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ISOLATION AND IDENTIFICATION OF THE ACID-SOLUBLE
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CHARLESII

DISSERTATION

Presented in Partial Fulfillment of the Requirements
for the Degree Doctor of Philosophy in the
Graduate School of The Ohio State
University

By

DONALD EARLE MAYNARD, B.A., M.S.

******

The Ohio State University
1961

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## CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>ILLUSTRATIONS</td>
<td>v</td>
</tr>
<tr>
<td>TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>vii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>REVIEW OF THE LITERATURE</td>
<td>5</td>
</tr>
<tr>
<td>A. Isolation, Characterization and Metabolism of Galactocarolose</td>
<td>5</td>
</tr>
<tr>
<td>B. Naturally Occurring Nucleosides</td>
<td>7</td>
</tr>
<tr>
<td>1. Crotidine</td>
<td>7</td>
</tr>
<tr>
<td>2. The Spongornucleosides</td>
<td>8</td>
</tr>
<tr>
<td>3. Psicofuranine</td>
<td>9</td>
</tr>
<tr>
<td>4. Adenine Glucoside</td>
<td>9</td>
</tr>
<tr>
<td>EXPERIMENTARY</td>
<td>11</td>
</tr>
<tr>
<td>A. Stock Cultures</td>
<td>11</td>
</tr>
<tr>
<td>B. Experimental Cultures</td>
<td>11</td>
</tr>
<tr>
<td>C. Perchloric Acid Extraction of Mycelial Pads</td>
<td>11</td>
</tr>
<tr>
<td>D. Changes in pH During \textit{P. charlesi} Growth</td>
<td>12</td>
</tr>
<tr>
<td>E. Transfer Experiments With Radioactive Substrates</td>
<td>13</td>
</tr>
<tr>
<td>1. Acetate-1-$^{14}$ Transfer Experiment</td>
<td>13</td>
</tr>
<tr>
<td>2. Glucose-U-$^{14}$ Transfer Experiment</td>
<td>14</td>
</tr>
<tr>
<td>F. Incorporation Studies With $^{14}$-labeled Purines and Pyrimidines</td>
<td>14</td>
</tr>
<tr>
<td>G. Isolation and Identification of the Acid-Soluble Nucleotides of \textit{P. charlesi}</td>
<td>15</td>
</tr>
</tbody>
</table>
# CONTENTS—(Continued)

<table>
<thead>
<tr>
<th></th>
<th>1. Preparative Chromatography</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2. Identification of Individual Nucleotides</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>3. Identification of Bases</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>4. Identification of the Sugar Components of Nucleotides</td>
<td>17</td>
</tr>
</tbody>
</table>

- **CHEMICAL SYNTHESSES** | 19

  - A. Preparation of N-Acetyl Adenine and Its Monochloromercuri Derivative | 19
  - B. Preparation of L-Galactose | 19
  - C. Acetylation of D-Galactose | 20
  - D. Preparation of 9-β-Galactopyranosyladenine | 21

- **RESULTS** | 25

  - A. Column Chromatography of Acid-Soluble Nucleotides | 25
  - B. Paper Chromatography of Nucleotide Material | 28

  1. Fraction A | 28
  2. Fraction A₂ | 28
  3. Fraction A₃ | 35
  4. Fractions B and C | 36
  5. Fraction D | 39
  6. Fraction E | 40

  C. Changes in pH During Growth | 41

  - D. Acetate-1-¹⁴ Transfer Experiment | 52
  - E. Glucose-U-¹⁴ Transfer Experiment | 57

  - F. Incorporation Studies with Radioactive Purines and Pyrimidines | 60

    1. Uracil-2-¹⁴ | 60
    2. Adenine-8-¹⁴ | 68
    3. Guanine-8-¹⁴ | 89

- **DISCUSSION** | 92

- **SUMMARY** | 109

- **BIBLIOGRAPHY** | 111

- **AUTOBIOGRAPHY** | 114
ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Structure of Galactocarlose</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>Typical Distribution of Acid-Soluble Nucleotides in P. charlesii</td>
<td>27</td>
</tr>
<tr>
<td>3</td>
<td>Ultraviolet Absorption Spectra of Compound A_x</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>Variation in pH with Age of P. charlesii Culture</td>
<td>42</td>
</tr>
<tr>
<td>5</td>
<td>Acid-Soluble Nucleotides in P. charlesii After Four Days Growth</td>
<td>44</td>
</tr>
<tr>
<td>6</td>
<td>Acid-Soluble Nucleotides in P. charlesii After 5.25 Days Growth</td>
<td>46</td>
</tr>
<tr>
<td>7</td>
<td>Acid-Soluble Nucleotides in P. charlesii After 10.25 Days Growth</td>
<td>48</td>
</tr>
<tr>
<td>8</td>
<td>Acid-Soluble Nucleotides in P. charlesii After 21 Days Growth</td>
<td>51</td>
</tr>
<tr>
<td>9</td>
<td>Acid-Soluble Nucleotides in P. charlesii Incorporating Acetate-1-C14</td>
<td>54</td>
</tr>
<tr>
<td>10</td>
<td>Reproduction of Chromatogram, Fraction A, Acetate-1-C14 Experiment, Showing Radioactivity Distribution</td>
<td>56</td>
</tr>
<tr>
<td>11</td>
<td>Acid-Soluble Nucleotides in P. charlesii Incorporating Glucose-U-C14</td>
<td>59</td>
</tr>
<tr>
<td>12</td>
<td>Reproduction of Chromatogram, Fraction A, Glucose-U-C14 Experiment, Showing Radioactivity Distribution</td>
<td>62</td>
</tr>
<tr>
<td>13</td>
<td>Acid-Soluble Nucleotides in P. charlesii Incorporating Uracil-2-C14</td>
<td>64</td>
</tr>
<tr>
<td>14</td>
<td>Reproduction of Chromatogram, Fraction A, Uracil-2-C14 Experiment, Showing Radioactivity Distribution</td>
<td>67</td>
</tr>
</tbody>
</table>
ILLUSTRATIONS— (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>Reproduction of Chromatogram, Fraction B, Uracil-2-$^{14}$C Experiment, Showing Radioactivity Distribution</td>
<td>70</td>
</tr>
<tr>
<td>16</td>
<td>Reproduction of Chromatogram, Fraction C, Uracil-2-$^{14}$C Experiment, Showing Radioactivity Distribution</td>
<td>72</td>
</tr>
<tr>
<td>17</td>
<td>Acid-Soluble Nucleotides in <em>P. charlesii</em> Incorporating Adenine-8-$^{14}$C</td>
<td>74</td>
</tr>
<tr>
<td>18</td>
<td>Reproduction of Chromatogram, Fraction A, Adenine-8-$^{14}$C Experiment, Showing Radioactivity Distribution</td>
<td>77</td>
</tr>
<tr>
<td>19</td>
<td>Reproduction of Chromatogram, Fraction A$_1$, Adenine-8-$^{14}$C Experiment, Showing Radioactivity Distribution</td>
<td>80</td>
</tr>
<tr>
<td>20</td>
<td>Reproduction of Chromatogram, Fraction B-C, Adenine-8-$^{14}$C Experiment, Showing Radioactivity Distribution</td>
<td>83</td>
</tr>
<tr>
<td>21</td>
<td>Reproduction of Chromatogram, Fraction D, Adenine-8-$^{14}$C Experiment, Showing Radioactivity Distribution</td>
<td>86</td>
</tr>
<tr>
<td>22</td>
<td>Reproduction of Chromatogram, Fraction E, Adenine-8-$^{14}$C Experiment, Showing Radioactivity Distribution</td>
<td>88</td>
</tr>
<tr>
<td>23</td>
<td>Acid-Soluble Nucleotides in <em>P. charlesii</em> Incorporating Guanine-8-$^{14}$C</td>
<td>91</td>
</tr>
<tr>
<td>24</td>
<td>Tetronic Acid Derivatives Produced by <em>P. charlesii</em> Grown on Raulin-Thom Glucose Medium</td>
<td>96</td>
</tr>
<tr>
<td>25</td>
<td>A Hypothetical Pathway For Galactocarbose Biosynthesis</td>
<td>107</td>
</tr>
</tbody>
</table>
### TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Relative Migration Rates of Various Nucleosides and Nucleotides</td>
<td>29</td>
</tr>
<tr>
<td>2</td>
<td>Relative Migration Rates of Aldoses and Their Phosphorylated Derivatives</td>
<td>34</td>
</tr>
<tr>
<td>3</td>
<td>Activities of Individual Fractions, Peak A, Uracil-2-C\textsuperscript{14} Experiment</td>
<td>65</td>
</tr>
<tr>
<td>4</td>
<td>Activities of Individual Fractions, Peak A, Adenine-8-C\textsuperscript{14} Experiment</td>
<td>75</td>
</tr>
<tr>
<td>5</td>
<td>Activities of Individual Fractions, Peak A\textsubscript{1}, Adenine-8-C\textsuperscript{14} Experiment</td>
<td>81</td>
</tr>
<tr>
<td>6</td>
<td>Factors Contributing to the Group-Transfer Potential of Various Glycosidic Bonds</td>
<td>101</td>
</tr>
</tbody>
</table>

### ABBREVIATIONS

The following abbreviations have been used in this dissertation: UMP, UDP, UTP: uridine mono-, di-, and triphosphate; UDPG: uridine diphosphate glucose; UDPGal: uridine diphosphate galactose; DPN and TPN: di- and triphosphopyridine nucleotide; ALTP, ADP, ATP: adenosine mono-, di-, and triphosphate; glucose-1-P: glucose-1-phosphate.
INTRODUCTION

During the past twenty years, a considerable number of investigators have devoted their efforts to defining the pathways by which some of the myriad naturally occurring oligo- and polysaccharides are formed. This work has necessarily been limited to those few compounds which can be obtained in relatively pure form, and whose structures have yielded to chemical, physical and/or biochemical analysis.

In the course of their experiments, those working in this broad area of biochemistry have so far established three general reactions by which oligo- and polysaccharides may be formed. These can be described by the following equations:

1) \[ \text{Gly-O-X} + \text{H-O-R} \rightleftharpoons \text{Gly-O-R} + \text{H-O-X}, \]

where Gly-O-X is a glycosyl phosphate and H-O-R is a carbohydrate residue of undefined chain length. The enzymes catalyzing this type of reaction, the phosphorylases, are primarily considered as degradative enzymes, but under certain conditions, the reaction equilibrium is such that polysaccharide synthesis is actually favored.

2) \[ \text{Gly-O-R} + \text{H-O-R'} \rightleftharpoons \text{Gly-O-R'} + \text{H-O-R}, \]

where R represents a glycosyl or aglycone moiety, and R' another carbohydrate residue. The transglycosylases are the most diverse of the three types.
3) Gly-O-PPX + H-O-R* → nucleoside → Gly-O-R* + XPP, diphosphate transglycosylases

where Gly-O-PPX is a nucleoside diphospho sugar and H-C-R* is a glycosyl residue which acts as an acceptor for the nucleotide-bound sugar moiety. This class of enzymes, the nucleoside diphosphate transglycosylases, is known to mediate the formation of many types of polymeric carbohydrates, including disaccharides and the high-molecular-weight substances such as glycogen and chitin.

