being accomplished in the course of heating with sulfuric acid. This reaction apparentlyoccasions minimal decomposition of the sugar, and the resulting fatty acid or its degradation products do not introduce any difficulties.

Table 2 demonstrates the recovery of glycolipide obtained with glycolipide solutions of known concentration as well as with culture media to which additional amounts of glycolipide had been added. Estimation of added glycolipide recovered was 96.7 ± 0.9 per cent.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>37°C</th>
<th>reciprocal shaking. Bactopeptone medium.</th>
<th>Glycolipide Isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol Content of Medium</td>
<td>Length of Run</td>
<td>Unused Glycerol</td>
<td>g/L</td>
</tr>
<tr>
<td>per cent</td>
<td>days</td>
<td>per cent</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>83</td>
<td>0.00</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>6</td>
<td>0.78</td>
</tr>
<tr>
<td>1.5</td>
<td>5</td>
<td>6</td>
<td>1.98</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>76</td>
<td>1.28</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>49</td>
<td>0.70</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>3</td>
<td>1.46</td>
</tr>
<tr>
<td>3*</td>
<td>5</td>
<td>3</td>
<td>3.16</td>
</tr>
<tr>
<td>3*</td>
<td>5</td>
<td>46</td>
<td>0.26</td>
</tr>
<tr>
<td>3*</td>
<td>5</td>
<td>70</td>
<td>0.10</td>
</tr>
</tbody>
</table>

* CO₂ collection attempted by sweeping sterile CO₂-free air through the flask and collecting the CO₂ produced by the culture in NaOH.

TABLE 1

Preliminary experiments. Numbers of early experiments were carried out in 2 L flasks containing 500 ml of bactopeptone medium each in order to confirm the findings of Jarvis and Johnson (1949) and to standardize an efficient method for the production and isolation of the glycolipide. Some representative results are shown in table 1. Since these experiments were carried out before the development of a method for the assay of glycolipide, column 4 represents the amounts of substance actually isolated. More than 5 g glycolipide per liter of medium has on occasion been produced; however, the variation from run to run is considerable.

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TABLE 2
Recovery of glycolipide and rhamnose by spectrophotometric assay

All results are expressed as rhamnose. Calculations were made on the basis of a molecular weight of 668 for the glycolipide (Jarvis and Johnson, 1949) and two moles of rhamnose per mole of glycolipide.

<table>
<thead>
<tr>
<th>Solution Assayed</th>
<th>Initial</th>
<th>Added</th>
<th>Recovered</th>
<th>Recovered per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycolipide solution</td>
<td>0.00</td>
<td>10.78</td>
<td>10.38</td>
<td>96.4</td>
</tr>
<tr>
<td>Rhamnose added to culture medium</td>
<td>0.70</td>
<td>16.66</td>
<td>16.55</td>
<td>99.3</td>
</tr>
<tr>
<td>Glycolipide added to culture medium</td>
<td>0.70</td>
<td>17.97</td>
<td>17.05</td>
<td>94.8</td>
</tr>
<tr>
<td>Glycolipide added to culture medium</td>
<td>16.82</td>
<td>8.63</td>
<td>8.28</td>
<td>95.9</td>
</tr>
</tbody>
</table>

TABLE 3
Confirmation of the glycolipide assay by the isotope dilution technique

The experimental procedure is described in the text. Results are expressed as mg glycolipide (GL).

<table>
<thead>
<tr>
<th>Experiment Number</th>
<th>GL in Medium by Assay (1)</th>
<th>C14-GL Added (2)</th>
<th>Total GL in Medium after Addition (1) + (2)</th>
<th>GL by Isotope Dilution</th>
<th>GL by Isotope Dilution × 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>125</td>
<td>11.3</td>
<td>136</td>
<td>115</td>
<td>85</td>
</tr>
<tr>
<td>2</td>
<td>125</td>
<td>22.6</td>
<td>148</td>
<td>129</td>
<td>87</td>
</tr>
<tr>
<td>3</td>
<td>125</td>
<td>16.9</td>
<td>142</td>
<td>154</td>
<td>108</td>
</tr>
<tr>
<td>4</td>
<td>109</td>
<td>24.4</td>
<td>133</td>
<td>135</td>
<td>101</td>
</tr>
<tr>
<td>5</td>
<td>109</td>
<td>24.4</td>
<td>133</td>
<td>117</td>
<td>88</td>
</tr>
<tr>
<td>6</td>
<td>109</td>
<td>48.8</td>
<td>158</td>
<td>148</td>
<td>94</td>
</tr>
<tr>
<td>7</td>
<td>109</td>
<td>48.8</td>
<td>158</td>
<td>148</td>
<td>94</td>
</tr>
<tr>
<td>Mean: 94 ± 3 per cent</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Recovery of rhamnose under the same conditions was 98.6 ± 0.5 per cent.

