HEMAGGLUTININ AND PROTEASE OF PATHOGENIC STRAINS OF BACTEROIDES MELANINOGENICUS

by

SALWA RASMY

B.Sc.(Hons.) University of Cairo

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY in THE FACULTY OF GRADUATE STUDIES DEPARTMENT OF MICROBIOLOGY

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

May, 1979

© Salwa Rasmy, 1979
In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the Head of my Department or by his representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Microbiology

The University of British Columbia
2075 Wesbrook Place
Vancouver, Canada
V6T 1W5

Date June 8, 1979
ABSTRACT

_Bacteroides melaninogenicus_ strains 2D and K110 were characterized with regard to their pathogenic, collagenolytic, proteolytic, hemagglutinating and metabolic activities. Both strains were members of the subspecies _B. melaninogenicus ss. asaccharolyticus_. They possessed a cell-bound oxygen-sensitive collagenase, a cell-bound and a soluble oxygen-sensitive hemagglutinin (HA), and a protease. Both strains produced butyric and phenylacetic acids and were infective in guinea pigs as characterized by their ability to produce necrotic lesions and to be transferred from one animal to another. Strain 2D required hemin for growth and its growth rate was influenced by the addition of free amino acids to the medium.

The hemagglutinating and proteolytic activities of strain 2D were investigated further to determine their relationship to infection. The soluble HA was reversibly inhibited by Hg and activity was restored in the presence of reducing agents. Iodoacetic acid caused irreversible inhibition. The HA was sensitive to heat and pronase treatment. Treatment of the red blood cells (RBC) with neuraminidase enhanced HA activity while the presence of galactose in the reaction mixture inhibited it, suggesting the involvement of galactose residues on the RBCs in the reaction. Adsorption of the HA to RBC followed by elution and gel filtration resulted in the recovery of 50% of the HA activity and a 52-fold purification.

Protease production by _B. melaninogenicus_ strain 2D was dependent on the growth rate of the organism. The protease was reversibly inhibited by HgCl₂ and irreversibly inhibited by iodoacetamide and iodoacetic acid.
The enzyme was insensitive to serine protease inhibitors and EDTA. The pH optimum for proteolytic activity was 7.0, which correlates with the pH of its natural environment, the gingival crevice. It is thus classified as a neutral sulfhydryl enzyme.

A 774-fold purification of the cellular protease of 2D, with a 160% recovery of activity, was accomplished by precipitation with 60% ethanol, ultracentrifugation and gel filtration through Sephadex G-100 and Sepharose 2B in the presence of urea.

Electrophoretic analysis of the protease on SDS-polyacrylamide gels revealed four distinct bands, each of which was shown to be associated with carbohydrate. In the absence of SDS only one band, which did not migrate into the gel, was obtained. Any attempts to further dissociate the protease resulted in the loss of activity. The protease was active against azocoll, azocasein, casein and N,N-dimethylcasein. No glycosidase, lipase, collagenase or HA activities were detected. Protein, carbohydrate and lipid were detected in the preparation.

The soluble protease which amounted to 20% of the cellular protease of strain 2D was subjected to gel filtration on Sephadex G-100 and eluted in a single peak at the void volume. The properties of the soluble protease were identical to those of the cell associated enzyme, suggesting the presence of a single proteolytic enzyme which was released into the culture medium with cell lysis or due to shedding of outer membrane fragments.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xi</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>xiv</td>
</tr>
<tr>
<td>I. INTRODUCTION</td>
<td></td>
</tr>
<tr>
<td>A. Bacteroides melaninogenicus</td>
<td>1</td>
</tr>
<tr>
<td>1. Biochemical characteristics of B. melaninogenicus</td>
<td>4</td>
</tr>
<tr>
<td>2. Pathogenic properties of B. melaninogenicus</td>
<td>10</td>
</tr>
<tr>
<td>a. Role of B. melaninogenicus in mixed anaerobic infections and periodontal diseases</td>
<td>10</td>
</tr>
<tr>
<td>b. Infectivity of B. melaninogenicus</td>
<td>12</td>
</tr>
<tr>
<td>c. Toxin production</td>
<td>14</td>
</tr>
<tr>
<td>3. Antibiotic sensitivity</td>
<td>15</td>
</tr>
<tr>
<td>4. Antigenic structure and serological heterogeneity</td>
<td>16</td>
</tr>
<tr>
<td>5. Genetic properties</td>
<td>17</td>
</tr>
<tr>
<td>6. Lipopolysaccharide and lipids of B. melaninogenicus</td>
<td>17</td>
</tr>
<tr>
<td>B. Hemagglutinating Activity and Adherence Properties of B. melaninogenicus</td>
<td>22</td>
</tr>
<tr>
<td>1. Adherence</td>
<td>22</td>
</tr>
<tr>
<td>2. Hemagglutinin</td>
<td>25</td>
</tr>
<tr>
<td>C. Proteolytic Activity of B. melaninogenicus</td>
<td>27</td>
</tr>
<tr>
<td>II. MATERIALS AND METHODS</td>
<td></td>
</tr>
<tr>
<td>A. Organisms</td>
<td>48</td>
</tr>
</tbody>
</table>
## II. MATERIALS AND METHODS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B. Growth</strong></td>
<td>48</td>
</tr>
<tr>
<td>1. Anaerobiosis</td>
<td>48</td>
</tr>
<tr>
<td>2. Media</td>
<td>49</td>
</tr>
<tr>
<td>3. Continuous cultures</td>
<td>50</td>
</tr>
<tr>
<td><strong>C. Protease</strong></td>
<td>51</td>
</tr>
<tr>
<td>1. Assays for proteolytic activity</td>
<td>51</td>
</tr>
<tr>
<td>2. Purification of the protease</td>
<td>56</td>
</tr>
<tr>
<td><strong>D. Hemagglutination</strong></td>
<td>66</td>
</tr>
<tr>
<td>1. Assay</td>
<td>66</td>
</tr>
<tr>
<td>2. Preparation of red blood cells</td>
<td>66</td>
</tr>
<tr>
<td>3. Determination of the effects of various reagents on HA</td>
<td>67</td>
</tr>
<tr>
<td>4. Adhesion and elution of hemagglutinin from RBC</td>
<td>67</td>
</tr>
<tr>
<td><strong>E. Infectivity</strong></td>
<td>68</td>
</tr>
<tr>
<td><strong>F. Metabolic End Product Analysis</strong></td>
<td>69</td>
</tr>
<tr>
<td><strong>G. Collagenase Assay</strong></td>
<td>70</td>
</tr>
<tr>
<td><strong>H. Protein Determination</strong></td>
<td>72</td>
</tr>
<tr>
<td><strong>I. Glucose Determination</strong></td>
<td>72</td>
</tr>
<tr>
<td><strong>J. Microdetermination of Lipids</strong></td>
<td>72</td>
</tr>
<tr>
<td><strong>K. Microdetermination of Phosphorous</strong></td>
<td>73</td>
</tr>
<tr>
<td><strong>L. Glucosidase Assay</strong></td>
<td>73</td>
</tr>
<tr>
<td><strong>M. Lipase Assay</strong></td>
<td>74</td>
</tr>
<tr>
<td><strong>N. Reagents and Chemicals</strong></td>
<td>74</td>
</tr>
</tbody>
</table>
### III. RESULTS