For the purposes of this discussion, the functions of these three classes of enzymes may be summarized as follows:

a. The phosphorylases are primarily enzymes which catalyze degradation of carbohydrate polymers to their simpler subunits.

b. The transglycosylases, as the term implies, are mainly involved in the transfer of glycosyl residues (one or more units at a time) from one glycosidic linkage to another, either with retention or inversion of configuration.

c. The nucleoside diphosphate transglycosylases catalyze reactions wherein nucleoside diphospho sugars act as donors of glycosyl units in polysaccharide formation. The products of this type of enzymic activity range from disaccharides, as in lactose synthesis, to very large molecules like glycogen and xylan.

In all cases known at present, the nucleoside diphosphate involved is uridine diphosphate. Since other nucleoside diphospho sugars are known to serve as intermediates in monosaccharide interconversions (1-4), however, it is probable that uridine diphosphate
is not the only nucleoside diphosphate with an important role in polysaccharide biosynthesis.

The research described in this dissertation was initiated in a study of the reactions by which the novel galactose oligosaccharide, galactocarolose, is formed by the organism Penicillium charlesii (5). Figure 1 shows the structure of galactocarolose. The most significant structural feature of this compound which must be considered in any attempt to propose a biosynthetic pathway is the presence of the \(\beta\)-1,5-linked galactofuranose units. In this structure, the hemiacetal oxygen joins carbons 1 and 4, making it quite unlikely that the furanose units arise via \(\alpha\)-D-glucopyranose-1-phosphate.

Thus, as suggested by Gander (6), at the time of formation of the glycosidic linkage the galactose molecule must already be in the furanose form. This proposal immediately makes questionable the participation of a known nucleoside diphospho sugar as the galactofuranosyl donor, since the only hexose units so far conclusively identified in this type of compound are known to possess the pyranose configuration (7).

This does not rule out the possibility that a nucleoside of some kind, rather than a nucleotide, might be capable of serving as a donor of galactose units which have already been built up in the desired ring structure. Available evidence indicates that all naturally occurring nucleosides, regardless of the sugar moiety, have the furanoside structure (8-11).
Fig. 1.—Structure of Galactocarolose

It was on this basis that the present investigation of the nucleosides and nucleotides of *P. charlesii* was undertaken.
REVIEW OF THE LITERATURE

A. Isolation, Characterization and Metabolism of Galactocarolose

Galactocarlose was first isolated by Clutterbuck et al. (5) from medium on which P. charlesii, G. Smith, had been grown. Glucose was the sole carbon source in the medium. The structure of galactocarlose was established by Haworth et al. (12) by means of methylation procedures. Additional evidence was presented at that time to show that galactocarlose was the only polygalactose compound synthesized by P. charlesii under these conditions.

The methods of isolation and identification were not sufficiently sensitive to determine conclusively whether the 9 or 10 galactose units present represent an average number of galactose units per molecule, or whether they are actually the only molecular species present.

No further investigation of the biosynthesis of this novel polygalactose was made until 1960, when Gander (6) reported the results of studies on the incorporation of C$^{14}$ from glucose-C$^{14}$ and DL-tartrate-1,4-C$^{14}$ into galactocarlose. The polygalactose was isolated from Rautilin-Thom glucose medium on which P. charlesii had grown in the presence of glucose-U-C$^{14}$. The galactose recovered after acid hydrolysis of the oligosaccharide had a specific activity one-third that of the glucose-U-C$^{14}$ in the medium.
This is tangible support for the theory that the galacto-furanosyl subunits are not synthesized by a pathway where glucose is epimerized to galactose without cleavage of the carbon chain.

Culture of \textit{P. oharlesii} on glucose-2-\textsuperscript{14}C resulted in the formation of labeled galactocarolose whose galactose subunits showed half the specific activity of the labeled glucose. This is interpreted to indicate that each mole of glucose is metabolized via two identical 2-carbon intermediates, each of which is condensed with some 4-carbon compound to yield two moles of galactose or a galactose derivative.

\textit{DL}-tartrate is an attractive candidate for the role of the 4-carbon intermediate, because it has the requisite steric configuration at carbons 2 and 3. Furthermore, the fact that four of the six carbons of galactose in these experiments must have been unlabeled, coupled with the presence of non-isotopic \textit{DL}-tartrate in the Raulin-Thom medium, make it logical to speculate that tartrate, or a metabolite of it, might furnish the 4-carbon unit for galactocarolose synthesis.

Evidence for the participation of tartrate was also presented by Gander (6), who showed that \textsuperscript{14}C from \textit{DL}-tartrate-1,4-\textsuperscript{14}C appeared in galactocarolose. No accurate calculation of dilution factors was made in this case, but it was assumed that only \textit{D}-tartrate was metabolized by \textit{P. oharlesii}.

Several interesting and valid pathways can be postulated for the formation of a 1,5-linked polygalactose by condensation of a 2- and a 4-carbon unit, but it must be remembered that some
structure in which carbon 5 is blocked is needed to maintain the desired furanose configuration in the desired intermediate. Two products fulfilling this condition would be 5-ketogalactose or galactofuranose-5-phosphate.

B. Naturally Occurring Nucleosides

The possibility of participation of a nucleoside in the formation of galactocarbohydrate necessitates a review of the known nucleosides other than those found in the nucleic acids. Some of the compounds to be discussed contain ribose; others contain different pentoses, or in some cases, hexoses.

1. Orotidine

This was the first naturally occurring pyrimidine nucleoside discovered other than those now associated with the nucleic acids. Orotic acid had been isolated by Biscaro and Belloni (12) in 1905, but an orotic acid nucleoside was not reported until 1951, when Michelson and his co-workers (9) isolated such a compound from the mycelia of a certain uridine-requiring mutant of Neurospora. The unexpected lability of the glycosidic bond in orotidine to acid hydrolysis was ascribed to the presence of the carboxyl group on the carbon adjacent to the nitrogen atom to which the carbohydrate was bonded.

Orotic acid and orotidine have both been shown to serve as precursors for orotidine-5'-phosphate, which is subsequently decarboxylated to uridine-5'-phosphate (14).
2. The Spongonucleosides

In the early 1950's, Bergmann and his co-workers (10, 15) isolated from the Caribbean sponge Cryptotethia cryptus several nucleosides not previously encountered in nature. The three compounds identified were given the trivial names spongothymidine, spongouridine, and spongosine.

Spongothymidine yielded thymine on strong acid degradation. The property of facile formation of a tribenzoate derivative and the results of periodate oxidation studies indicated the presence of a pentofuranose structure, but it wasn't until the compound had been reduced by the procedure of Burke (16) that the carbohydrate moiety of spongothymidine was conclusively identified as D-arabinose.

An analogous series of investigations on spongouridine established it as an arabinosyl nucleoside as well. The structures assigned to these two compounds were 1-β-arabinofuranosylthymine and 1-β-arabinofuranosyluracil, respectively. These structures have been confirmed by synthesis (17, 18).

The third spongonucleoside, spongosine, proved to be a purine, rather than a pyrimidine derivative. Both degradative studies (19) and chemical synthesis (20) have established the structure of spongosine as 6-amino-2-methoxy-9-(β-D-ribofuranosyl)-purine, or 2-methoxy-adenine.

The interesting feature of the first two spongonucleosides, in the light of the present discussion, is the presence of pentoses, other than D-ribose, which nonetheless are in the furanose form.
Several other compounds, such as the antibiotics nebularine (21), cordycepin (22), and puromycin (23, 24) have been shown to have nucleoside structures. In these compounds, with the exception of nebularine whose sugar is D-ribose, the sugar moieties are quite complex, but the basic furanose ring structure is present, despite substitution or chain-branching in the sugar itself.

3. Psicofuranine

The first instance of a naturally occurring hexose nucleoside was reported by Schroeder and Hoeksema (8). These investigators were able to isolate a compound with antibiotic activity from the culture medium of Streptomyces hygroscopicus var. decoyinine, and established the structure of the compound as 6-amin-9-β-D-psicofuranosylpurine, or psicofuranine.

This novel compound has a ketohexose as its carbohydrate moiety, but the presence of the furanose structure was shown conclusively by periodate oxidation studies. Confirmation of the reported structure was later obtained by synthesis and comparison of the synthetic with the natural product. Only in one other case has psicose been reported to be a naturally occurring sugar (25).

4. Adenine Glucoside

The most recent report of a non-ribose nucleoside derivative was made by Plaisted (26), who isolated a compound containing only adenine, glucose and phosphate from a perchloric acid extract of cotton leaves. This particular compound may actually be a nucleotide, but the presence of glucose in place of ribose is noteworthy. The phosphate was found to be acid-stable,
and to be present in the proportion of 1 mole per mole of adenine. No data concerning the ring form of the glucosyl moiety has yet appeared in the literature.
A. Stock Cultures

*P. charlesii* A.T.C.C. strain 1887 were maintained on plates containing Czapek-Dox medium-2% agar (12) with 5% glucose as the sole carbon source. Fresh plates were inoculated with mature *P. charlesii* spores every 4 to 6 weeks.

B. Experimental Cultures

500-ml. wide-mouth Erlenmeyer flasks, containing 150 ml. each of Raulin-Thom medium (5) with 50 mg./ml. glucose and 7.2 mg./ml. tartrate as carbon sources, were inoculated with spores from stock cultures of *P. charlesii*. These flasks were kept at 25-30°C. for periods up to 28 days, when the mycelial pads were collected, washed and treated as described below.

C. Perchloric Acid Extraction of Mycelial Pads

Mycelial pads of the desired age were separated from the growth medium by filtration through several layers of cheesecloth. The pads were squeezed dry, washed several times with distilled water, and dried again. The pads were then frozen with liquid nitrogen and ground with mortar and pestle or powdered in a Waring blender.

The ground pads were extracted by stirring 1 hour at 4°C. with 10 ml./gm. of 0.25M perchloric acid. The extract was filtered
through cheesecloth to remove most of the cell debris and titrated to pH 6.5-7.0 with cold 1.0M potassium hydroxide. The neutral extract was stored at 0-5°C, for several hours before removal of insoluble KClO₄ and cell debris by filtering in the cold.

The clear yellow filtrate was lyophilized, taken up in a small volume of water and filtered to remove the last traces of insoluble salts. The pH of the extract was adjusted to 8-9 with ammonium hydroxide and the solution was added to a Dowex-1-formate column prepared according to Hurlbert et al. (27).

The column was eluted by the gradient method, using reservoir solutions of water, 1.0M ammonium formate and 2.0M ammonium formate, in that order. The mixing volume was 500 ml., and each fraction collected was 10 ml.

D. Changes in pH During P. charlesii Growth

Forty flasks of Raulin-Thom glucose medium were prepared, autoclaved and inoculated with P. charlesii spores. One flask was arbitrarily selected to be sampled for pH determinations, and at intervals ranging from 6 to 24 hours, 0.5 ml. aliquots of well-mixed medium were removed with a sterile pipet. The pH of these samples was determined with a Beckman Zeromatic pH-meter, using a special sample holder.

To investigate possible variations in nucleotide distribution with culture age and/or pH of the medium, ten mycelial pads were harvested at 4, 5.25, 10.25, and 21 days after inoculation. Each sample was powdered, extracted and chromatographed on a Dowex-1-formate column as described above.
E. Transfer Experiments with Radioactive Substrates

In each experiment, ten 150-ml. samples of sterile Raunin-Thom glucose medium were inoculated with P. charlesii spores and incubated in the usual manner. During incubation, the pH of the medium was periodically checked by withdrawing a 1-ml. aliquot of well-mixed medium with a sterile pipet and measuring its pH on a Beckman Zeromatic pH-meter.