In addition to ascertaining the completeness of recovery of added glycolipide it was necessary to demonstrate that the reaction when applied to culture media would indeed measure glycolipide rather than free rhamnose or other substances. Using the chromatographic technique described above, no free rhamnose could be detected in the concentrated supernatant medium of the bacterial cultures after five days' growth.

Confirmation of the applicability of the assay was achieved by means of the isotope dilution technique. C14 was incorporated into glycolipide by using α-labeled C14-glycerol in the culture medium and isolating the glycolipide five days after inoculation. After recrystallisation to constant activity it counted 120,000 cpm/mm2, an activity suitable for isotope dilution experiments.

P. aeruginosa was grown as usual in both the peptone and synthetic glycerol containing media. To 100 ml of the supernatant after removal of the organisms by centrifugation, weighed amounts of radioglycolipide of known activity, dissolved in ethyl alcohol, were added. The media were thoroughly mixed to insure homogeneous distribution of the radioactive substance, acidified, and the material isolated and recrystallised in the usual way. Glycolipide was counted directly and as BaCO3 after combustion to CO2. The results are summarized in table 3.

Despite some variation, mainly due to counting error, it is evident that the method yields a reliable and reproducible index of the amount of glycolipide produced in a given medium. The presence of minute amounts of free rhamnose, undetectable on the paper chromatogram or by isotope dilution, cannot, however, be excluded.

Characteristics of growth and glycolipide production. Since preliminary experiments indicated that a delay occurs between the time at which growth of P. aeruginosa becomes visible in the medium and the appearance of demonstrable amounts of glycolipide in the supernatant medium, it seemed desirable to observe this phenomenon in greater detail.

Glycerol disappearance, glycolipide appearance, and bacterial growth were measured with time in peptone containing and synthetic media with 3 per cent glycerol, as well as in 1 per cent glycerol synthetic media. In all cases duplicate experiments were carried out, and agreement was found to be excellent. At frequent intervals 5 ml samples were removed aseptically, centri-
Figure 1. Glycolipide appearance, glycerol disappearance, and bacterial growth in bactopeptone—3 per cent glycerol media.

● = glycolipide (GL), △ = glycerol (gly), ○ = viable organisms (V), △ = turbidity (T) expressed as corrected Klett readings (no. 56 filter). All curves represent the average of duplicate runs. Temperature 31 C; reciprocal shaking.

Figure 2. Glycolipide appearance, glycerol disappearance, and bacterial growth in synthetic media containing 1 per cent glycerol.

● = glycolipide (GL), △ = glycerol (gly), △ = turbidity (T) expressed as corrected Klett readings (no. 56 filter). All curves represent the average of duplicate runs. Temperature 31 C to 34 C; reciprocal shaking.

fuged, and the supernatant suitably diluted for the various determinations. The data are plotted in figures 1, 2, and 3.

The suspicion of a lag-period in the formation of glycolipide is corroborated by these data. In all cases, regardless of the type of medium or glycerol concentration, no glycolipide was detectable until 20 to 30 hours after a sharp increase in the turbidity of the medium, due to bacterial multiplication, occurred. During the
logarithmic phase of growth, essentially no glycolipide appeared. Not until the organisms approached the maximum stationary phase could the presence of glycolipide be demonstrated in the supernatant medium. This does not seem to be the result of liberation of the material from the bacteria by lysis since only negligible amounts could be extracted from washed cells, and the concentration in the medium did not increase during the phase of decreasing bacterial population (figure 2).

In the peptone containing medium (figure 1), glycerol began to disappear only a short time before it was possible to demonstrate glycolipide, indicating a preference of the organism for energy sources other than glycerol. In synthetic media (figures 2 and 3), glycerol was utilized as soon as growth began since it was the sole carbon source. Figure 2 shows clearly the interdependence of raw material and product; the exhaustion of the supply of the former halts the synthesis of the latter.

The rates of production differed considerably in natural and synthetic media. On bactopeptone the maximal rate was 37 mg glycolipide per hr, while on synthetic media it ranged around one-third of that amount (11–15 mg/hr). The absolute linearity of the glycolipide production curve between 40 and 110 hours in the case of experiments with 1 per cent glycerol in the medium is particularly noteworthy (figure 2). One of the cultures grown on 3 per cent glycerol containing synthetic media (figure 3) indicates an interesting phenomenon; glycerol content diminished quite slowly and glycolipide was still rising when the experiment was terminated after 33 days.