<table>
<thead>
<tr>
<th>A. Characterization of <em>B. melaninogenicus</em></th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Fatty acid production</td>
<td>76</td>
</tr>
<tr>
<td>2. Collagenase activity</td>
<td>81</td>
</tr>
<tr>
<td>3. Pathogenicity</td>
<td>81</td>
</tr>
<tr>
<td>a. Infectivity</td>
<td>81</td>
</tr>
<tr>
<td>b. Vascular permeability</td>
<td>82</td>
</tr>
<tr>
<td>4. Growth of strain 2D <em>B. melaninogenicus</em></td>
<td>84</td>
</tr>
<tr>
<td>a. Hemin requirement</td>
<td>84</td>
</tr>
<tr>
<td>b. Growth response to amino acids</td>
<td>87</td>
</tr>
<tr>
<td>5. Hemagglutinin and protease activity of <em>B. melaninogenicus</em></td>
<td>87</td>
</tr>
<tr>
<td>6. Effect of washing 2D cells on the HA and protease</td>
<td>91</td>
</tr>
<tr>
<td>7. Release of periplasmic enzymes from 2D cells</td>
<td>94</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. Soluble Hemagglutinin of <em>B. melaninogenicus</em></th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Adherence of <em>B. melaninogenicus</em> 2D cells to formalinized human RBC</td>
<td>96</td>
</tr>
<tr>
<td>2. Determination of optimal conditions for the hemagglutinin assay</td>
<td>96</td>
</tr>
<tr>
<td>3. Relationship of HA to culture age</td>
<td>106</td>
</tr>
<tr>
<td>4. Effects of RBC modification on HA</td>
<td>106</td>
</tr>
<tr>
<td>5. Modification of the HA</td>
<td>106</td>
</tr>
<tr>
<td>6. Effect of carbohydrates on HA</td>
<td>110</td>
</tr>
<tr>
<td>7. Stability of the soluble HA</td>
<td>110</td>
</tr>
<tr>
<td>8. Oxygen sensitivity of the soluble HA</td>
<td>111</td>
</tr>
<tr>
<td>9. Effect of sulfhydryl modifiers on HA</td>
<td>111</td>
</tr>
<tr>
<td>10. Ultracentrifugation of soluble HA</td>
<td>111</td>
</tr>
</tbody>
</table>
### III. RESULTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>11. Partial purification of soluble HA</td>
<td>115</td>
</tr>
<tr>
<td>a. Concentration of the HA</td>
<td>115</td>
</tr>
<tr>
<td>b. Chromatography</td>
<td>115</td>
</tr>
<tr>
<td>c. Binding to Millipore filters</td>
<td>121</td>
</tr>
<tr>
<td>C. Protease of <em>B. melaninogenicus</em></td>
<td>122</td>
</tr>
<tr>
<td>1. Protease assays</td>
<td>122</td>
</tr>
<tr>
<td>2. Relationship of protease to culture age</td>
<td>128</td>
</tr>
<tr>
<td>3. Cell-bound protease of <em>B. melaninogenicus</em></td>
<td>128</td>
</tr>
<tr>
<td>a. Effect of passaging 2D cells in guinea pigs on protease production</td>
<td>133</td>
</tr>
<tr>
<td>b. Effect of hemin concentration on growth and protease production</td>
<td>134</td>
</tr>
<tr>
<td>c. Protease production in the presence of succinate</td>
<td>134</td>
</tr>
<tr>
<td>d. Effect of amino acids on protease production</td>
<td>140</td>
</tr>
<tr>
<td>e. Growth rate and protease production</td>
<td>142</td>
</tr>
<tr>
<td>f. Production of the protease at different concentrations of hemin</td>
<td>142</td>
</tr>
<tr>
<td>g. Preliminary characterization of the cellular protease of <em>B. melaninogenicus</em></td>
<td>146</td>
</tr>
<tr>
<td>h. Purification of the cellular protease of <em>B. melaninogenicus</em></td>
<td>150</td>
</tr>
<tr>
<td>i. Characterization of the purified protease</td>
<td>184</td>
</tr>
<tr>
<td>4. Soluble protease of <em>B. melaninogenicus</em></td>
<td>202</td>
</tr>
<tr>
<td>a. Demonstration of an extracellular protease</td>
<td>202</td>
</tr>
<tr>
<td>b. Preliminary characterization of the extracellular protease</td>
<td>203</td>
</tr>
<tr>
<td>c. Partial purification of the soluble protease</td>
<td>208</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>--------------------------</td>
<td>------</td>
</tr>
<tr>
<td>IV. DISCUSSION</td>
<td>215</td>
</tr>
<tr>
<td>V. LITERATURE CITED</td>
<td>232</td>
</tr>
<tr>
<td>TABLE</td>
<td>Title</td>
</tr>
<tr>
<td>-------</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>1.</td>
<td>Collagenase assay</td>
</tr>
<tr>
<td>2.</td>
<td>Vascular permeability test</td>
</tr>
<tr>
<td>3.</td>
<td>Effect of the addition of amino acids on growth of <em>B. melaninogenicus</em> ss. <em>asaccharolyticus</em> 2D in TYH medium.</td>
</tr>
<tr>
<td>4.</td>
<td>Hemagglutinin and protease of 2D and K110.</td>
</tr>
<tr>
<td>5.</td>
<td>Effect of washing 2D cells on the HA.</td>
</tr>
<tr>
<td>6.</td>
<td>Release of the HA from 2D cells by treatment with Polymyxin B.</td>
</tr>
<tr>
<td>7.</td>
<td>Influence of culture age on the adherence of 2D to FRBC.</td>
</tr>
<tr>
<td>8.</td>
<td>Effect of the source of red blood cells on HA activity.</td>
</tr>
<tr>
<td>9.</td>
<td>Effect of treatment of RBC on their ability to hemagglutinate with soluble HA.</td>
</tr>
<tr>
<td>10.</td>
<td>Effect of aeration on soluble HA.</td>
</tr>
<tr>
<td>11.</td>
<td>Effect of sulfhydryl modifiers on HA.</td>
</tr>
<tr>
<td>12.</td>
<td>Ultracentrifugation of soluble HA.</td>
</tr>
<tr>
<td>13.</td>
<td>Characteristics of the HA eluted from RBC with urea.</td>
</tr>
<tr>
<td>14.</td>
<td>Analysis of the HA purification procedures.</td>
</tr>
<tr>
<td>15.</td>
<td>Effect of passaging 2D on protease activity.</td>
</tr>
<tr>
<td>16.</td>
<td>Effect of additions of amino acids to TYH medium on the proteolytic activity of <em>B. melaninogenicus</em>.</td>
</tr>
<tr>
<td>17.</td>
<td>Effect of hemin concentration on protease production.</td>
</tr>
<tr>
<td>TABLE</td>
<td>Title</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------</td>
</tr>
<tr>
<td>18.</td>
<td>Effect of reducing agents on the cellular protease</td>
</tr>
<tr>
<td>19.</td>
<td>Thermoliability of the proteolytic activity in the cell-extract</td>
</tr>
<tr>
<td>20.</td>
<td>Comparison of methods for liberating protease</td>
</tr>
<tr>
<td>21.</td>
<td>Ethanol precipitation of protease from cell-extract</td>
</tr>
<tr>
<td>22.</td>
<td>Purification of protease from B. melaninogenicus-2D</td>
</tr>
<tr>
<td>23.</td>
<td>Chemical composition of the purified protease preparation</td>
</tr>
<tr>
<td>24.</td>
<td>Gas-liquid chromatographic analysis of fatty acids in the purified enzyme preparation</td>
</tr>
<tr>
<td>25.</td>
<td>Modification of the purified protease</td>
</tr>
<tr>
<td>26.</td>
<td>Inhibition of soluble protease</td>
</tr>
<tr>
<td>27.</td>
<td>Partial purification of B. melaninogenicus extracellular protease</td>
</tr>
<tr>
<td>FIGURE</td>
<td>Title</td>
</tr>
<tr>
<td>--------</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>1.</td>
<td>General patterns of enzyme production in continuous culture</td>
</tr>
<tr>
<td>2.</td>
<td>The effect of dilution rate on the synthesis of amidase by <em>Pseudomonas aeruginosa</em> growing in a chemostat under steady-state conditions</td>
</tr>
<tr>
<td>3.</td>
<td>Gas chromatography of volatile fatty acids produced by <em>B. melaninogenicus</em></td>
</tr>
<tr>
<td>4.</td>
<td>Gas chromatography of non-volatile fatty acids produced by <em>B. melaninogenicus</em></td>
</tr>
<tr>
<td>5.</td>
<td>Response of <em>B. melaninogenicus</em> (strain 2D) to hemin</td>
</tr>
<tr>
<td>6.</td>
<td>Effect of glutamic acid on growth of <em>B. melaninogenicus</em></td>
</tr>
<tr>
<td>7.</td>
<td>Effect of pH on HA of <em>B. melaninogenicus</em></td>
</tr>
<tr>
<td>8.</td>
<td>Optimal erythrocyte concentration for microtiter hemagglutination assay</td>
</tr>
<tr>
<td>9.</td>
<td>Effects of incubation temperature on HA</td>
</tr>
<tr>
<td>10.</td>
<td>Relationship of HA to culture age</td>
</tr>
<tr>
<td>11.</td>
<td>Sephadex G-100 gel filtration of the soluble HA</td>
</tr>
<tr>
<td>12.</td>
<td>Hydrolysis of azocasein by <em>B. melaninogenicus</em> protease</td>
</tr>
<tr>
<td>13.</td>
<td>Effect of azocasein concentration</td>
</tr>
<tr>
<td>14.</td>
<td>Effect of enzyme concentration on the azocasein assay</td>
</tr>
<tr>
<td>15.</td>
<td>Relationship of protease to culture age</td>
</tr>
<tr>
<td>16.</td>
<td>Effect of hemin concentration on growth and protease production of 2D</td>
</tr>
<tr>
<td>FIGURE</td>
<td>Title</td>
</tr>
<tr>
<td>--------</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>17.</td>
<td>Protease production in hemin and succinate media.</td>
</tr>
<tr>
<td>18.</td>
<td>Effect of dilution rate (D) on protease production by B. melaninogenicus</td>
</tr>
<tr>
<td>19.</td>
<td>Gel filtration of the ethanol precipitated protease.</td>
</tr>
<tr>
<td>20.</td>
<td>Fractionation on Sepharose-2B.</td>
</tr>
<tr>
<td>21.</td>
<td>Chromatography on thiol Sepharose-4B.</td>
</tr>
<tr>
<td>22.</td>
<td>Sepharose mercury chromatography.</td>
</tr>
<tr>
<td>23.</td>
<td>Polyacrylamide gel electrophoresis in tris-glycine-SDS buffer of the purified protease</td>
</tr>
<tr>
<td>24.</td>
<td>Polyacrylamide gel electrophoresis in tris-glycine-SDS buffer of protease fractions obtained during various steps in the purification process</td>
</tr>
<tr>
<td>25.</td>
<td>Polyacrylamide gel electrophoresis of protease fractions obtained from the different purification procedures</td>
</tr>
<tr>
<td>26.</td>
<td>Polyacrylamide gel electrophoresis in tris-glycine buffer without SDS</td>
</tr>
<tr>
<td>27.</td>
<td>Polyacrylamide electrophoresis of fraction A and B stained for lipids</td>
</tr>
<tr>
<td>28.</td>
<td>Diagram of glycoprotein and protein bands on slab gels following electrophoresis of purified protease</td>
</tr>
<tr>
<td>29.</td>
<td>Effect of pH on the purified protease activity</td>
</tr>
<tr>
<td>30.</td>
<td>HgCl₂ inhibition of the purified protease</td>
</tr>
<tr>
<td>31.</td>
<td>Inhibition of the purified protease by iodoacetamide</td>
</tr>
<tr>
<td>32.</td>
<td>Effect of guanidine hydrochloride, lithium chloride and NaCl on the purified protease</td>
</tr>
<tr>
<td>FIGURE</td>
<td>Title</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>33.</td>
<td>pH optimum of the soluble protease</td>
</tr>
<tr>
<td>34.</td>
<td>Sephadex G-100 gel filtration of the soluble protease</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I wish to convey my sincere gratitude to Dr. J.J.R. Campbell, whose constant guidance and encouragement made this work possible. His warm fatherly attitude has helped me during the critical stages of the entire program and is gratefully acknowledged.