In order to transfer the mycelial pads to a smaller volume of medium before adding the labeled material, the medium was decanted and pooled, with care being taken not to rupture the pads. Transfer was made 5-6 days after inoculation, when the pH had reached a minimum. After filtration and re-sterilization, 50-ml. aliquots of the medium were pipetted back into the flasks containing the intact pads, and the required amount of radioactive substrate was then added to one or more of the ten flasks.

After three days incubation under normal conditions, the radioactive medium was decanted, combined with the unlabeled medium and refrigerated for further study. Both radioactive and non-radioactive pads were combined, washed, frozen, powdered, and extracted in the usual manner.

1. Acetate-1-C\textsuperscript{14} Transfer Experiment

At the stated point in the above procedure, 100 mc. of sodium acetate-1-C\textsuperscript{14} were added to each of three flasks. At the end of the three-day incubation, a perchloric acid extract of the pads was prepared as previously described and submitted to ion-exchange separation on a Dowex-1-formate column.
The first ten fractions, eluted from the column with distilled water, were combined as fraction A. The radio activity of this pooled material was determined; it was then treated with charcoal, which was eluted with 10% pyridine in 50% ethanol (28). After removal of the eluting solvents and solution of the residue in a small amount of water, another radio activity determination was made. The material was then chromatographed in the isobutyric acid solvent, and scanned to locate any radioactive and/or ultraviolet-absorbing areas. Radioactivity was detected by means of a Forro strip-scanner, adjusted to show fullscale deflection at 300 cpm. All other scans were made at this setting, unless otherwise noted.

2. Glucose-U-C\textsuperscript{14} Transfer Experiment

In this experiment, 100 mc. of uniformly labeled glucose-\textsuperscript{14} were added to each of two flasks. The remainder of the procedure was the same as outlined in the previous section, with the exception that aliquots from each of the first six fractions were plated and counted before these fractions were pooled and treated with charcoal.

F. Incorporation Studies with C\textsubscript{14}-labeled Purines and Pyrimidines

Three separate experiments, designed to study the incorporation into \textit{P. charlesii} of uracil-2-C\textsubscript{14}, adenine-8-C\textsubscript{14}, and guanine-8-C\textsubscript{14}, respectively, were carried out.

Sufficient Raulin-Thom glucose medium was prepared for ten flasks. The salt mixture and the glucose were autoclaved
separately, 100 c. of the desired \( ^{14}C \)-labeled base being added to the glucose solution shortly before sterilization.

The two solutions were combined under sterile conditions and the flasks were inoculated with \( P. \) charlesii spores. After 14 days, the pads were harvested, washed, and extracted in the usual manner and submitted to column chromatography as previously described.

Samples from each tube under the first large peak eluted from the column were plated and counted in a Nuclear-Chicago Model 181A gas-flow counter. Later peaks were pooled and desalted by charcoal treatment. In this series of experiments, the charcoal was washed successively with water, 0.01M versene (pH 7), and again with water, before elution with 50% ethanol, according to Pontis et al. (29).

These pooled materials were chromatographed on paper in the isobutyric acid solvent, as described in section G-2. After the radioactive areas had been located by scanning on a Forro strip-scanner, they were eluted with water and samples were plated and counted for determination of specific activities of the individual compounds. All counts were corrected to 100% counting efficiency.

G. Isolation and Identification of the Acid-Soluble Nucleotides of \( P. \) charlesii Mycelial Pads

After the column separation of the acid-soluble nucleotides was completed, the tubes representing the various ultraviolet-
absorbing peaks were pooled, and shaken with 10 mg./ml. of acid-washed Darco-G activated charcoal for one hour at room temperature. The charcoal was collected by centrifugation, washed twice with distilled water and finally eluted by shaking with 10% pyridine in 50% ethanol for 1.5 hours at room temperature.

The ethanolic pyridine solutions of nucleotide material were evaporated to dryness in vacuo, redissolved in a small amount of water and re-evaporated to remove the last traces of pyridine.

Solution of the residue in a minimum amount of water provided samples which could be further purified by paper chromatography before undergoing various chemical and spectral analyses.

1. **Preparative Chromatography**

Crude samples of each of the major UV-absorbing peaks obtained by column chromatography were stripped on sheets of Whatman 3MM filter paper for ascending chromatography in isobutyric acid:water:ammonia, 66:33:1.

After 16-24 hours, the sheets were removed from the tank, vigorously aerated to remove as much isobutyric acid as possible and examined under a Lineralite lamp (2537 Å) to locate the nucleotide bands. After outlining, the ultraviolet-absorbing bands were cut out in addition to an appropriate area of blank strip taken from ahead of the fastest-migrating component, and eluted with distilled water in a capillary eluting apparatus (30).

2. **Identification of Individual Nucleotides**

Ultraviolet spectra were obtained for each fraction at pH 2, 7, and 11, and an indication of the purine or pyrimidine base involved was obtained from the spectral characteristics.
The individual components were then rechromatographed along with authentic samples of all available members of the base-nucleoside-nucleotide series to which they were thought to belong. The solvent systems employed in this procedure were 1) isobutyric acid:water:ammonia, 66:33:1; 2) 95% ethanol:1.0M ammonium acetate, pH 7.5, 7:3; 3) 0.1M potassium dihydrogen phosphate, pH 6.5:ammonium sulfate:isopropanol, 100:60:2 (31).

3. Identification of Bases

Purine bases were identified by hydrolysis of nucleosides and nucleotides in 2.5N sulfuric acid for 2 hours at 100°C., followed by adsorption onto and elution from charcoal, evaporation of eluent, re-solution in distilled water and chromatography with authentic bases in nucleotide solvents 1, 2, and 3.

Pyrimidine nucleosides and nucleotides, hydrolysis of which is somewhat more difficult, required evaporation of the sample to dryness, solution in 60% perchloric acid, hydrolysis for one hour at 100°C., charcoal treatment, and chromatography as described in the previous paragraph.

4. Identification of the Sugar Components of Nucleotides

The principal method used to identify the sugar moieties in the nucleotides was comparative ascending chromatography, with authentic samples as controls. Following acid hydrolysis, the sugar samples were freed of salts by electrodialysis and spotted on Whatman 3MM paper for chromatography in the following solvent systems: 1) butanol:pyridine:water, 6:4:3; 2) methanol:formic acid:water, 80:15:5; 3) ethyl acetate:pyridine:water, 36:10:15 (32).
In several cases, semi-quantitative assays for galactose were made by use of the characteristic spectrum produced by this sugar on reaction with orcinol in the presence of concentrated sulfuric acid (33). A much more sensitive and accurate galactose assay was also employed (34). This involved a coupled enzyme system composed of galactokinase-phosphoenol pyruvate kinase-lactic dehydrogenase, in which galactose concentration is reflected in the characteristic disappearance of absorption at 340m\(\mu\) on oxidation of DPNH.

The galactokinase assay system has been shown to provide a 1:1 stoichiometric relationship between galactose phosphorylation and DPNH oxidation. The system had also been thought to possess a high degree of specificity for galactose (34), but experiments presented later in this dissertation show that both D- and L-altrose, as well as an unknown sugar, are also phosphorylated by this system. A small amount of hexokinase present in the galactokinase preparation also results in the formation of glucose-6-phosphate, but this can readily be distinguished from the sugar-1-phosphates.
CHEMICAL SYNTHESSES

A. Preparation of N-Acetyl adenine and Its Monochloromercuri Derivative

Five grams of adenine were acetylated and then converted to the N-acetyl monochloromercuri derivative according to the procedure of Davoll and Lowy (35). The latter product, 6-acetamido-9-chloromercuripurine, was obtained in 56% yield.

B. Preparation of L-Galactose (36)

L-galactose was prepared for use in an investigation of the specificity of the coupled galactokinase assay system. Substitution of this sugar for its D-isomer in the assay system failed to result in phosphorylation by galactokinase, as indicated by the absence of the characteristic decrease in OD₃₄₀ due to DPNH oxidation.

L-galactose was prepared by sodium amalgam reduction of D-galacturonic acid. The intermediate L-galactonolactone was not isolated, but was reduced directly to L-galactose.

After removal of salts and in vacuo evaporation of solvent, L-galactose was obtained as a thin, colorless sirup. Repeated solution in anhydrous methanol and evaporation under reduced pressure resulted in the formation of a white powder, M.P. 158-160°C. This product was not crystallized, but on chromatography in butanol:pyridine:water and methanol:formic acid:water proved to
be chromatographically homogeneous when detected by means of ammoniacal silver nitrate and periodate-benzidine spray reagents.

The L-galactose, as anticipated, was inseparable from its D-isomer in both solvents, but on the basis of the synthetic procedure employed, its melting point, its migration at the same rate as D-galactose in both solvents during paper chromatography, and its failure to serve as a substrate for galactokinase, there is little question that the product is L-galactose.

C. Acetylation of D-Galactose

Anhydrous D-galactose was acetylated according to the procedure of Schlubach and Prochownick (37). On cooling of the ethanolic solution, several crops of crude 1,2,3,4,6-penta-O-acetylated D-galactopyranose were obtained. These were combined, and on recrystallization from 95% ethanol yielded white crystals, m.p. 142.5-143°C.

The combined mother liquors were concentrated in vacuo to about 250 ml., and seeded with a crystal of the 1,2,3,5,6-penta-O-acetylated D-galactofuranose, then stored at 4°C. for several days. No crystallization was effected; only a brown sirup separated from the ethanolic solution.

This sirup was redissolved in ethanol, seeded once more with a crystal of galactofuranose pentaacetate and stored at 12-16°C. After 6-8 days at this temperature, a solid layer of light-brown crystals formed on the bottom and sides of the container. After decolorization with Darco-G charcoal and two re-
crystallizations from 95% ethanol, a pure-white crystalline product, M.P. 98.5-99.5°C., was obtained in 17% yield. The melting points obtained for both pentaacetates are in close agreement with the values appearing in the literature (37, 38).

D. Preparation of 9-α-Galactopyranosyladenine

This compound was prepared by a modification of the procedure used by Wolfrom et al. (39) for synthesis of maltose and cellobiose nucleosides.

Five grams of 1,2,3,4,6-penta-O-acetylad-α-D-galactopyranose were converted to 2,3,4,6-tetra-O-acetylad-β-D-galactopyranosyl bromide (40). One and one-half grams of the sirupy product was dissolved in ten milliliters of toluene and utilized in the reaction described below.

One gram of 6-acetamido-9-chloromercuripurine and one gram of cadmium carbonate were suspended in 35 ml. of toluene in a 50-ml. micro flask fitted with mechanical stirrer and distillation apparatus. The mixture was dried by azeotropic distillation of 10 ml. of solvent. The toluene solution of the tetraacetylgalactopyranosyl bromide was added and the mixture was refluxed for three hours with vigorous stirring.

After cooling, the clear yellow solution was decanted and diluted with five volumes of petroleum ether (B.P. 30-60°C.), whereupon an amorphous yellow-white precipitate formed. The product was stored at 5°C. for 0.5 hour, then filtered. The solid product was extracted with several portions of hot chloroform,
after which the combined chloroform extracts were washed successively with 30% aqueous potassium iodide, then water, and dried overnight over sodium sulfate.

The solvent was evaporated in vacuo, leaving a thin yellow sirup which was dissolved in 50 ml. of a methanolic solution containing 15 ml. of n-butylamine and refluxed for three hours.

On removal of the methanol-n-butylamine under reduced pressure, a dark sirup was obtained. This was dissolved in water-saturated 1-butanol and evaporated in vacuo in a Rinco flash-evaporator. A granular solid was deposited on the walls of the evaporating flask as 1-butanol was removed; on completion of evaporation, the solid was dissolved in a few ml. of water and allowed to evaporate in an open vessel at room temperature.