An experiment was performed in which half of the organisms produced in a 20 hour period were transferred to a freshly prepared medium, after washing. Approximately 15 hours elapsed before glycolipide was formed. Since this lag was somewhat shorter than under the usual conditions, the operation of an adaptive mechanism was considered. Enzyme production by an aging bacterial population under conditions favorable for adaptation is not unknown. Gale (1946) found that the bacterial amino acid decarboxylases, for example, which form as growth proceeds, are fully developed within the organism only at the end of active cell division. The variation in activity with the age of the culture seems to be due to some as yet undefined change in the medium brought about by the metabolic activities of the organism. Experiments to investigate the possibility of adaptation by the whole culture are at present being undertaken. The source of the inoculum may affect the final concentration of glycolipide, as is shown in
GLYCOLIPIDE PRODUCTION BY PSEUDOMONAS

As may be seen from columns (1) and (2) where adequate amounts of all components of the medium were present, no significant difference was observed whether the inoculum was glycerol grown or not. In column (3), where the concentration of salt mixture in the medium was one-half that normally used, the final glycolipide level reached after inoculation with a glycerol grown culture was about 60 per cent more than that reached after inoculation from a tryptic digest agar slant. Thus it appears that, with the former inoculum, a given level of glycolipide may be attained sooner after inoculation, possibly because of the early presence of enzymes concerned in glycolipide biosynthesis. A difference in final levels was, however, demonstrated when the concentration of inorganic salts was the growth limiting factor, as is presumably the case in column (3).

Influence of various substances on glycolipide production. Fructose, glucose, and citrate were able to serve as sole carbon sources in the synthetic medium described above (Materials and Methods). When the medium contained only acetate as carbon source, no growth occurred, in contrast to findings by Campbell and Stokes (1951) who used a somewhat different salt mixture. Of the above organic substrates, fructose furnished glycolipide in yields comparable to those obtained on glycerol. In the case of citrate no glycolipide was formed. Minute quantities were detected in the case of glucose. Since circumstances are known where glucose exerts an inhibitory effect (e.g., Magasanik, 1953; Gale, 1938) on adaptive enzyme formation, an analogous action on a component of the glycolipide synthesizing system might be operative in this instance. Table 5 shows the depression of glycolipide production to one-half by an amount of glucose equimolar in carbon to the glycerol present. (The effect of ethylenediaminetetraacetic acid will be discussed below.)

Other compounds also reduced glycolipide formation as shown in table 6. Succinate, citrate, and acetate produced this effect. The organism was clearly able to utilize these substances for its metabolic activity, and they exerted a glycerol sparing action. A similar inhibitory effect of these compounds on the production of acid from glucose by P. aeruginosa in synthetic medium has been noted by Liu (1952).

TABLE 4
Effect of dilution of the stock salt mixture and source of the bacterial inoculum

<table>
<thead>
<tr>
<th>Source of Inoculum</th>
<th>Dilution of Stock Salt Mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(1) 3:20</td>
</tr>
<tr>
<td>Tryptic digest nutrient agar slant</td>
<td>935</td>
</tr>
<tr>
<td>Glycerol-containing synthetic medium</td>
<td>982</td>
</tr>
</tbody>
</table>

TABLE 5
Inhibition of glycolipide production from glycerol by glucose and ethylenediamine tetraacetic acid

Each flask contained 7 mm glycerol in 50 ml of medium. Temperature 32 C to 35 C; rotary shaking for 138 hours.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Glycolipide Produced mg/L</th>
<th>per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2,303</td>
<td>100</td>
</tr>
<tr>
<td>3.5 mm glucose</td>
<td>1,070</td>
<td>46</td>
</tr>
<tr>
<td>0.15 mm EDTA</td>
<td>415</td>
<td>18</td>
</tr>
</tbody>
</table>

TABLE 6
Inhibition of glycolipide production from glycerol by organic acid salts

All flasks contained 50 ml of stock salt solution diluted 1:10, 34 mm of glycerol carbon and 8 mm of "inhibitor" carbon. Incubation at 31 C to 34 C for 126 hours.

<table>
<thead>
<tr>
<th>Additions</th>
<th>None</th>
<th>Sodium succinate</th>
<th>Sodium citrate</th>
<th>Sodium acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycolipide (per cent of amount formed in glycerol medium)</td>
<td>100</td>
<td>53</td>
<td>33</td>
<td>50</td>
</tr>
<tr>
<td>Glycerol used (per cent of total)</td>
<td>51</td>
<td>35</td>
<td>41</td>
<td>—</td>
</tr>
</tbody>
</table>

(Porter, 1946), and acetic acid was once considered a "specific" disinfectant for P. aeruginosa. 0.34 N acetic acid (pH 2.7) has been reported to be bactericidal, and 0.004 N (pH 5.11) inhibitory
for these bacteria (Reid, 1932) with the acetate anion playing some role in this action.