I extend my appreciation and eternal thanks to my research advisor Dr. B.C. McBride. His invaluable academic assistance, understanding, criticism and extremely amiable attitude were necessary to complete this research.

I also wish to thank Dr. Antonio Weerkamp and Mary Gisslow for their helpful comments and suggestions.

Last but certainly not least, I want to record my deep feeling of gratitude to the immeasurable support and sacrifices rendered me by my immediate family, children, husband and mother, who were always there to lean on whenever necessary. This thesis is dedicated to them.
I. INTRODUCTION

A. *Bacteroides melaninogenicus*

The role of anaerobic bacteria in the etiology of infection and their function in the microbial ecology of man, has recently been of increased interest (2,58,78,79,80,117,150,158,212,217,220). Anaerobic bacteria are present throughout the body as constituents of the normal flora (58) and under certain conditions, particularly in compromised patients, these organisms can invade any organ of the body and cause a variety of illnesses (56). A commonly encountered species in human infection is *B. melaninogenicus* (4,25,57,138,202,230,231) which is frequently isolated from abscesses in the upper half of the body.

*B. melaninogenicus* is a strictly anaerobic, Gram-negative, non-motile, non-sporulating rod with rounded or pointed ends. The cells vary in size and shape from small coccoid forms to long filamentous organisms. *B. melaninogenicus* is normally found in small numbers in the human intestine (25), on male and female external genitalia (25,78), in the throat (25,163), in the oral cavity where it is found in large numbers in the gingival crevice (25,68,69,197,204), and in supragingival human dental plaque (69,85,130,207). In the gingival crevice it may account for as much as 5% of the total cultivable flora. It is also present in the mouths of dogs but is not found in the mouths of rodents. It has been isolated in association with other bacteria from various types of clinical infections: tooth abscesses (25,104), soft tissue infections (158), liver
abscesses (182), brain and lung abscesses (25,32,192), bite wounds and infected surgical wounds (163,231), urine from a suspected infected kidney (163), appendicitis peritonitis (3,4,154), and the uterus and blood in patients with puerperal infections (188,212,220). The organism has been implicated in periodontal disease (137,138,208,217,230) but this relationship has not been proven. Characteristically, the organism is associated with abscesses populated by a mixture of non-sporulating anaerobes and/or facultative anaerobes (4,25,54,57,79,80,148,192,202,208).

The occurrence of \textit{B. melaninogenicus} in clinical specimens was described as early as 1921, but the organism is still often undetected in clinical specimens. This may be due to improper collection of the specimens, the lengthy delay between specimen collection and culture, failure to use the proper culture medium and failure to incubate the cultures under strictly anaerobic conditions and for sufficient time to permit growth and pigment production.

For many years, \textit{B. melaninogenicus} has been of interest to a number of investigators, but detailed analyses concerning the biochemical properties of the organism have been impeded by difficulties in growing it in pure culture (26,54). Furthermore, isolates which are considered to be \textit{B. melaninogenicus} represent a heterogeneous group.

Sawyer et al. (185) reported that although biochemical differences existed among various strains of the organism, for example, carbohydrate fermentation patterns and menadione requirement, none of the strain differences were related. Thus, it was thought that \textit{B. melaninogenicus} should remain a single species. Later, Moore and Holdeman (155) divided the strains of this organism into the following subspecies based on
characteristic fermentation patterns and volatile fatty acids produced during growth:

B. melaninogenicus subsp. melaninogenicus
B. melaninogenicus subsp. intermedius
B. melaninogenicus subsp. asaccharolyticus

However, the clinical and epidemiological significance of this differentiation among these subspecies has not yet been determined. Several techniques and tests, nonetheless, have been developed for routine separation of the three subspecies of B. melaninogenicus (94,128,196,213, 214,235) and they clearly show the substantial differences among the subspecies (93,189). The subspecies differ from each other in cell wall composition, guanine plus cytosine content and some biochemical tests such as esculin and starch hydrolysis (189,235). More recently, a fluorescent antibody technique was developed (115) for the identification of B. melaninogenicus and it provided support for the biochemical separation of this organism into the three subspecies proposed by Moore and Holdeman (155). It also provided a rapid procedure for identification of these organisms in the clinical laboratory as opposed to the slower biochemical methods.

More recently, the subcommittee on Gram-negative anaerobic rods of the International Committee on Systematic Bacteriology proposed that asaccharolytic strains of B. melaninogenicus should be reclassified as a separate species, Bacteroides asaccharolyticus whereas the saccharolytic strains should retain their current designation (98).

For the purpose of the present work, the former nomenclature, B. melaninogenicus ss. asaccharolyticus will be used.
1. Biochemical Characteristics of \textit{B. melaninogenicus}

\textit{B. melaninogenicus} produces a cell-associated black pigment when grown in the presence of excess heme. The usual procedure is to grow the organism anaerobically on blood agar medium containing laked blood. Pigmentation begins about the third day and becomes dark olive brown in a week, and finally black in 5-14 days. Often this is the sole criterion for identifying a member of the \textit{Bacteroides} genus as \textit{B. melaninogenicus}, as the characteristic pigmentation remains the basic criterion for differentiation from other \textit{Bacteroides} species (95).