The product was deposited as 0.093 gms. of a glassy yellow-brown solid, which was pulverized and submitted to further purification by paper chromatography as described below.

A portion of the crude product was redissolved in water and stripped on a 16 x 16-inch sheet of Whatman 3MM filter paper for ascending chromatography in isobutyric acid:water:ammonia, 66:33:1 for 16-24 hours at room temperature. The chromatogram was vigorously aerated to remove isobutyric acid, and the nucleoside material was located by observation under a Mineralite lamp (2537 Å).

The ultraviolet-absorbing area, Rf 0.62, was cut from the sheet and eluted with distilled water. The aqueous solution was again allowed to evaporate from a watch glass, but repeated attempts
failed to yield a crystalline product. Purification by means of
the picrate derivative was then attempted.

Twenty-five milligrams of the amorphous adenine galacto-
pyranose were dissolved in 2 ml. of 95% ethanol to which sufficient
water was added to complete solution. The solution was warmed on
the steam bath and 2 ml. of a saturated ethanolic picric acid
solution were added.

After the solution had been heated to boiling, it was
quickly cooled, whereupon a yellow solid formed. This was stored
overnight at 4°C., filtered and air-dried. The yellow solid melted
over the range 225–230°C., but attempts to recrystallize it from
aqueous ethanol were unsuccessful.

The nucleoside was regenerated from the picrate by stirring
an aqueous solution of the derivative with a suspension of Dowex-
1-carbonate resin until the solution became colorless. This so-
lution was filtered, concentrated, rechromatographed in the iso-
butyric acid solvent and submitted to partial characterization as
described in the succeeding paragraphs.

Hydrolysis of a sample of the material in 2.5M HCl for two
hours at 100°C. released a sugar migrating like authentic D-galac-
tose in three different solvents. When assayed by the coupled
galactokinase system, the sugar caused a decrease in OD_{340},
indicating it was galactose.

Another sample of the adenine galactopyranose was treated
with 0.001M periodic acid in 0.1M acetate buffer, pH 4.25 (41).
Consumption of periodate was followed by measurement of the decrease in optical density at 265nm.

After 24 hours, the molar ratio of periodate consumed to adenine galactopyranose was 2:1. Excess periodic acid was destroyed by the addition of 0.05 ml. of 0.01M sodium arsenite, and aliquots of the reaction mixture were assayed for formaldehyde by the chromotropic acid method (42), using glycerol, treated with periodate, as a standard.

Formaldehyde production was less than 0.1 mole per mole of adenine galactoside. This is evidence of a hexopyranose, though because of the small quantities of material available, no determination of formic acid production was made, making an unequivocal identification of a pyranoside impossible.

These data substantiate the identity of the synthetic material as adenine galactopyranose.
RESULTS

A. Column Chromatography of Acid-Soluble Nucleotides

Figure 2 shows a typical elution pattern obtained with an ammonium formate gradient system employing a Dowex-1-formate column (27); each tube represents a volume of 10 ml. Peaks \( A \) and \( A_1 \) were eluted with water; peaks \( B \) and \( C \) were eluted with 1.0M ammonium formate in the reservoir, peaks \( D \) and \( E \) appeared after the reservoir solution had been changed to 2.0M ammonium formate. Once peak \( E \) had been eluted, no further nucleotide material could be brought off the column, even at higher concentrations of ammonium formate, both with and without added formic acid in the reservoir.

As will be seen in later figures, only minor variations in the nucleotide elution patterns occurred as the mycelial pads of different ages were investigated. However, there was some variation in the relative quantities of some nucleotides in these experiments.

In the experiments employing radioactive purines and pyrimidines, some previously unidentified compounds were detected, but proved to be either non-nucleotide materials, or to be present in such small quantities as to make identification impractical.
Fig. 2.—Typical Distribution of Acid-Soluble Nucleotides in *P. charlesii*.
B. Paper Chromatography of Nucleotide Material

1. Fraction A

Chromatography of crude fraction A in the isobutyric acid solvent resulted in the separation of two major and three minor ultraviolet-absorbing bands. One major band migrated at Rf 0.88, and on spectral analysis and rechromatography against authentic samples in three different solvents was shown to be adenine.

The fastest-migrating minor band was located at Rf 0.57, and was identified as uracil by virtue of its spectral and chromatographic properties.

The second major area of ultraviolet-absorbing material, hereafter referred to as compound A_x, was the most pronounced in intensity and was found to migrate at Rf 0.49. The identification of this substance is described more fully in the succeeding section.

Two more minor bands were located at Rf 0.40 and 0.33, respectively, but neither occurred in sufficient quantity even to provide spectral data. These two fractions were not identified.

2. Fraction A_x

The band designated as A_x was eluted from the chromatogram of crude fraction A with distilled water, concentrated and rechromatographed in the isobutyric acid solvent. This purified material was again eluted, along with a blank strip. A sample of each was evaporated to dryness under an air-stream, dissolved in 60% perchloric acid and heated in a boiling water-bath for one hour.
Chromatography of the hydrolyzate after it had been freed of salts, using authentic samples of purines and pyrimidines as controls, clearly showed the base component of $A_x$ to be uracil. Confirmatory evidence for the presence of uracil was obtained from the ultraviolet absorption spectra of unhydrolyzed $A_x$, which are shown in Figure 3.

Table 1 summarizes the chromatographic behavior of both PCA-hydrolyzed and unhydrolyzed samples of $A_x$ in the various nucleotide solvents.

**Table 1**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Solvent 1</th>
<th>Solvent 2</th>
<th>Solvent 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_x$ (hydrolyzed)</td>
<td>.62</td>
<td>.66</td>
<td>.54</td>
</tr>
<tr>
<td>$A_x$ (unhydrolyzed)</td>
<td>.49</td>
<td>.46</td>
<td>.57</td>
</tr>
<tr>
<td>Uracil</td>
<td>.62</td>
<td>.65</td>
<td>.54</td>
</tr>
<tr>
<td>Uridine</td>
<td>.46</td>
<td>.49</td>
<td>.60</td>
</tr>
<tr>
<td>Deoxyuridine</td>
<td>.54</td>
<td>.77</td>
<td>--</td>
</tr>
<tr>
<td>UMP</td>
<td>.22</td>
<td>.11</td>
<td>.73</td>
</tr>
</tbody>
</table>

Although the position of elution from the Dowex-1-formate column suggested that $A_x$ would probably not contain phosphate, determination of acid-labile phosphate was made by the method of Chen *et al.* (43), after hydrolysis in 1.0N HCl for 15 minutes at 100°C. An additional sample was ashed by the procedure of Ames
and Dubin (44) and analyzed for total phosphate. No significant amount of phosphate was detected by either analysis.

Fig. 3.—Ultraviolet Absorption Spectra of Compound Aₘ
To identify the sugar component of A₁, a sample was hydrolyzed in 2.5N sulfuric acid for two hours at 100°C.; a second sample was treated with 2.5N HCl under identical conditions. The hydrolyzates were neutralized, electrodialyzed and chromatographed in the three sugar solvent systems. As anticipated, both A₁ and several known nucleosides and sugars underwent degradation as a result of this harsh treatment, as indicated by the appearance of several anomalous carbohydrate spots on the chromatograms.

Successful release of the carbohydrate moiety was effected when an aqueous solution of the material was first treated with 3% sodium amalgam at room temperature for 45 hours to reduce the pyrimidine ring (16). After separation from the mercury, the solution was adjusted to pH 2 with HCl, heated in a boiling water bath for 30 minutes, cooled and neutralized.

The supernatant solution after charcoal treatment was electrodialyzed, concentrated, and spotted on Whatman 3MM paper along with several authentic carbohydrate control samples. The chromatogram was ascended three times in ethyl acetate:pyridine:water. Development with periodate-benzidine spray reagent showed three carbohydrate spots, at R₆ 0.83, 2.47, and 3.73, respectively, plus an additional carbohydrate-positive spot at the starting line.

Development of the reverse side of the same chromatogram with ammoniacal silver nitrate showed the same four spots, with the area at R₆ 0.83 the most prominent. Galactose migrated at exactly the same R₆ as this spot on the chromatogram. On another
chromatogram run in the same solvent system, only the spots at $R_{\text{glu}} 0.83$ and at the starting line appeared when the chromatogram was developed with ammoniacal silver nitrate.

In an attempt to identify the material migrating at $R_{\text{glu}} 0.83$, a sample of the solution obtained on elution of the material, from the chromatogram, along with the appropriate blank, was submitted to analysis by the coupled galactokinase assay system, in which ATP$^{32}$, terminally-labeled by the exchange method (45), was included.

After the decrease in $OD_{340}$ had reached a maximum, the reaction mixture was deproteinized by heating in a water bath at 100°C for two minutes, cooled and centrifuged. The supernatant was treated with charcoal to remove unreacted ATP$^{32}$, filtered and concentrated in vacuo at room temperature. This solution, with similar solutions obtained from control samples of glucose, galactose, and the reduction blank, was stripped on Whatman 3MM paper and ascended in methanol:formic acid:water. After drying, strips from each chromatogram were cut out and scanned for radioactivity.

As anticipated, both the glucose control and the reduction blank showed radioactivity at $R_f 0.55$, where glucose-6-phosphate is known to migrate in this solvent. The galactose control, apparently contaminated with glucose, showed activity at $R_f 0.15$, 0.32 (galactose-1-phosphate), and 0.55.

An anomalous radioactive area was noted at $R_f 0.46$ on the chromatogram of the phosphorylated $A_x$ hydrolyzate. This area
showed an appreciable amount of radioactivity so it was eluted with water, and concentrated for rechromatography.

To check the position of galactose-1-phosphate, a sample of galactose-1-C\textsuperscript{14} was phosphorylated with the coupled enzyme system, using unlabeled ATP. Samples of D- and L-altrose were phosphorylated with ATP\textsuperscript{32} in like manner, and after deproteination and purification, samples of these three reaction mixtures, plus the unknown sugar phosphate, were chromatographed in the methanol:formic acid:water system.

A scan of the C\textsuperscript{14}-galactose reaction products confirmed the position of galactose-1-phosphate at R\textsubscript{f} 0.32. Galactose-1-C\textsuperscript{14} was found at R\textsubscript{f} 0.46. Phosphorylated derivatives of L- and D-altrose were detected at R\textsubscript{f} 0.44 and 0.46–0.52, respectively. The width of the radioactive band in the D-altrose experiment may be due to contamination, though chromatography of the sugar in ethyl acetate:pyridine:water showed only one silver nitrate-positive area.

The phosphorylated derivative of the A\textsubscript{x} sugar moiety migrated at R\textsubscript{f} 0.50, distinguishing it from the L-altrose derivative and making its identity with the D-altrose phosphate questionable. The possibility of D-altrose as the unknown sugar was ruled out altogether when it was shown to migrate at R\textsubscript{glu} 1.09 in the ethyl acetate:pyridine:water system.