The above findings may be compared with the results of studies on the effect of addition of varying amounts of sodium acetate to glycerol containing media plotted in figure 4. While small amounts of acetate did not affect glycolipide production and possibly even enhanced it slightly, a contribution to the total carbon in the medium of more than 9.5 per cent in mm by acetate carbon resulted in a sharp decrease in glycolipide production which fell to zero when about one-third of the carbon present was furnished by acetate.

That this phenomenon is not due merely to the inhibition of growth by acetate is demonstrated by the fact that good growth occurred in all flasks except those with the highest acetate concentration. No turbidity could be observed in these flasks (62 per cent acetate carbon) even after six days. At all other acetate concentrations

**Figure 4.** Inhibition of glycolipide formation from glycerol by sodium acetate.

O = flasks containing only glycerol, ● = flasks containing both glycerol and acetate (total: 42 mm of carbon), + = ratio of O/●, expressed as per cent.

Volume in each flask 50 ml; temperature 32 C to 35 C; length of incubation 120 hours; rotary shaking. Each point represents the average of determinations on duplicate flasks.

a pronounced delay occurred in the initiation of growth, as much as 48 hours in those containing 6 mm of acetate, constituting 20 per cent of the total carbon. At the end of the experiment the turbidity in acetate flasks was as great as that in the controls, however. A similar lag occurred in pigment production. Pigment was formed later in acetate flasks and became more intense and much yellower than the green coloration in the glycerol flasks. This effect became stronger with higher acetate concentrations.

In figure 5 are plotted the results of an experiment to exclude the possibility that in those flasks where growth had been delayed a further glycolipide accumulation, approaching that in the control flasks, might occur. Both sets of flasks (one containing glycerol alone, the other glycerol and acetate) showed a marked increase in glycolipide content during a period of ten days after the usual five day incubation. However, amounts in the acetate inhibited flasks dropped from 34 per cent (after five days) to 29 per cent (after fifteen days) of those formed on glycerol alone. The comparative over-all rates of production during the period from the fifth to the
fifteenth day are 1.72 mg per liter an hr to 12.3 mg per liter an hr, thus differing by a factor of seven.

It was observed that while cultures grown on glycerol containing media yielded free flowing supernatants, the introduction of sodium acetate caused a rather viscous slime to be formed, much like that obtained by growth on potassium gluconate (Haynes, 1951). Frequently “oyster” formation also took place. Similar conditions prevailed in succinate grown cultures, as well as those grown on citrate, where they were most pronounced.

Identification of intermediates in the biosynthesis of glycolipide from glycerol would be of great interest. To date, however, attempts to have the organism grow with one of a number of 3-C compounds other than glycerol as the sole source of carbon have been unsuccessful. Dihydroxyacetone, propanediol, allyl alcohol, and methylglyoxal were tried individually and in various combinations. Of these only propanediol was able to initiate growth although 36 hours elapsed between inoculation and visible turbidity.

When additions of these substances were made to cultures in synthetic media containing 0.67 per cent glucose, which had been incubated with shaking for 18 hours, growth proceeded normally. In no case, however, could glycolipide formation be shown. It should be noted, however, that glucose depressed glycolipide production even from glycerol.

The nonmetabolizable cation-binding compound ethylenediaminetetraacetic acid also exerts a very marked effect on glycolipide production from glycerol. Although good growth was obtained at the concentration used, relatively little glycolipide was made (table 5). Some of the enzymes involved in its synthesis are undoubtedly activated by metal cations.

Some difficulty was experienced with the assay method where dihydroxyacetone, allyl alcohol, and methylglyoxal were used. Some of the residual substrates carbonized on heating with sulfuric acid. An extraction procedure was standardized which, in numerous control analyses, yielded results of 50 ± 10 per cent of those prior to extraction. The acidified culture supernatants were exhaustively extracted with ether, the ethereal extracts washed with distilled water, and a bicarbonate extract of this solution subjected to analysis.

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SUMMARY

Glycolipide was produced by a strain of Pseudomonas aeruginosa on both a bactopeptone and a synthetic medium. Fructose as well as glycerol could act as carbon source for its synthesis, and atmospheric carbon dioxide plays a role.

A satisfactory assay has been established, and its validity confirmed by the isotope dilution technique. The results of the assay are consistent with the structure previously proposed by other workers.

Glycolipide formation appears to be a function of an aging bacterial population with its onset following the logarithmic phase of growth.

Glucose, citrate, succinate, and acetate additions sharply reduce glycolipide production in the presence of glycerol.

In the case of acetate, inhibition begins when 10 per cent and is complete when 35 per cent of the carbon source is furnished by acetate carbon. Both the rate of formation and total accumulation are affected.

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