The pigment of \textit{B. melaninogenicus} was identified as extracellular melanin by Oliver and Wherry (163), and later claimed to be intracellular colloidal ferrous sulphide by Tracy (222). Schwabacher \textit{et al.} (187), and more recently Duerden (44) presented data showing that the pigment was hematin. Formation of pigment is dependent upon cultural conditions such as age of the culture, presence of heme or heme-containing compounds in the growth medium and other factors. Therefore, the final identification of \textit{B. melaninogenicus} should not be based on pigmentation characteristics alone (203).

Most strains of \textit{B. melaninogenicus} require a complex growth medium. They have an obligate requirement for hemin and for peptides. Many strains also have a requirement for vitamin K or a related naphthaquinone for growth (66,119). It was also found that a number of biosynthetic precursors of vitamin K could act as growth promoters for \textit{B. melaninogenicus} (179).

Since the black pigment formed by the bacterium is a hemin derivative, and the extent of production is dependent upon the amount
of hemin present in the medium (187), it is tempting to speculate that pigment formation represents a storage mechanism for this required nutrient. The observation that deeply pigmented colonies subcultured from blood agar plates develop well when initially transferred to hemin-free media but fail to grow upon subsequent transfer (66), supports this view.

The fact that both heme and vitamin K are required for growth of B. melaninogenicus suggests that an electron transport system may be involved in energy metabolism. Rizza et al. (177) found that 99% of the cytochromes were located in a partially purified membrane fraction. Whether or not the electron transport system functions in the metabolism of B. melaninogenicus remains undefined.

Lev and Milford (121,122) reported that vitamin K had a specific effect on sphingolipid biosynthesis. It induced the formation of 3-keto dihydrosphingosine synthetase (123) and thus stimulated the synthesis of this novel microbial lipid.

Sodium succinate was found to be an additional growth factor for B. melaninogenicus in that it could replace the required heme in the presence of vitamin K, allowing good growth of the organism. Succinate can also partially replace the required vitamin K in the presence of heme (120). The addition of succinate to a medium supplemented with both vitamin K and heme increased the growth rate of the culture. These results demonstrate a central role for succinate in the metabolism of B. melaninogenicus and suggest that there are two pathways of succinate metabolism, mediated by heme and vitamin K, respectively.
The relationship between heme, vitamin K and succinate in *B. melaninogenicus* is not understood. It is not known whether strains of *B. melaninogenicus* which do not require vitamin K synthesize the molecule *de novo* or whether they have evolved alternate metabolic systems which do not require the molecule. It has been reported that growth of *B. melaninogenicus* was dependent on the presence of large quantities of succinic acid suggesting that this compound was used in energy metabolism and was not incorporated into cellular carbon (146). This assumption was supported by the observation that only 0.5% of the succinate carbon could be found in the cell, the remainder of the metabolized succinate was excreted as butyrate. It was also found that hemin blocked the metabolism of succinate and that the fatty acid metabolites are qualitatively similar but quantitatively different in cells grown in hemin free succinate medium (146).

Thirty-one strains of *B. melaninogenicus* were studied by Sawyer et al. (185). All the strains were actively proteolytic and collagenolytic, attacking reconstituted neutral salt-extracted collagen and gelatin, and produced $\text{H}_2\text{S}$. All the strains required or were stimulated by hemin and when given an excess of hemin, produced black colonies (185). None of the strains reduced nitrates, none formed catalase and three distinctly different fermentative patterns were observed. A similar study done by Courant and Gibbons (35) showed the same results. Werner and Reichertz (232) characterized ten strains of *B. melaninogenicus* with regard to the inability to produce ammonia or propionate from threonine and the lack of glutamate decarboxylase activity. Burdon (25) reported on strains of *B. melaninogenicus* which
were highly proteolytic, attacking gelatin, coagulated serum egg albumin and milk, but which did not ferment carbohydrates. Later, Finegold (59) reported that *B. melaninogenicus ss asaccharolyticus* hydrolyzed esculin, was indole negative, clotted milk, and did not possess lipase activity. While differences have been found between strains with regards to enzyme activity, indole, esculin and starch hydrolysis, \( \text{NH}_3 \) and \( \text{H}_2\text{S} \) production, fermentation patterns and end product analysis, no correlation between any of the above and pathogenicity has been established. We have found in our laboratory a very relevant classification scheme which is based on the pathogenicity of the *B. melaninogenicus* (146). In this scheme collagenase, protease, fatty acid production, hemagglutination and pathogenicity are related. After screening 200 new strains as well as strains 2D and K110, it was concluded that irrespective of the source, the isolates could be separated into two groups. The pathogenic strains produce collagenase, high levels of protease, butyrate and phenylacetate and they agglutinate red blood cells. The non-pathogenic strains produce succinate instead of phenylacetate and do not produce the other compounds. No exceptions were reported in the 200 strains isolated in the laboratory. This suggested that a study of some of these properties in greater detail, would be valuable.

Many strains of *B. melaninogenicus* were reported to produce a collagenase (67,83,84,106,131). Okuda and Takazoe (161) found that twenty nine out of fifty nine strains of *B. melaninogenicus* studied had hemagglutinating activity. Recently, Slots and Gibbons (199) reported that forty seven out of forty eight asaccharolytic strains of *B. melaninogenicus* agglutinated human erythrocytes, whereas none of 20 fermentative strains were active. It has been shown by Reichertz
et al. (175) that anaerobic Gram-negative, non-sporing rods belonging to the genus *Bacteroides* were unable to degrade the amino acids valine and leucine completely and therefore accumulate isobutyric and isovaleric acid. They also characterized *B. melaninogenicus* in a more recent report (232), according to the results obtained with ten strains, by the production of acetic, propionic, isobutyric, butyric and isovaleric acids in peptone-yeast extract-glucose media. *B. melaninogenicus* was separated from other *Bacteroides* strains which exhibit a similar pattern of acid products by the relatively great amount of butyrate and the outcome of the glutamate decarboxylase test.

The acid end products of *B. melaninogenicus* were also analyzed by Sawyer et al. (185) who found that saccharolytic strains produced mostly lactic, succinic and acetic acids, whereas non-saccharolytic strains produced large amounts of propionic and butyric acids.

It was found that the addition of glucose to tryppticase-yeast extract medium did not enhance growth nor was the glucose metabolized to volatile acid end products (228). Studies by Finegold and Barnes (59) revealed basic differences in the acidic end products of the two fermentative groups of *B. melaninogenicus*. Isobutyric acid was produced by both groups, whereas n-butyric acid was produced only by asaccharolytic strains, and succinic acid was produced only by saccharolytic strains. Similar results were obtained by Williams and Bowden (235).

Biochemical studies (35,185) have shown that most strains of *B. melaninogenicus* grow well in sugar-free peptide-containing broth.
In addition, this growth decreased when the trypticase concentration of the medium was reduced suggesting that the fermentation of proteinaceous constituents plays an important role in the metabolism of \textit{B. melaninogenicus} and that the organisms have the potential to ferment amino acids. Experiments using labelled proteins (228) indicated that strains of \textit{B. melaninogenicus} readily fermented amino acids when they were present as peptides. This suggested that peptides were more easily transported into the cell than were most free amino acids.

More recently (149), it was found that the addition of individual amino acids to a trypticase-yeast extract-hemin medium affected growth rates and the final yield of saccharolytic and asaccharolytic strains of \textit{B. melaninogenicus}. Some amino acids enhanced growth, and others inhibited it. The significant stimulation of growth by certain amino acids in the presence of tryptic peptides in this study suggested that for some strains of \textit{B. melaninogenicus} a few amino acids are taken up as readily as peptides. The mechanism of growth inhibition by amino acids is not known.

Lev and Milford (124) found that growth of \textit{B. melaninogenicus} was inhibited by the addition of certain monosaccharides to trypticase-hemin medium. The major inhibitory effect of the sugar was to prolong the transition from the lag to logarithmic growth phase. They ascribed this to an effect on enzyme induction of which the inhibition of 3-keto dihydrosphingosine synthetase activity was one example. In actively growing cultures, addition of sugar slowed the growth rate and did not appear to be related to the activity of the synthetase enzyme. It was also possible that other enzymes were affected by the inhibitory
monosaccharides, contributing to a retardation of the growth rate. They also noted that sugars did not inhibit enzyme activity in vitro.