A comparison of the chromatographic characteristics of the various aldoses and their phosphorylated derivatives is presented in Table 2. The solvent systems used are the same as those described earlier in this section.
TABLE 2
RELATIVE MIGRATION RATES OF ALDOSES AND THEIR PHOSPHORYLATED DERIVATIVES

<table>
<thead>
<tr>
<th></th>
<th>Free Sugar (R&lt;sub&gt;g&lt;/sub&gt;&lt;sub&gt;lu&lt;/sub&gt;)</th>
<th>Sugar Phosphate (R&lt;sub&gt;f&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1.00</td>
<td>0.54 (6-P)</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.83</td>
<td>0.32 (1-P)</td>
</tr>
<tr>
<td>L-Altrose</td>
<td>1.09</td>
<td>0.44</td>
</tr>
<tr>
<td>D-Altrose</td>
<td>1.09</td>
<td>0.46-52</td>
</tr>
<tr>
<td>A&lt;sub&gt;x&lt;/sub&gt;</td>
<td>0.83</td>
<td>0.50</td>
</tr>
<tr>
<td>Guloose</td>
<td>1.50</td>
<td>---</td>
</tr>
<tr>
<td>Talose</td>
<td>0.95</td>
<td>---</td>
</tr>
</tbody>
</table>

It can be seen that the characteristics of the unknown carbohydrate and its phosphate derivative do not agree with those of any of the known hexoses or their phosphates. Mannose is not phosphorylated by galactokinase; allose was not available for investigation, but due to structural differences between it and galactose, its function as a galactokinase substrate is doubtful.

A sample of unhydrolyzed, unreduced A<sub>x</sub> was oxidized with 0.001M periodic acid in 0.1M acetate buffer (40) at room temperature for 24 hours in a stoppered cuvette. Observation of the decrease in optical density at 265m indicated the consumption of 2.05 moles of periodate per mole of A<sub>x</sub> (based on a molar extinction coefficient of 10,000 for uracil) (30).
After addition of 0.05 ml. 0.01M sodium arsenite to the reaction mixture, an aliquot was assayed for formaldehyde by the chromotropio acid method (41). Formaldehyde was found to be present in the ratio of one mole per mole of A\textsubscript{x} oxidized by periodate. Due to the small samples available, no determinations of formic acid were made.

A sample of the carbohydrate material released from A\textsubscript{x}, after enzymic phosphorylation and chromatography, was treated with periodate in a like manner. After 24 hours, chromotropic acid assay of the reaction mixture showed the presence of formaldehyde, indicating the sugar moiety was still in the furanoside configuration established by periodate oxidation studies on the unhydrolyzed A\textsubscript{x} sample.

These data indicate that A\textsubscript{x} is a uracil hexofuranoside. The similarity of the ultraviolet spectra of A\textsubscript{x} to those of uridine and deoxyuridine suggest that the carbohydrate residue is probably attached to the nitrogen at position 3 in the pyrimidine ring.

The other carbohydrate compounds found in the hydrolyzate of the amalgam-treated sample were not identified. No UV-absorption was detected in any of these areas before the chromatograms were developed.

3. Fraction A\textsubscript{1}

The peak A\textsubscript{1}, which in this experiment was partially separated from peak A during elution of the column with water, was shown by comparative paper chromatography to consist mainly of adenosine. Traces of compound A\textsubscript{x} and the slower-moving components
of fraction A (R_f 0.40 and 0.33) were also detected after A had been chromatographed in the isobutyric acid solvent system.

4. Fractions B and C

These two fractions, which were never completely separated by ion-exchange chromatography, were initially collected and analyzed separately. However, because the nucleosides and nucleotides isolated from both fractions proved to be the same, the procedures employed for separation and identification will be considered in the same section.

After the two fractions had been pooled, freed of salt by charcoal absorption and concentrated, paper chromatography in the isobutyric acid solvent showed a total of six distinct ultraviolet-absorbing bands in each fraction.

In fraction B, the major component showed an R_f value of 0.10. Two slightly less pronounced bands were detected at R_f 0.23 and 0.34. Three minor bands appeared at R_f 0.44, 0.48, and 0.57. In fraction C, the same six bands were identified, but the most intense bands in this case were those at R_f 0.23 and 0.34.

After elution from the chromatograms, spectra were obtained for each component, and each was rechromatographed in the three solvents along with authentic samples of the group of compounds possessing the most similar spectral characteristics.

The individual compounds were hydrolyzed in acid and after purification with charcoal were co-chromatographed with known bases in the three solvents mentioned previously.
When materials from fractions B and C migrating with equal R_f values showed identical characteristics on rechromatography both before and after acid hydrolysis, they were combined. All further studies were done on the combined samples, hereafter designated as B-1 through B-6, in order of ascending R_f values in the isobutyric acid solvent.

Compound B-1 showed the spectral and chromatographic characteristics of a uracil nucleotide, although it was difficult to distinguish in this way between UTP, UDP, UDPG, and UDPGal. Therefore, a sample of B-1 was hydrolyzed in 0.01N HCl for ten minutes at 100^\circ C. This treatment is sufficient to free acid-labile phosphates from nucleoside di- and triphosphates, as well as to release sugars from any nucleoside diphospho sugars present.

Mild acid hydrolysis of B-1 liberates a mixture of ultraviolet-absorbing compounds which migrate at R_f values equal to those of UTP and UDP in the solvents used. Earlier, it had been shown that hydrolysis of B-1 in 60% perchloric acid for one hour at 100^\circ C. released uracil as the sole nitrogenous base.

Additional evidence for the identity of B-1 was obtained by enzymic assay. A sample of purified B-1 was assayed by the UDPG-dehydrogenase enzyme system (46), and the increase in optical density at 340 nm was sufficiently great to indicate that UDPG is the sole component of B-1.

The only known compounds whose chromatographic behavior and spectral properties consistently resembled those of compounds B-2 and B-3 were TPN and DPN, respectively. Consequently, these
two compounds were assayed by enzyme systems known to be specific for each of the pyridine nucleotides.

A sample of B-2 was substituted for TPN in the glucose-6-phosphate dehydrogenase system (47). A known sample of TPN was run separately as a control, and an increase in optical density at 340 mμ was considered evidence for the presence of TPN in the unknown sample.

A similar procedure for the identification of B-3 involved the use of yeast glyceraldehyde-3-phosphate dehydrogenase (48), known to possess a high degree of specificity for DPN. Substitution of B-3 for DPN, along with a suitable control, produced a sufficient increase in optical density at 340 mμ to establish the identity of B-3 as DPN.

Confirmatory evidence for the identities of these two compounds were obtained by chromatographic identification of adenine as the nitrogenous base in both compounds following acid hydrolysis, by showing the release of ribose by acidic hydrolysis of both compounds, and by demonstrating the presence of phosphate in both compounds by use of the Bandurski phosphate spray reagent directly on the paper chromatograms.

The minor components B-4, B-5, and B-6 were successfully identified by means of comparative chromatography in the various solvents. B-4 was shown to migrate at Rf values very similar to those of authentic samples of adenosine-5'-monophosphate. This compound also yielded adenine and ribose on hydrolysis in mineral acids, and demonstrated the typical spectral characteristics of the adenine nucleotides.
The minor components B-5 and B-6 were identified by their spectra, which were typical of the uracil series, and by chromatography with authentic samples, as uridine and uracil, respectively.

5. Fraction D

The initial paper chromatography of fraction D in the isobutyric acid system showed three ultraviolet-absorbing compounds, at $R_f$ 0.20, 0.32, and 0.47. These were designated as D-1, D-2, and D-3, the most intense absorption being associated with D-2.

Ultraviolet spectra were obtained on the eluted materials, and were indicative of the presence of adenine in all three compounds. When each was rechromatographed in the three nucleotide solvents along with the complete series of adenine nucleosides and nucleotides, D-1, D-2, and D-3 were tentatively identified as ATP, ADP, and AMP, respectively.

The major component, D-2, was conclusively identified as ADP by assaying it in a coupled phosphoenol pyruvate kinase-lactic dehydrogenase system, where the presence of ADP is reflected in the typical disappearance of absorption at 340 nm on oxidation of DPNH. The phosphoenol pyruvate kinase enzyme has also been shown to utilize UDP, at a much lower rate than ADP (49). However, in conjunction with the other available evidence, this can be considered as confirmatory evidence for the identification of ADP as the major component of fraction D. The ATP present is believed to be due to incomplete separation of peaks D and E during column
chromatography, the ATP to spontaneous degradation occurring during the isolation procedures.

6. Fraction E

This fraction was separated into four bands by the isobutyric acid solvent. By far the most prominent absorption was shown by the band at $R_f$ 0.18-0.20, designated as E-1. The less intense bands, located at $R_f$ 0.30, 0.46, and 0.74 were subsequently referred to as E-2, E-3, and E-4.

Once again, all four bands exhibited spectra in the ultraviolet range consistent with the presence of adenine in their structures. Hydrolysis of each in 2.5N sulfuric acid for two hours resulted in the release of adenine as the only detectable base upon paper chromatography in the three solvents employed.

Compound E-1 was identified as ATP by the use of the coupled galactokinase-PEP kinase-lactic dehydrogenase system. This has been described in an earlier section as a quantitative method for determination of galactose, but by providing known galactose and substituting E-1 for ATP in the incubation mixture, it is possible to show the decrease in optical density at 340nm associated with galactose phosphorylation in the complete system.

As in fraction D, compound E-2, tentatively identified as ADP through its chromatographic behavior, was identified as the expected nucleotide with the PEP kinase-lactic dehydrogenase system discussed in the previous section.

The minor components E-2 and E-4 were identified as ATP and adenosine by the previously described procedures. It seems,
in view of the relative amounts of the four components, and the position at which fraction E was eluted from the column, that ATP can be considered the principal compound of this fraction. ADP, ATP, and adenosine are then assumed to be primarily the result of overlap with peak D (in the case of ADP) and degradation of ATP during isolation and purification.

C. Changes in pH During Growth

The changes in the pH of Raulin-Thom glucose medium over a 21-day period of incubation with P. charlesii are shown in Figure 4.

The pH, 4.25 before inoculation, decreased steadily during the first five days of growth. Ten mycelial pads were harvested at four days to check the nucleotide distribution during the period when the medium was becoming more acidic. The result of ion-exchange separation of the extract of four-day pads is shown in Figure 5.

After 5.25 days of incubation, the pH had apparently reached a minimum, about 2.80. Another ten pads were harvested, and the extract of these pads gave the nucleotide distribution shown in Figure 6.

The pH remained nearly constant over the next 5-6 days, showing an increase again on the tenth day. Column chromatography of an extract of pads collected at 10.25 days gave the nucleotide pattern depicted in Figure 7.

The steady increase in pH continued for an additional ten days, surpassing the value obtained for the sterile medium before
Fig. 4.—Variation in pH with Age of *P. charlesii* Culture
Fig. 5.—Acid-Soluble Nucleotides in *P. charlesii* After Four Days Growth.
Acid-Soluble Nucleotides in P. charlesii After 5.25 Days Growth.
Fig. 7.—Acid-Soluble Nucleotides in *P. charlesii* After 10.25 Days Growth.
inoculation and reaching a maximum of 6.40 on the twentieth day of growth. The remaining ten pads were harvested at 21 days, and their nucleotide distribution is recorded in Figure 8.

Paper chromatographic investigation of the individual fractions obtained in the column separation of these four extracts showed no apparent differences in the types of compounds present at various ages. Each chromatogram showed UV-absorbing bands corresponding to those shown in the respective fractions of previous experiments.

The relative amounts of $A_x$, to which particular attention was paid, seemed to increase as the culture aged. However, no measurement of the absolute concentrations of $A_x$ were made, so it is impossible to determine whether this apparent increase is a function of the different amounts of mycelial material extracted in a given experiment, or whether it reflects a significant change in the metabolic behavior of P. charlesii during the experiment.