2. Pathogenic Properties of *B. melaninogenicus*

   a. Role of *B. melaninogenicus* in mixed anaerobic infections and periodontal diseases.

   In the oral cavity, mixed populations of anaerobes can be isolated from a variety of necrotic lesions, including mucous membrane abscesses, dry socket and cellulitis. Anaerobic bacteria comprise a large percentage of the gingival microflora and it is not unreasonable to assume that they may be involved in the initiation of pathogenic processes. The possible importance of anaerobic organisms in the etiology of periodontal diseases has been noted by a number of authors (134,137,138,205,206,208,217,230). Thus, the oral flora not only possesses pathogenic potential, but this potential is often realized when a suitable environment is available. *B. melaninogenicus* is almost always present in the human mouth (25,68,69,197,204) and is commonly found in mixed anaerobic infections (4,25,148,192,202). Antigenic components of *B. melaninogenicus* have also been demonstrated in diseased gingival tissue (34). *B. melaninogenicus* of oral origin has been reported to be involved in anaerobic infections of the lung and brain (25,32,192).

   In a recent report (198), Gram-negative anaerobic rods were shown to comprise approximately 75% of the cultivable bacteria in plaque which was removed from the base of deep gingival pockets of adults. *B. melaninogenicus* constituted almost half of
these Gram-negative isolates, and most of the *B. melaninogenicus* strains were non-saccharolytic and appeared to belong to the sub-species *asaccharolyticus* (198).

Mixtures of pure cultures of anaerobic human gingival crevice bacteria were shown to be pathogenic when injected subcutaneously into the groin of a guinea pig (134). Macdonald extended this observation to show that an infection could be initiated by a combination of *B. melaninogenicus*, two other bacteroides and a diphtheroid (135). The principal role played by *B. melaninogenicus* in the etiology of the infection was shown by Socransky and Gibbons (205). It was found that no infection occurred when *B. melaninogenicus* was omitted from the mixture.

The role of the "helper" organisms has only been partially defined (66,205,206,211). It can be assumed that "helper" organisms assist anaerobes by using up the oxygen, decreasing the Eh and/or producing catalase. Other factors that might be involved are the ability to force entry into tissues, resistance to host defences, the production of substances that block humoral antimicrobial action or nutritional dependency (66,205,206,211). Evidence for the latter was provided in an experiment where the *B. melaninogenicus* used did not require vitamin K, thus the diphtheroid and one of the bacteroides were omitted without altering the pathogenicity. It was also reported that the infectivity of *B. melaninogenicus asaccharolyticus* was dependent on a "helper" organism to produce a required growth factor which was shown to be succinate (146). The need for the second
organism could be eliminated by inoculating \textit{B. melaninogenicus} together with agar-immobilized succinate (146).

On the assumption that elucidation of the pathogenic mechanisms involved in experimental mixed infections might provide some understanding of the mechanisms involved in the pathogenicity of clinical anaerobic infections, investigators became concerned with the production of potentially damaging metabolites, toxins, or other factors which would explain how \textit{B. melaninogenicus} and associated organisms were able to cause infections. Although Macdonald had suggested earlier that perhaps mixed infections were bacterially nonspecific but biochemically specific in terms of toxins, lytic enzymes and other damaging factors produced by the mixed population (135,136), the demonstration of the essential role of \textit{B. melaninogenicus} in the experimental system suggested that the "nonspecific" infections were, in fact, dependent on the presence of \textit{B. melaninogenicus} and that the role of other organisms was one of supporting and enhancing the \textit{in vivo} growth of the primary pathogen (139,205). Evidence thus implicated \textit{B. melaninogenicus} as the primary pathogen in mixed infections of soft tissues; consequently, \textit{B. melaninogenicus} was examined for pathogenic properties and infectivity.

b. Infectivity of \textit{B. melaninogenicus}.

The criteria used for defining a typical transmissible infection were summarized by Socransky and Gibbons (205). A successful infection occurs when: (i) inoculation results in visible necrosis, either spreading or confined to a pustular abscess; (ii) exudate is infective when inoculated into another
Two types of experimental mixed anaerobic infections are observed: (1) A fatal, rapidly spreading necrotic infection which penetrates the peritoneal cavity and/or perforates the skin within 18 hours. The animal loses hair and necrosis of the skin occurs in the abdominal region. The fascia connecting the skin to the abdominal wall is loosened and the cavity created becomes filled with an exudate containing bacteria, white blood cells and eventually, red blood cells. (2) A walled-off localized abscess containing foul-smelling exudate which can be used to transmit the infection to a second animal. Successful transmissible infections in animals inoculated with defined mixtures of microorganisms were reported by several investigators (4, 89, 105, 136, 138, 139, 203).

It was shown (215) that a pathogenic strain of \textit{B. melaninogenicus} was infective when a pure culture was injected intradermally in rabbits and guinea pigs. During a study on the immunological characterization of \textit{B. melaninogenicus} (215), it was found that a vaccine prepared by phenol treatment from one strain was so harmful that rabbits frequently died during the immunization period. Therefore, the potential pathogenicity of the strain was suspected. \textit{B. melaninogenicus}, except for two reported cases (215, 142), does not possess any known capsular material or anti-phagocytic surface components. However, some strains can elaborate cell-associated and extracellular enzymes which may enhance their invasive properties. These enzymes include collagenase (69), proteases and
hyaluronidase (1). Kestenbaum (106) demonstrated a positive correlation between collagenase activity and infectivity for four *B. melanogenicus* strains in a guinea pig system. Collagen degradation is a feature of periodontal disease (131,206) and although *B. melanogenicus* is the only organism indigenous to the oral cavity known to produce a collagenase (67,83,84), the relationship between collagenase production and the pathogenicity of the organism either in oral lesions or in other mixed anaerobic infections remains unclear. Whether factors other than collagenase were involved in Kestenbaum's system is not known. Enhancement of a fusobacterial infection in rabbits by simultaneous injection of a crude cell-free preparation of *B. melanogenicus* collagenase was demonstrated by Kaufman (101). Heating eliminated both enzyme activity and the effects of the extract on infection, suggesting that collagenase may play a role in the organism's pathogenicity. This does not exclude the possibility that other heat-sensitive factors such as protease or hemagglutinin may contribute to the pathogenicity of the cells. This also suggests that endotoxin was not involved since its effect cannot be removed by heating.

c. Toxin production.

Some mixtures of bacteria containing *B. melanogenicus* when injected subcutaneously into the groin of a guinea pig cause a rapid and spreading infection (134) resulting in extensive fluid accumulation. A pathogenic strain of *B. melanogenicus* ss. *asaccharolyticus* was reported to possess a heat
sensitive toxin which induced fluid accumulation in ligated mouse ileal loops (146). *Vibrio cholerae* toxin induces a similar response in the gut (168). Preliminary experiments by B.C. McBride (personal communication) have indicated that culture supernatants obtained from *B. melaninogenicus* ss. *asaccharolyticus* possess a cholera toxin-like activity as measured by the vascular permeability assay described by Craig (36). The activity is not as strong as that of *V. cholerae* and is lost when the cells are subcultured repeatedly. Toxin is injected intracutaneously into the skin of a shaved guinea pig, and after a suitable interval, a blue dye is injected intracardially. The dye complexes with serum proteins and passes through capillary walls in areas where vascular permeability has been increased by toxin. The resulting blue area around the site of inoculation is then measured. The assay is simple and sensitive.

3. **Antibiotic Sensitivity**

A study on antibiotic susceptibility (232) showed that *B. melaninogenicus* ss. *asaccharolyticus* was sensitive to penicillin, cephalosporins, bacitracin, chlorotetracyclin, chloramphenicol, erythromycin and rifampicin and resistant to streptomycin, colistin, polymyxin B and neomycin. Finegold (55) has found stock strains of *B. melaninogenicus* to be uniquely resistant to kanamycin and vancomycin and has suggested that the isolation of *B. melaninogenicus* from heavily contaminated source material would be facilitated by the incorporation of these antibiotics in the media. Loesche and Hockett (129) reported the resistance of certain strains of *B. melaninogenicus* to kanamycin.
and that the addition of this antibiotic to a culture medium facilitated the primary isolation of the organism from source material such as dental plaque. A more recent study (189) showed that sensitivity to vancomycin and colistin will differentiate between saccharolytic and asaccharolytic strains. Most asaccharolytic strains were sensitive to vancomycin and resistant to colistin whereas both saccharolytic groups were resistant to vancomycin and sensitive to colistin.

4. **Antigenic Structure and Serological Heterogeneity**

The antigenic composition of bacteroides cells has been shown to be species-specific and potentially useful in speciation (39). However, only few studies have attempted to group strains of *B. melaninogenicus* on the basis of serology. Weiss (230) extracted protein from two strains of *B. melaninogenicus* which were immunologically distinct. Shevky et al. (192) reported that several strains of *B. melaninogenicus* reacted with a single antiserum and stated that there was "no reason to postulate the existence of a wide variety of serologically heterogeneous strains". The antigenicity of thirteen strains of *B. melaninogenicus* isolated from various sources was studied by Courant and Gibbons (35) who concluded that *B. melaninogenicus* strains were serologically heterogeneous, and seemed to represent a spectrum of serotypes. More recently, a fluorescent antibody procedure was developed (115) which showed that human *B. melaninogenicus* strains could be divided into three specific serogroups according to the biochemical subspecies already known. The fluorescent antibody conjugates were specific and no cross-reaction occurred with other anaerobes or aerobes tested.
Rabbits and guinea pigs immunized with *B. melaninogenicus* were examined for their humoral and cellular antibody (162). The results indicated that *B. melaninogenicus* resident in the gingival crevice has an ability to induce delayed hypersensitivity with the result that the area becomes susceptible to infection by the microorganism.

5. Genetic Properties

Genetic studies on *B. melaninogenicus* are scarce and no specific details have been published concerning the genetic properties and variability of the organism. Colony form is usually smooth but rough variants are found (215). Pigmentation is also noticeably variable being generally black, but different shades of brown can be observed. Other properties, such as the requirement for heme and vitamin K, hemagglutinin, collagenase and protease production might also reflect some genetic variation, although this has not been proven. *B. melaninogenicus* has not been found to possess plasmids nor has it been shown to acquire plasmids from different Gram-negative species (27). Antibiotic resistance is not related to the presence of plasmids (41). Bacteriophages capable of infecting *B. melaninogenicus* have not been isolated (103).

6. Outer Membrane of *B. melaninogenicus*

Lipopolysaccharides (LPS) are located in the cell wall of Gram-negative bacteria where they form, along with lipids and proteins, the outer membrane of the cell. They represent the O antigen and the endotoxins of these organisms (99). Endotoxic LPS, consisting of three major components, i.e. fatty acids, saccharides and sometimes bound amino
acids, is considered to include three main regions of contrasting chemical and biological properties. The O-specific polysaccharide (region I), carrying the main serologic specificity, is linked to the core polysaccharide (region II), which is relatively group specific. The core is linked through 2-keto-3-deoxyoctonate (KDO) to lipid (region III) termed lipid A (133).

Lipopolysaccharides, also called endotoxins from aerobic Gram-negative bacteria have been the subject of detailed investigation for many years. Considerable attention has been directed toward definition of the chemical structure, biologic activity, and immunogenicity of these outer cell membrane-localized antigens (17,99,133). The LPS of *Salmonella* is an important virulence factor; loss of its O-specific side chains results in loss of virulence but has no effect on endotoxic properties. Endotoxins exert their multiple biological and immunologic effects only after liberation from bacteria. Such bacterial and immunologic effects become evident after adsorption of endotoxin onto the host cellular membrane (17).

Lipopolysaccharide (LPS) antigens of anaerobic Gram-negative bacteria have received far less study, because the importance of these bacteria in clinical infections was not fully appreciated until recently when their isolation from clinical specimens became technically more feasible. Some anaerobic Gram-negative bacteria contain LPS which is chemically and biologically similar to the endotoxins of aerobic bacteria (92). However, it is of interest that *Bacteroides fragilis* and *B. melaninogenicus* appear to have rather unusual LPS(92,100). In studies of these two species, Hofstad has noted the predominance
of fatty acids and neutral sugars with the absence of the sugars 2-keto-3-deoxyoctonate (KDO) and heptose, which are found uniformly in the LPS of aerobic Gram-negative bacteria.

The isolation and purification of the outer membrane complex of \textit{B. melaninogenicus} subspecies \textit{asaccharolyticus} was studied by Mansheim and Kasper (141). Morphologic study by electron microscopy disclosed the presence of a capsule and a cell wall structure otherwise typical of a Gram-negative organism. With the use of gentle techniques of heat, EDTA treatment, shearing, and differential centrifugation, the outer membrane was isolated. A relatively pure preparation was suggested by the absence of nucleic acids and muramic acids, the existence of relatively few peptide bands on SDS-polyacrylamide gel electrophoresis, morphologic studies by electron microscopy, and the presence of a single band on a sucrose density gradient (141).

Fractionation by gel filtration of the outer membrane after deoxycholate treatment revealed two major components. The first consisted primarily of a large molecular weight protein-polysaccharide complex with loosely bound lipid (26%). Antigenicity of this first component was demonstrated by agar gel diffusion. Analysis of the protein by SDS-polyacrylamide gel electrophoresis of three strains revealed strain specificity. Further purification of this fraction showed that the polysaccharide component cross-reacted with antiserum to another strain of the same subspecies. This component probably represents the capsular antigen and may prove to be the basis of serogrouping.

The second membrane fraction differed chemically from the first fraction and represents the lipopolysaccharide component of the
outer membrane. It consisted mainly of loosely bound lipid (62%), protein (5%), and polysaccharide which was clearly distinct from that of the first fraction. Notably, this component lacked 2-keto-3-deoxyoctonate, one of the backbone components of aerobic, Gram-negative lipopolysaccharides. Purification of the outer membrane of *B. melaninogenicus* and identification of the outer membrane antigens will provide an opportunity for better study of the mechanisms of immunity to infections involving this organism. Demonstration of serologic cross-reactivity between capsular antigens may form the basis for serogrouping within the species *B. melaninogenicus*.

Recently, the lipopolysaccharide component was isolated from the outer membrane complex of *B. melaninogenicus* ss. *asaccharolyticus* (142) by gel chromatography using sodium deoxycholate (NaD), a disaggregating detergent, in the running buffer. The LPS was composed of loosely bound lipid (62%) and carbohydrate (32%), with less than 5% protein. Glucose, galactose, and glucosamine were the major sugars as detected by gas-liquid chromatography (GLC). Heptose and KDO were not observed by colorimetric analysis. Long chain fatty acid analysis by GLC disclosed an unusual pattern; β-OH myristic acid, a common component of aerobic Gram-negative LPS, was absent. Furthermore, two unknown peaks, which may be cyclic or odd chain fatty acids, were detected.

*B. melaninogenicus* LPS preparation did not induce skin reactions in rabbits when administered in doses of up to 1 mg, compared to *Salmonella typhi* endotoxin which elicited a positive reaction in doses of 12.5 μg. Little or no endotoxic activity was demonstrated.
These findings are compatible with previously noted observations on LPS in anaerobic bacteria (90,100), all of which stand in sharp contrast to the widely known biologic activity of aerobic Gram-negative LPS. This may explain the rarity of septic shock in patients infected with anaerobic organisms. It is intriguing to speculate that the unusual pattern of fatty acids in the lipid A, may be responsible for the biologic impotence of the LPS of B. melaninogenicus (145). The presence of capsular polysaccharide contaminating the phenol/water-extracted LPS was reported (142), and may explain partially the serologic heterogeneity which has been described previously in studies of the LPS of this organism (91). The factors within the LPS which determine serologic activity are extremely complex. Characterization (142) of a relatively homogeneous LPS may make further investigation of the pathogenic mechanisms and immune response to B. melaninogenicus somewhat more clear.