Regardless of the cause, the level of $A_x$, as indicated by the intensity of its UV-absorption on the paper chromatograms, seemed to become constant after the fifth day, although the relative size of peak $A$ itself continued to increase throughout the incubation period.
Fig. 8.—Acid-Soluble Nucleotides in *P. charlesii* After 21 Days Growth.
Figure 9 shows the nucleotide elution pattern obtained on column chromatography of the extract of the P. charlesi mycelial pads obtained from this experiment.

Fractions 1-10, which were pooled as fraction A, showed a total activity of $1.91 \times 10^6$ cpm., before adsorption on charcoal. This fraction, 100 ml. in volume, was concentrated in vacuo to 10 ml. and treated with charcoal. After elution with pyridine-ethanol and removal of the volatile solvents, the residue was taken up in a small volume of water and aliquots were plated and counted.

As a result of this treatment, the total activity in the purified fraction A fell off to $3.27 \times 10^4$ cpm. By concentrating the supernatant solution from the charcoal treatment and chromatographing it with samples of acetate-1-$^{14}$C in various solvents, it was found that the majority of the activity remaining in the supernatant was unreacted acetate-1-$^{14}$C, along with traces of other labeled carbohydrate materials.

In Figure 10, it is seen that chromatography of purified fraction A in the isobutyric acid solvent shows that a small amount of radioactivity is associated with the UV-absorbing band at the $R_f$ established for compound $A_x$. Another radioactive area appears at $R_f 0.40$, where a UV-absorbing compound, $A_y$, has been observed. Attempts to determine spectra of $A_y$ failed to show typical nucleotide curves at several pH values, and no further identification of the compound was attempted.
Fig. 9.—Acid-Soluble Nucleotides in *P. charlesii* Incorporating Acetate-1-Cl4.
Fig. 10.—Reproduction of Chromatogram, Fraction A, Acetate-\textsuperscript{14}C Experiment, Showing Radioactivity Distribution.
The third area of radioactivity appears at \( R_f \) 0.57, and on this basis was assumed to be uracil, though there was insufficient material on the chromatogram to observe UV absorption. No further identification of this band was made.

It is important to note that this investigation makes no attempt to differentiate between incorporation of \( ^{14}C \) into the nitrogenous base or sugar moiety, or both, of the nucleosides and nucleotides. In the case of \( A_x \), known to contain uracil, the presence of isotope in both portions of the molecule is a distinct possibility.

Because the amount of material available was quite small, no further work was done on this fraction, nor were the other fractions given more than a cursory examination for radioactivity. No appreciable amounts of activity were detected in any of the other fractions.

E. Glucose-\( ^{14}C \) Transfer Experiment

The nucleotide elution sequence in this experiment is shown in Figure 11. The crude pooled fraction A, before charcoal treatment, contained \( 3.3 \times 10^6 \) cpm. After charcoal adsorption and elution, recovery of radioactivity amounted to only 27%, or a total of \( 9.4 \times 10^4 \) cpm.

Once again, because such small amounts of each fraction were available, only fraction A was submitted to paper chromatography in the isobutyric acid solvent and subsequent scanning to locate any radioactive areas.
Fig. 11.—Acid-Soluble Nucleotides in P. charlesii Incorporating Glucose-U-C14.
Figure 12 shows a reproduction of both the chromatogram and its radioactivity distribution. It can be seen that radioactivity is present in the \( A_x \) band, as well as in the band migrating at \( R_f 0.57 \), presumed to be uracil. In this case, band \( A_y \) was not detected by examination under UV light or by the presence of radioactivity. Here again, it was not determined whether \(^{14}C \) had been incorporated into the base or the sugar portions of the \( A_x \) molecule.

The limited quantities of material prevented further identification studies on \( A_x \), but when it and the uracil fraction were eluted and the eluents were diluted to equal volumes, the total radioactivity in the \( A_x \) solution was found to be 2.58 times that of the uracil fraction.

No other detectable radioactive areas were found on this chromatogram. The other fractions obtained by column separation were not examined.

F. Incorporation Studies with Radioactive Purines and Pyrimidines

1. Uracil-2-\(^{14}C\)

The nucleotide elution patterns from this experiment appear in Figure 13. Shown in Table 3 are the relative activities of the individual fractions of peak A before pooling. These were combined, charcoal-treated, and chromatographed in the isobutyric acid solvent.
Fig. 12.—Reproduction of Chromatogram, Fraction A, Glucose-U-\(^{14}\)C Experiment, Showing Radioactivity Distribution.
Fig. 13.—Acid-Soluble Nucleotides in *P. charlesii* Incorporating Uracil-2-Cl4.
### TABLE 3
ACTIVITIES OF INDIVIDUAL FRACTIONS, PEAK A, URACIL-2-\(^{14}\)C EXPERIMENT

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Activity (cpm. x 10(^{-3}))</th>
<th>Tube No.</th>
<th>Activity (cpm. x 10(^{-3}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0</td>
<td>6</td>
<td>39.2</td>
</tr>
<tr>
<td>2</td>
<td>19.0</td>
<td>7</td>
<td>21.7</td>
</tr>
<tr>
<td>3</td>
<td>16.5</td>
<td>8</td>
<td>7.4</td>
</tr>
<tr>
<td>4</td>
<td>45.4</td>
<td>9</td>
<td>3.0</td>
</tr>
<tr>
<td>5</td>
<td>57.4</td>
<td>10</td>
<td>2.1</td>
</tr>
</tbody>
</table>

A reproduction of the chromatogram of fraction A and its radioactivity scan are shown in Figure 14. The UV-absorbing A\(_x\) band exactly corresponds to the slower-migrating radioactive area, which substantiates the previously-described identification of uracil as the base component of A\(_x\). The faster-migrating compound was identified by comparative chromatography as uracil. However, it is not possible to determine whether it had been incorporated and not metabolized, or whether it had adhered to the outside of the pads in spite of washing before extraction.

Both the A\(_x\) and uracil fractions were eluted and their specific activities were determined. A\(_x\) had a specific activity of 3.17 \(\times\) 10\(^4\) cpm/\(\mu\)M; uracil, 6.6 \(\times\) 10\(^5\) cpm/\(\mu\)M, as compared to the specific activity of the uracil administered, 1.69 \(\times\) 10\(^7\) cpm/\(\mu\)M. This can be calculated as an approximate dilution of 25-fold for uracil-2-\(^{14}\)C, and a 500-fold dilution of the uracil-2-\(^{14}\)C in A\(_x\).
Fig. 14.—Reproduction of Chromatogram, Fraction A, Uracil-2-C\textsuperscript{14} Experiment, Showing Radioactivity Distribution.
The other fractions, B, C, D, and E, were desalted and aliquots of each were plated and counted. Only B and C showed sufficient radioactivity to warrant chromatographic examination. Figure 15 shows the distribution of both UV-absorbing and radioactive materials on the chromatogram of fraction B. As anticipated, the radioactivity coincides with the very intense UV-absorbing band already shown to be UDPG. The other UV band was either non-radioactive or of such low activity as to make detection impossible.

The distribution of nucleotides and radioactivity in fraction C, shown in Figure 16, again reveals radioactivity, though in small amount, in the UV band at Rf 0.10. This is, no doubt, the result of incomplete separation of the UDPG component of peak B from peak C during column fractionation. UDPG is definitely a minor component of fraction C.

The absence of uracil in either fraction B or C, when it had been identified in these fractions in earlier experiments, is believed due to the failure of uridine nucleotides to be degraded during isolation in the present experiment.

2. Adenine-8-Cl4

The nucleotide elution pattern obtained in this experiment closely resembles those obtained in previous experiments, as can be seen in Figure 17. In this particular case, a somewhat clearer separation of peaks A and A1 was obtained.

Samples of the individual fractions of peak A were plated and counted. The activities determined in this manner appear in Table 4.
Fig. 15.—Reproduction of Chromatogram, Fraction B, Uracil-2-C¹⁴ Experiment, Showing Radioactivity Distribution.
Fig. 16.—Reproduction of Chromatogram, Fraction C, Uraoil-2-Cl4 Experiment Showing Radioactivity Distribution.
Fig. 17.—Acid-Soluble Nucleotides in *P. charlesii* Incorporating Adenine-6-C$^{14}$. 
TABLE 4
ACTIVITIES OF INDIVIDUAL FRACTIONS,
PEAK A, ADENINE-8-C¹⁴ EXPERIMENT

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Activity cpm x 10⁻³</th>
<th>Tube No.</th>
<th>Activity cpm x 10⁻³</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>5</td>
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<td>6</td>
<td>82</td>
</tr>
<tr>
<td>3</td>
<td>220</td>
<td>7</td>
<td>68</td>
</tr>
<tr>
<td>4</td>
<td>239</td>
<td>8</td>
<td>48</td>
</tr>
</tbody>
</table>

On chromatography of fraction A in the isobutyric acid solvent, the distribution of UV-absorbing material and radioactivity depicted in Figure 18 was obtained. The only radioactive area noted was located at R_f 0.40, where compound A_y had been observed in earlier experiments. A_y was not visible under UV light on this chromatogram, and though it had not been possible to obtain satisfactory spectra for it in previous experiments, it is assumed that the presence of radioactivity in this area can be interpreted as evidence that A_y is an adenine derivative. In this solvent system, adenine, adenosine and deoxyadenosine migrate at R_f 0.88, 0.76, and 0.74, respectively.

Also present in fraction A was a new UV-absorbing compound, migrating at R_f 0.20 and designated as A_z. The scan of the chromatogram showed that there was no radioactivity associated with A_z. This band had not been observed in previous experiments, and does not appear at an R_f value corresponding to any unidentified radioactive area in other isotope experiments.
Fig. 18.—Reproduction of Chromatogram, Fraction A, Adenine-8-Cl\textsuperscript{4} Experiment, Showing Radioactivity Distribution.
The  band was eluted along with a suitable blank, and attempts were made to obtain UV spectra. No conclusions could be drawn from these spectra. Samples of the material were then submitted to acid hydrolysis under two different sets of conditions: 60% perchloric acid for one hour at 100°C., and 2.5N HCl for two hours at 100°C. Chromatography of the desalted hydrolyzates showed that  was unchanged, which was considered good evidence that  is non-nucleotide material, since these hydrolytic procedures are known to release pyrimidines and purines, respectively, from their nucleosides and nucleotides.

The isobutyric acid chromatogram and the radioactivity distribution of fraction  are reproduced in Figure 19. Since peak  was of considerable magnitude in this experiment, samples from each tube were plated and counted as had been done for peak . The activities are summarized in Table 5.

Chromatography of fraction  in the isobutyric acid system shows three bands of UV-absorbing material and two radioactive areas. UV-absorption and radioactivity coincide in the band at 0.76, which was identified as adenosine by comparative chromatography.

 is again present, probably due to incomplete separation of  and  on the column. The radioactive  band and  are also present for the same reason.