The lipids and related compounds in the cell envelopes of B. melaninogenicus were studied by two groups of investigators. Parker and White (167) and Rizza et al. (178) reported that nearly half of the phospholipids isolated from B. melaninogenicus are phosphosphingolipids. The two major phosphosphingolipids have been characterized as ceramide phosphorylethanolamine (CPE) and ceramide phosphorylglycerol (CPG). The finding of phosphosphingolipids in bacteria is exceedingly rare, although another anaerobe, Bacteroides ruminicola, has been reported to contain an ethanolamine-containing sphingolipid (133).
It was also found that the lipid composition of *B. melaninogenicus* was similar to that of other Gram-negative bacteria in that part of the extractable fatty acids was present as phospholipid and that phosphatidyl ethanolamine was the predominant diacyl phospholipid. *B. melaninogenicus* is unusual in that it contains only a small amount of non-extractable fatty acids which are usually found to be associated with the polysaccharide, and in the absence of β-hydroxy fatty acids usually found in lipid A of the outer membrane of Gram-negative bacteria. The diacylphospholipids of *B. melaninogenicus* consist of phosphatidylethanolamine, phosphatidyl glycerol, phosphatidyl serine, phosphatidic acid and cardiolipin. It has been reported that these bacteria do not contain glucolipids (167). Vitamin K₂ isoprenologues make up the bulk of neutral lipids (178) which represent a small portion of the extractable fatty acids. The lipids seem to be localized in the membrane fraction of *B. melaninogenicus* along with cytochrome c. The phospholipid found in the supernatant fraction might represent very small membrane fragments (178). There was not much change in the total amount of vitamin K₂ and phospholipid in the membrane of *B. melaninogenicus* grown with different levels of protoheme supplementation in the medium (178).

B. Hemagglutinating Activity and Adherence Properties of *B. melaninogenicus*

1. Adherence

Adherence has recently been found to be an important ecologic-al factor in specific disease processes (52,62,70,143,236). The
ability of many organisms to adhere to a particular surface of their host is one requirement for colonization and in some cases facilitates the invasion of that host (73). It has been recognized that *Vibrio cholerae* adhere to the intestinal mucosa (61) and that the inability to do so results in a reduction in virulence (63, 186). Ellen and Gibbons demonstrated that virulent strains of *Streptococcus pyogenes* adhered well to epithelial cells whereas an avirulent strain lacked this ability (51).

Adherence is also an important ecological determinant which influences the colonization of bacteria in environments subject to the flow of liquids (73). It was shown, as early as 1954, that certain oral bacterial species preferentially colonize different sites within the mouth (112). Gibbons has shown that this selective colonization of oral bacteria is correlated with selective bacterial adherence (70). Despite the recognition of the selective adherence of oral bacteria, there is limited information concerning the mechanism of adherence. Tentative conclusions as to the nature of the adherence of bacteria were based on either direct microscopic examination or on the results of enzymatic, physical and chemical pretreatments (125, 209). However, one must be careful interpreting these data because of the gross nature of the various treatments. It was postulated that for some organisms, adherence is mediated by proteinaceous surface components (51, 52, 125), while for others a lipid component may be involved (71, 159). There are also reports of bacterial adherence to epithelial cells with the possible involvement of neuraminic acid receptors on the host cells (209), and in other cases the possible involvement of
carbohydrate and teichoic acid moieties on the bacterial cells (64).

Adherence can generally be classified according to:
(i) attachment to a body surface, i.e. buccal epithelium or tooth
(ii) attachment to other bacteria, i.e. plaque. Attachment involves
a specific, unique receptor site on the bacterial cell surface and a
 corresponding site on the substrate to which the organism adheres.

The relatively high proportions of \textit{B. melaninogenicus}
ss. \textit{asaccharolyticus} found in periodontal pockets (198) and the
potential of this organism to synergistically produce mixed anaerobic
infections in experimental animals (72,134,139,205), suggest that it
may play an important role in the etiology and pathogenesis of peri­
dontitis. Little is known about the parameters which influence the
colonization of \textit{B. melaninogenicus} in periodontal pockets or in other
sites of the mouth, and little information is available about the
mechanisms of \textit{B. melaninogenicus} retention and prevalence in the oral
cavity. Recently, Slots and Gibbons (199) and Edwards (50) initiated
studies to determine the ability of \textit{B. melaninogenicus} ss. \textit{asaccharo­}
lyticus to attach to various oral surfaces, and to evaluate the role
that adherence might play in its oral and subgingival colonization.
They found that \textit{B. melaninogenicus} cells suspended in phosphate-
buffered saline adhered well to buccal epithelial cells and to the
surfaces of certain Gram-negative bacteria that are prominent in human
dental plaque. They also reported that of forty eight asaccharolytic
strains of \textit{B. melaninogenicus}, forty seven agglutinated human
erthrocytes (199). Their data indicated that certain Gram-positive
organisms found in dental plaque possess receptors for the attachment
of B. melaninogenicus cells and that these receptors are different from those present on buccal epithelial cells and erythrocytes.

2. Hemagglutinin

Macromolecules which react with specific components of red blood cell membranes leading to agglutination of these cells are not uncommon among certain bacterial species.

For organizational purposes, the literature survey of bacterial hemagglutination is considered separately from studies using cells and surfaces other than erythrocytes. Although recent reviews on bacterial adherence have often overlooked the literature on bacterial hemagglutination, there is no evidence to suggest that adherence of bacteria to cells other than erythrocytes is a different phenomenon from bacterial hemagglutination (72,73). In fact, studies using hemagglutination assume that this phenomenon is an index of bacterial attachment and, at least in some cases, adherence to epithelial cells has exactly the same characteristics as bacterial hemagglutination (160). Therefore, one way of studying adherence is to evaluate the hemagglutinating activity of an organism using model adherence systems of erythrocytes as relatively simple, well-characterized natural surfaces.

The ability of certain bacteria to agglutinate red blood cells was demonstrated as early as 1955 (45). In later studies, a great deal of significance was placed on the role of pili in hemagglutination (28,48,49,72,160,191). From studies using piliated and non-piliated enterobacteria, it appeared that the presence of pili did not always seem to be necessary in order for hemagglutination to occur (46,47,224). It was concluded that there are several different characteristics
between a piliated and a non-piliated hemagglutination reaction. Among these there are differences in agglutination range of erythrocyte species, elution of bacteria from erythrocytes at high temperature, and inhibitory effects of D-mannose (46,47,224). Although much work has been done on the adherence of oral bacteria to epithelial cells and other surfaces, little has been done on the hemagglutination activity of the organisms. Recently, it was reported that

*Streptococcus sanguis*, *Streptococcus mitis*, and *Actinomyces viscosus* all agglutinate human red blood cells (180). Studies on the hemagglutinating properties of anaerobic bacteria have been minimal. Okuda and Takazoe (161) found that twenty nine of fifty-nine strains of *B. melaninogenicus* studied had hemagglutinating activity that seemed to be mediated by surface pili. They suggested that these structures might also mediate the attachment of *B. melaninogenicus* cells to oral mucosa. Slots and Gibbons (199) reported that forty-seven of forty-eight asaccharolytic strains of *B. melaninogenicus* representing fresh isolates from subgingival plaque and tonsillar swabbings agglutinated human erythrocytes, whereas none of twenty fermentative strains, which included reference cultures of the subspecies *intermedius* and *melaninogenicus* were active. Electron microscopy indicated that both asaccharolytic and fermentative strains possessed pili. These workers also found that the non-hemagglutinating strains of *B. melaninogenicus* containing pili attached well to buccal epithelial cells. Thus, no clear relationship exists between the hemagglutinating activity of strains of *B. melaninogenicus* and their ability to attach to buccal epithelial cells. The observations further suggested that several
types of pili exist on different strains and subspecies of
B. melaninogenicus (199).

C. Proteolytic Activity of B. melaninogenicus

It has been known for many years that many microorganisms produce
appreciable amounts of proteolytic enzymes. The bacterial proteases are
instrumental in the degradation of complex protein substrates to amino
acids and peptides in nature. The water soluble products with lower
molecular weights are assimilable, thus supporting cell growth.

Studies on protease enzymes are not always easy to undertake. The
reason for this may be that some proteolytic enzymes are unstable and
susceptible to autodigestion; consequently, purification procedures must
be carried out with great care under defined conditions. A few procedures
have been shown to be useful with the majority of enzyme systems and
these have been used routinely. These procedures include the following:
fractional precipitation by pH changes (5), fractional denaturation by heat,
fractional precipitation by salts (11), fractional precipitation with organic
solvents (9), fractional adsorption (166), column chromatography (5,11) and
crystallization (9). The sequence in which several or all of these steps
are used is determined by the enzyme under investigation.