Owing to the overlapping of peaks B and C on column separation, they were combined and chromatographed as described in earlier sections. A reproduction of the chromatogram, run in the
Fig. 19.—Reproduction of Chromatogram, Fraction A₁, Adenine-8-Cl⁴ Experiment, Showing Radioactivity Distribution.
UV ABSORPTION

A\textsubscript{Z}  A\textsubscript{Y}  A\textsubscript{X}  ADENOSINE
TABLE 5
ACTIVITIES OF INDIVIDUAL FRACTIONS,
PEAK A₁, ADENINE-6-Cl4 EXPERIMENT

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Activity (cpm $\times 10^{-3}$)</th>
<th>Tube No.</th>
<th>Activity (cpm $\times 10^{-3}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>31.9</td>
<td>16</td>
<td>44.0</td>
</tr>
<tr>
<td>10</td>
<td>33.7</td>
<td>17</td>
<td>35.0</td>
</tr>
<tr>
<td>11</td>
<td>38.6</td>
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<td>29.5</td>
</tr>
<tr>
<td>12</td>
<td>67.6</td>
<td>19</td>
<td>23.1</td>
</tr>
<tr>
<td>13</td>
<td>52.1</td>
<td>20</td>
<td>17.2</td>
</tr>
<tr>
<td>14</td>
<td>50.3</td>
<td>21</td>
<td>13.8</td>
</tr>
<tr>
<td>15</td>
<td>48.2</td>
<td>22</td>
<td>10.0</td>
</tr>
</tbody>
</table>

isobutyric acid solvent, and the radioactivity pattern appear in Figure 20.

The nucleotide materials identified differ somewhat from those seen in earlier experiments, although the two radioactive peaks appear in predictable positions. The very strong band at $R_f$ 0.10 is UDPG, but in this case, the material at $R_f$ 0.23 was shown to be UDP, rather than TPN, which was found at this position in earlier work.

It was thought that perhaps a mixture of UDP and TPN might be involved, but the absence of radioactivity at this point on the chromatogram makes it unlikely that any appreciable amount of TPN is present.

In contrast, a very strongly radioactive area appears at $R_f$ 0.35, where DPN had previously been identified. The specific
Fig. 20.—Reproduction of Chromatogram, Fraction B-C, Adenine-6-C$^{14}$ Experiment, Showing Radioactivity Distribution.
activity, though not actually determined, was thought to be quite high, because no UV band was visible in spite of the high activity revealed in the scan.

At $R_f 0.45$, a small amount of radioactivity appeared, which was anticipated as a result of the prior identification of AMP at this point. Apparently the specific activity of this nucleotide is low, since a visible band provides such a low activity compared to that obtained for DPN.

The distribution of the UV-absorbing and radioactive materials in fraction D, shown in Figure 21, follows the pattern anticipated in the light of results reported earlier in this dissertation. Three bands were observed, each of which is seen to correspond to a radioactive area.

The fastest-migrating band, at $R_f 0.45$, is ADP, presumably formed through degradation of ADP and ATP during isolation and chromatography.

The major band occurs at $R_f 0.29$, and was identified as ADP. Apparently the ATP present at $R_f 0.20$ is due to the incomplete separation of peak D from peak E during column chromatography.

Fraction E, whose radioactivity distribution and isobutyric acid chromatogram are represented in Figure 22, showed the same three bands of nucleotide material as did fraction D, each corresponding to a peak on the radioactivity scan. However, ATP is the major component in this fraction, as had been shown earlier.
Fig. 21.—Reproduction of Chromatogram, Fraction D, Adenine-8-Cl4 Experiment, Showing Radioactivity Distribution.
Fig. 22.—Reproduction of Chromatogram, Fraction E, Adenine-8-Cl4 Experiment, Showing Radioactivity Distribution.
3. Guanine-8-C\textsuperscript{14}

The pattern of nucleotide distribution obtained from an extract of \textit{P. charlesii} grown on guanine-8-C\textsuperscript{14} is shown in Figure 23. The elution sequence and location of peaks was quite similar to that obtained in other column separations.

The radioactivity level present in the extract before placing it on the column was only $1.5 \times 10^5$ cpm. This was extremely low compared to any of the other isotope experiments. As a result, the activities of the individual fractions under peaks A and A\textsubscript{1} were not determined before pooling and paper chromatography.

The apparent failure to incorporate guanine-8-C\textsuperscript{14} to any appreciable extent is borne out in the chromatographic studies employing the isobutyric acid system. Only fraction A showed a detectable radioactive area. This was of very low activity and did not correspond to any visible UV-absorbing area on the paper chromatogram. The $R_f$ value of the radioactive material, 0.40, suggests that the compound might be guanosine, which is reported to have an $R_f$ of 0.43 in this solvent system.

No other fractions showed any radioactivity, although the distribution of the UV-absorbing compounds closely paralleled that seen in the adenine-8-C\textsuperscript{14} experiment, even to the presence of an intense A\textsubscript{z} band.

Inasmuch as no guanine nucleosides or nucleotides had been identified in any of the other experiments, the extremely low level of incorporation of guanine-8-C\textsuperscript{14} is not surprising. However, the results of the previous C\textsuperscript{14}-base experiments might lead one to expect a higher degree of free base incorporation.
Fig. 23.—Acid-Soluble Nucleotides in *P. charlesii* Incorporating Guanine-8-C¹⁴.
DISCUSSION

Studies on the acid-soluble nucleotide distribution of P. charlesii under various growth conditions have led to the discovery of several new compounds worthy of further investigation.

The most interesting and best-characterized of these new compounds is the material designated as \( A_x \), which has been shown to be a uracil furanoside. Although the carbohydrate moiety of the compound has not yet been conclusively identified, the presence of any of the known hexoses or pentoses in \( A_x \) has been virtually eliminated from consideration. The possible significance of this compound in terms of the biosynthesis of galactocarlose will be discussed in a later section.

Comparison of the specific activities of \( A_x \) and uracil isolated from mycelia after incubation with uracil-2-\( C^{14} \) show that there is a dilution of some 20-fold of activity of \( A_x \) compared to uracil. This can be considered evidence for at least two pathways to \( A_x \) formation.

In one, glucose and/or tartrate would be metabolized to form a sizable pool of unlabeled uracil or uracil derivatives, from which \( A_x \) is eventually synthesized. This process would no doubt be carried on from the time of inoculation, since transfer of mycelial pads to acetate- or glucose-\( C^{14} \) media after 5-6 days of growth results in so little incorporation of \( C^{14} \) into \( A_x \) during the short-term incubation with labeled medium. Proposal of such a
pathway suggests that future investigations might well include a search for an orotic acid nucleoside capable of releasing formaldehyde on periodate oxidation.

In the second pathway, uracil, incorporated directly from the culture medium, could be converted to the nucleoside via a reaction or series of reactions in which it is not in equilibrium with the uracil synthesized de novo by the organism. This would explain the considerable isotope dilution observed in the uracil-2-C\(^{14}\) experiment.

The apparent preponderance of uracil in the metabolic pool of the organism is reflected in the distribution of nucleosides and nucleotides in the perhydrochloric acid extracts. The nucleotide spectrum, other than fraction A, shows little variation from the general patterns established in previous investigations. Adenine and uracil nucleosides and nucleotides predominate; no traces of either guanine or cytosine compounds were found.

This may indicate that neither type of compound is present in the nucleotide pool of the organism, but more likely is a reflection of steady-state concentrations below the limits of sensitivity of the identification procedures employed. An earlier report of a preponderance of adenine and uracil compounds was made by Bergkvist (50), during studies on Polyergus squamosus.

A second new compound, referred to as A\(_y\) and as yet undefined, is apparently an adenine derivative, as shown by the high level of radioactivity associated with it when isolated from P. charlesii grown on adenine-8-C\(^{14}\). Its chromatographic behavior
distinguishes it from adenine, adenosine and deoxyadenosine, as well as the various adenine nucleotides, but no other information on its structure has been obtained.

A third compound, \( A_z \), was noted only in the experiments where \( {^{14}C} \)-labeled purines were included in the culture medium. In neither case was any detectable radioactivity associated with \( A_z \), and attempts to obtain spectral data on samples of the compound gave no conclusive results.

It may also be noted that the distribution of the types of nucleosides and nucleotides remains quite constant, regardless of culture age or carbon source. The only notable exceptions were the three compounds described in the preceding paragraphs.

Another intriguing facet of the present investigation was uncovered during examination of pH changes in the growth medium during culture of \( P. \ charlesii \). The initial sharp decline in pH, noted during the first five to six days following inoculation, coincides with an equally precipitous drop in the glucose concentration of the medium. Other studies carried out in this laboratory indicate that approximately 75% of the glucose has been metabolized by the time the pH has reached a minimal value (51).

The time of the inflection point in the pH curve also has been found to correspond to the time of appearance of the first detectable amounts of galactocarolose in the medium.

The reasons for the increase in acidity of the medium remain undefined, but a combination of at least two factors is very likely involved. First, the original Raulin-Thom medium
contains a tartaric acid-ammonium tartrate buffer system, with additional ammonium salts present in low concentrations. It is conceivable, that as ammonium ion is incorporated into the organism, the buffer capacity of the medium is decreased to such an extent that pH decrease is inevitable.

In addition, it has been shown by Raistrick's group (52) that culture of *P. charlesii* on Raulin-Thom glucose medium results in the formation of the tetronic acid derivatives shown in Figure 24. The expulsion of these by-products of glucose metabolism, coupled with ammonium ion uptake, could well produce the observed pH decrease.

Two schools of thought exist concerning the place of these tetronic acids in the pathway of glucose metabolism in this organism. One (53) holds that the principal metabolic process in the organism is the formation of the oligosaccharide, galactocarolose, and that the acids are derived by catabolism of the furanose units. It can readily be seen that the structure of each of these acids is closely related to the furanose structure in which galactose is present in the polymer.

The opposing opinion (54) suggests that the various acids are intermediates in the synthesis of the galactofuranose units, rather than degradation products of these units. This view certainly finds much wider support in studies of oligo- and polysaccharide synthesis in other organisms, where the principal polymer often has little or no structural relation to the majority of the other important metabolites.
Fig. 24.—Tetronic Acid Derivatives Produced by *P. charlesii* Grown on Raulin-Thom Glucose Medium.
It appears that the latter theory gains additional support from the present study of pH changes. The decrease in pH is probably greater than could be expected as a result of the incorporation of the components of the buffer system of the medium. The added effect of the excretion of these acids by the organism would probably contribute greatly to the decrease in pH, in spite of the relatively low acid-strength of the individual compounds.

Attention is also directed to the portion of the pH curve where acidity remains at the minimal level for 4-5 days. Galactocarolose production is increasing during this period, and since it cannot be formed from glucose (which has been almost completely depleted by this time), it is logical to postulate that the organism shifts its metabolism to utilize the preformed "furancoid" units found in the tetronic acids to continue oligosaccharide formation.

This line of reasoning offers a simple explanation of the sharp increase in pH from the 10th to the 20th day, where pH rises far above its initial value. By this time, not only the original tartrate of the medium, but also the acidic by-products have been metabolized, most likely to form the polymer, but also probably to some extent to provide an energy source.

The reason for the formation of the oligosaccharide is obscure. It is not known whether it is a structural component or merely a storage form of carbohydrate. How much is present within the mycelial structure is unknown; the only quantitative studies have been made on samples of the growth medium.
It has been shown, however, that the polymer itself is a poor substrate for growth of *P. charlesii* (55) which may, of course, be the simple result of failure to incorporate the complex molecule. Regardless of the function of the oligosaccharide, the reasons for excretion of such large quantities are unexplainable on the basis of present information.

Before leaving the topic of the tetronic acid derivatives, it is worthwhile to point out that studies on the biosynthesis of carolic and carlosic acids (56, 57) tend to substantiate the $C_2-C_4$ condensation proposed by Gander (6) as a pathway to formation of the galactofuranose units. Bentley (57) has shown that carbons 2 and 3 of succinate are incorporated into positions 5 and 10 of carolic acid, while neither carbon is significantly labeled by acetate- or malonate-$C^{14}$. Both the latter substrates will label carbon atoms 2, 3, 6, 7, 8, and 9 of carolic acid in such a manner that the "head-to-tail" condensation of acetate (with itself or with malonate) can be proposed as the biosynthetic pathway to the side chain of the carolic acid molecule.

No definitive studies of $C^{14}$-distribution in the galactofuranose sub-units have yet been made, but the possibility that the condensation of tartrate or a metabolite of it with acetate, followed by a series of hydrations and reductions, is the route to galactofuranose is well worth investigation.

The significance of the nucleotide identification studies in relation to galactocarlose biosynthesis lies primarily in the isolation and partial characterization of the new uracil furanoside.
Attempts to identify the carbohydrate moiety failed to show that this portion of the nucleoside was identical to any of the known hexoses whose structures could reasonably be related to galactofuranose. The basis for implication of galactose or another hexose was the behavior of the unknown carbohydrate during paper chromatography.

Its relative migration, identical to that of galactose, proved to be a false lead, when the phosphorylated derivative produced by the galactokinase reaction was shown to migrate quite differently from the anticipated galactose-1-phosphate. None of the hexoses available for comparison matched the $R_f$ of the unknown, either in free form or after phosphorylation.

However, consideration must be given to the possibility that the carbohydrate moiety under investigation may be some as yet unidentified metabolite of one of the tetronic acid derivatives. If the lactone structure of such a compound were reduced to a hemiacetal, the resultant structure could be considered a substituted hexose. In such a case, all data obtained so far would agree with a structure in which a two-carbon side-chain attached at position 5 in the tetronic acid ring system would have either vicinal hydroxyls or an $\alpha$-hydroxyketone structure, capable of yielding formaldehyde on periodate oxidation.

The uracil furanoside has been shown to incorporate isotope from both acetate-1-\(^{14}\)C and glucose-U-\(^{14}\)C. These results are not striking in themselves, but do warrant investigation of isotope distribution in the compound, to determine whether \(^{14}\)C is present
in the pyrimidine or the carbohydrate, or both. Not only this, but a role for this uracil furanoside as a potential donor of "galactosyl" units during formation of the oligosaccharide can readily be suggested.

Before attempting to postulate the participation of uracil furanoside as the furanosyl donor in the reaction

\[
\text{uracil furanoside} + (\text{acceptor-furanose})_n \rightarrow \text{uracil} + (\text{acceptor-furanose})_{n+1},
\]

it is necessary to examine the factors which might provide the requisite free energy to cause the reaction to proceed in favor of oligosaccharide synthesis.

The important factors contributing to the "group-transfer potential" of the uracil-furanoside bond are summarized in Table 6. In each case, the influence of a particular factor, or lack of it, is signified by a plus or minus sign. An asterisk indicates a small but significant contribution.

The contribution of resonance stabilization in the phosphate compounds is a result of the additional resonance hybrids which become available to the phosphate radical when the C-O bond is broken. It has been shown by Lipmann (58) and Pullman and Pullman (59) that the contribution of these additional structures to the free energy of the phosphate compounds is of the order of two kilocalories.

In uracil furanoside, no phosphate is involved, but it can be shown that four possible resonance hybrids exist for the uracil in the nucleoside structure. However, on transfer of the glycosyl
<table>
<thead>
<tr>
<th>Compound</th>
<th>Group Transferred</th>
<th>Acceptor</th>
<th>Resonance Stabilization</th>
<th>Opposing Resonance</th>
<th>Tautomerism</th>
<th>Free Energy of Neutralization</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDPG glucose</td>
<td>ROH</td>
<td>+</td>
<td>+</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>HOH</td>
<td>+</td>
<td>+</td>
<td>*</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>α-Amylose glucose</td>
<td>ROH</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td></td>
<td>HOH</td>
<td>-</td>
<td>-</td>
<td></td>
<td>*</td>
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</tr>
<tr>
<td>Glucose-1-P glucose</td>
<td>ROH</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>HOH</td>
<td>+</td>
<td>-</td>
<td></td>
<td>*</td>
<td>+</td>
</tr>
<tr>
<td>Galacto- carolose</td>
<td>ROH</td>
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<td>-</td>
</tr>
<tr>
<td></td>
<td>HOH</td>
<td>-</td>
<td>-</td>
<td></td>
<td>*</td>
<td>-</td>
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<td>Uracil furanoside</td>
<td>ROH</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>HOH</td>
<td>+</td>
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<td></td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
residue to an acceptor and replacement of it by a hydrogen atom, an additional nine hybrid structures become available. Obviously, a significant contribution to the free energy can be anticipated as a result of more than tripling the number of potential resonance structures which a molecule can assume.

The second factor is opposing resonance, a term employed by Pullman and Pullman (59) to describe the effect on the bond stability of the close proximity of at least three centers of positive charge. Thus, in the P-O-P-O-C structure of UDPG, there are five positive centers in succession, although the influence of the carbon atom is not appreciable. This subjects the molecule to considerable internal instability due to mutual repulsion.

Opposing resonance is far less important, in fact negligible, in glucose-1-phosphate, since only two positive centers of any consequence are involved. It is not a significant factor in the uracil furanoside.

Two different types of tautomerism must be considered in discussion of the third factor. In each of the five compounds listed in Table 6, the possibility exists that when the glycosidic bond is broken, an equilibrium between the furanose and pyranose forms of the free sugar may be set up. In those compounds where the sugar moiety is originally in the pyranose form, UDPG, α-amyllose and glucose-1-phosphate, the contribution of this equilibrium would be small even in a hydrolytic reaction, because the pyranose is by far the more favored structure.
On the other hand, this predominance of the pyranose form over the furanose would provide a considerable contribution to the free energy on hydrolysis of either of the furanosyl compounds. This discussion is not pertinent in the reaction under consideration, however, because if the furanosyl moiety is transferred to form a new glycosidic bond without alteration of ring structure, the free energy of this tautomerization is conserved and cannot be a factor.

The additional possibility of tautomerism in the uracil molecule will no doubt add a tangible amount to the total free energy driving the reaction. It is impossible for both keto groups of uracil to assume simultaneously the enol structure when the molecule is a member of a glycosidic bond. If this bond is broken, however, the simultaneous tautomerism of the keto groups lends an extra measure of stability to the uracil molecule, thereby increasing still further the free energy available to drive the transfer reaction.

Finally, let us consider the free energy of neutralization which is involved in both the phosphate compounds and the proposed nucleoside intermediate. This factor was also mentioned in the discussions of high-energy bond and is known to contribute as much as a kilocalorie to the free energy of reactions involving phosphate.

In the case of uracil furanoside, the neutralization of the ring nitrogen of uracil may actually require a consumption of free energy. If we assume a physiological pH of 7 for the
occurrence of the transfer reaction, and a pK of about 9 for the uracil nitrogen, a value as high as +2800 calories for the free energy of neutralization could be calculated, as was done by Kalckar (60) for hypoxanthine.

An additional factor in estimation of the free energy of the glycosyl transfer has not yet been mentioned. The free energy of hydrolysis of the glycosidic bond, although not known for the (1,5)-furanose bond of galactocarolose, is probably of the order of 4-5 kilocalories, as are most of the values reported for such bonds (61-64). In this context however, as explained in consideration of the furanose-pyranose equilibrium, the fact that the furanose bond is conserved rather than hydrolyzed effectively negates any appreciable contribution this free energy value might make to the overall free energy of the transfer reaction.

In summary, allowing a contribution of -3000 calories due to resonance stabilization, -2000 calories as a consequence of tautomerism, and a consumption of 2500 calories for the free energy of neutralization of uracil, a value of -2500 calories is obtained for the change in free energy accompanying synthesis of galactocarolose by the proposed reaction.

By calculation of the equilibrium constant from this value, it can be shown that synthesis of the oligosaccharide by this reaction would be favored by a factor of at least 60 to 1. Theoretically, this would allow a uracil glycoside to participate in a transglycosylation in which the equilibrium would lie far toward galactocarolose formation.
Having established the thermodynamic feasibility of the
nucleoside as a glycosyl donor, it is interesting to speculate
on the reactions which both precede and follow it in the pathway
from glucose to galactocarolose.

A hypothetical scheme is depicted in Figure 25. The con-
densation of undefined 2- and 4-carbon units, followed by the
requisite oxidations, reductions and other conversions, leads to
the formation of a galactofuranose derivative. This may be the
previously proposed tetronic acid derivative, or a derivative of
galactose in which the 5-position is blocked in such a manner as
to force formation of the furanose configuration. Potentially
available structures of this type include 5-keto- or 5-phospho-
galactofuranose.

Once the galactofuranose derivative has been formed, it
must be activated before combination with uracil. Many schemes
can be postulated for this activation, but three which gain a
measure of credibility from previous studies of other enzymic
systems are phosphorylation to the 1-phosphate, pyrophosphory-
lation at the same position or a transfer reaction with uridine,
in which uracil has already been activated by glycosidic bonding
with the ring nitrogen of the pyrimidine.

When the proposed intermediate, $A_x$, has been formed, one
can only guess whether any further alteration in the structure of
the carbohydrate moiety must occur before transfer of galactose to
an acceptor to form the polymer. The stages at which the oxidations,
Fig. 25.—A Hypothetical Pathway For Galactocarolose Biosynthesis.
"C_2" + "C_4" → GALACTOFURANOSE DERIVATIVE → A_k → URACIL GALACTOFURANOSE

(POLYGALACTOFURANOSE)_n → URACIL

(POLYGALACTOFURANOSE)_n + 1
phosphorylations and other conversions occur, if they do occur, really have little bearing on the discussion, since it is necessarily so hypothetical at this point.

The entire proposal has been based on the isolation of a single possible intermediate. Regardless of the logic of the arguments advanced to implicate it as an intermediate in the formation of galactocarolose, much more must be learned about the other metabolites involved in this complex series of reactions before any degree of validity can be given this pathway.
SUMMARY

The results of an investigation of the acid-soluble nucleosides and nucleotides in Penicillium charlesii show that at least three new compounds are present in perchloric acid extracts of the organism, after culture on various carbon sources.

The most interesting of these compounds, A₁, was shown to be a uracil nucleoside. Investigation of the structure of the carbohydrate portion of the molecule indicated that it is a hexo-furanoside. Its rate of migration during paper chromatography is identical to that of galactose, but a phosphorylated derivative prepared by the action of galactokinase does not migrate at the same position as authentic galactose-1-phosphate. None of the other known hexoses examined showed properties identical with the unknown compound.

Oxidation of the unknown nucleoside by periodic acid resulted in the uptake of two moles of periodate per mole of nucleoside, with the concomitant release of one mole of formaldehyde per mole of nucleoside. This limits the structure of the sugar residue to the furanose configuration, and rules out any known pentoses.

The unknown sugar reduces ammoniacal silver nitrate and gives a positive reaction with the periodate-benzidine spray reagent. Periodate oxidation of the free carbohydrate after cleavage
of its glycosidic bond with uracil again showed release of formaldehyde, indication that the furanose configuration is maintained.

The structures of the other two compounds, $A_y$ and $A_z$, were not determined, but on the basis of its incorporation of adenine-8-$^{14}$C, $A_y$ was shown to be an adenine derivative.

A possible function of the uracil furanoside as a donor of "galactosyl" sub-units to the oligosaccharide galactocarolose was discussed, and the thermodynamic implications of such a donor reaction were explored.

The distribution of nucleosides and nucleotides other than the three compounds described appeared to be quite constant, in spite of variations in the carbon sources and growth conditions employed. A predominance of adenine and uracil derivatives was well established.

A theoretical discussion of a possible pathway from glucose to galactocarolose was presented.
BIBLIOGRAPHY

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