There are many published reports on the purification of bacterial
proteases. The following covers a few selected examples concerning the
purification of cellular and extracellular proteases produced by different
organisms.

The extracellular protease of Pseudomonas maltophilia was partially
purified by ammonium sulfate precipitation and chromatography on Sephadex
G-75 and Bio-rex 70. Gel electrophoresis revealed minor impurities (16).
The cell-bound protease of *Bacteroides amylophilus* H18 was liberated from the mechanically ruptured cell envelopes by n-butanol treatment and was purified 80-fold by (NH$_4$)$_2$SO$_4$ precipitation, electrophoresis and gel filtration through Sephadex G-200 (15).

Once a proteolytic enzyme is discovered and partially purified, it is interesting to investigate its chemical and physical properties. Information in these areas is important in order to classify the enzyme and to determine its function in vivo. In the past, proteolytic enzymes have been classified by several criteria. One classification grouped proteolytic enzymes into the categories pepsin-like, trypsin-like or cathepsin-like. This system is based mainly on the pH optimum of the enzyme. Extracellular proteases from microorganisms have been classified into three groups by their pH optima i.e., acid, neutral and alkaline proteases (81). Bergmann (10) proposed a system which grouped proteolytic enzymes according to their action on synthetic substrates. A system such as this tells the investigator something of the mode of action of a particular enzyme but this method also has its limitations. Enzymes of different origins may produce essentially the same action on synthetic substrates but may differ in their reactivity towards natural substrates. Another system was proposed (6) which divided the proteolytic enzymes into categories based on their behaviour toward a number of proteolytic inhibitors. Systems such as those mentioned have their inherent shortcomings. For example, if a particular enzyme was classified as trypsin-like, one might assume that it possessed all the other chemical and physical properties of trypsin whereas in reality it did not. It is necessary therefore to characterize the proteolytic enzyme in as many ways as possible.
Characterization of a protease should include the determination of the site of hydrolytic attack on natural substrates, the action of the enzyme on various synthetic substrates, the nature of the reactive site of the enzyme, the response to inhibitors, the pH and the temperature stability, as well as the electrophoretic properties and the molecular weight of the enzyme.

Generally, if the enzyme under investigation shows a specificity like, or is affected by one of the inhibitors of the well-known and thoroughly studied groups of enzymes, it is preliminary referred to as belonging to this class of enzymes. It should be noted, however, that some proteolytic enzymes do not fall into the major categories which are based on mechanism of action rather than origin of physiological action. These categories include four principal classes of enzymes.

The first of such main groups of enzymes is the serine proteases, which are distinguished by a serine residue in the active site (86). A common test for these enzymes is the inhibition of their hydrolase activity by the reaction of this serine residue with diisopropylphosphorofluoridate (DFP). Examples include enzymes isolated from B. subtilis and related strains (subtilisins) as well as proteolytic enzymes isolated from organisms such as Streptomyces griseus (227).

The second group of proteolytic enzymes are dependent on sulfhydryl groups for their catalytic activity. Activation of these enzymes is usually achieved by mild reducing agents such as cysteine, sulfide and sulfite, which liberate a free thiol group on the enzyme. Little, if any, conformational change is associated with reduction (8). Optimum activation was found to occur upon simultaneous application of a thiol compound such as cysteine or thioglycolate and a heavy metal-binding agent like EDTA (108),
or by the addition of 2,3-dimercaptopropanol, a compound which combines the functions of both a reducing agent and a metal binder (210). The enzymes are reversibly inactivated in the presence of air and can be reactivated by addition of reducing agent. Heavy metal ions such as Cd$^{2+}$, Zn$^{2+}$, Fe$^{2+}$, Cu$^{2+}$, Hg$^{2+}$ and Pb$^{2+}$ are inhibitory. The metal inactivated enzymes can be totally reactivated by addition of a reducing agent and a chelating agent. The readily reversible formation of a stable inactivate complex with mercury has been utilized as a useful step in the purification of the well known sulfhydryl enzyme, papain (21,108). All sulfhydryl-binding reagents can act as sulfhydryl enzyme inhibitors. Thus, p-chloromercuribenzoate forms a stable complex with the enzyme and can serve for titration of the free-SH group (60). Iodoacetic acid or iodoacetamide also react with the free sulfhydryl group, causing thereby irreversible inactivation (109,190). Papain was found to react with the chloromethyl ketones of phenylalanine and lysine (TPCK and TLCK) with total loss of activity (233). In this case, the reagents act specifically on the active sulfhydryl group of the enzyme rather than on the imidazole group of particular histidyl residues as they do in the case of trypsin and chymotrypsin, and thus the inactivation of papain is a stoichiometric reaction. Examples of bacterial sulfhydryl enzymes are the streptococcal proteinases (53,114).

Another group of proteolytic enzymes includes the acidic proteases. The presence of proteolytic enzymes with a pH optimum in the acid pH range (pH 1-5) has been reported in a variety of microorganisms where they occur both intracellularly and extracellularly. Several strains of Clostridium (C. acetobutylicum and C. butyricum) (225) and Lactobacilli (18) have been shown to produce weak proteinase activity with an acid pH optimum.
There is also the group of metal proteinases which includes enzymes specific for releasing NH₂-terminal amino acids, such as aminopeptidase-P. This enzyme is an exopeptidase cleaving the bond between any N-terminal amino acid residue and a following proline residue (241) and is isolated from Escherichia coli. Thermophilic aminopeptidase-AP1, produced by Bacillus stearothermophilus splits all amino acids from the amino end of a polypeptide; preferentially hydrolyzing peptides containing leucine, valine, and those with aromatic amino acid residues.

The following are some reported examples of protease characterization. The subtilisins are alkaline proteases of broad specificity produced by strains of B. subtilis. Three of these enzymes have been studied in considerable detail and are probably the best known of all microbial proteases. The subtilisins are specifically and stoichiometrically inactivated by DFP (144), indicating that they are serine proteases. The striking feature of the subtilisins may be that their sequences bear no significant relationship to those of the pancreatic serine proteases, whereas their status as serine proteases carries the obvious implication that the active sites are in some way similar in structure to those of the pancreatic enzymes. It is nevertheless clear that there are considerable differences in the specificities of the subtilisins and the pancreatic serine proteases. Studies of the hydrolysis of ester substrates (76) suggest that, in contrast to the high specificity of chymotrypsin and trypsin, the subtilisins have rather low specificity.

A number of interesting DFP-sensitive proteases from strains of Staphylococcus aureus have been studied by Drapeau and co-workers (42) and are reported to have a high specificity for glutamyl and aspartyl residues.
The staphylococcal enzymes thus do not resemble any of the previously known serine proteases.

Streptococcal proteinase is a sulfhydryl enzyme which is elaborated by group A streptococci. It is excreted into the medium as a zymogen which is transformed into an active enzyme by proteolysis followed by reduction. Both the zymogen and the enzyme contain only a single half-cystine residue per molecule (53,114). The reduced, active enzyme can be readily inactivated by reagents known to react with sulfhydryl groups, such as iodoacetic acid, iodoacetamide, p-chloromercuribenzoate, Hg$_2^+$, and atmospheric oxygen. Streptococcal proteinase thus appears to be a classic sulfhydryl enzyme.

There are comparatively few reports of extracellular proteases from Gram-negative bacteria, and fewer still of DFP-sensitive proteases. A survey of published work indicated that the Gram-negative bacteria known to secrete extracellular proteases are largely confined to the pseudomonads. This situation is also true of exoenzymes in general, and may be a reflection of the difference in complexity of the cell envelope between Gram-negative and Gram-positive bacteria (170,82). Several of the proteases from Gram-negative bacteria appear to be metallo-proteases (152,16,157,151). Extensive studies on the enzymes produced by *Aeromonas proteolytica* showed that the organism excretes two proteolytic enzymes, an endopeptidase and an amino peptidase. Both enzymes are metal proteinases which are inactivated by EDTA and possess molecular weights of 34,800 and 29,500 respectively (107). Boethling (16) described the purification and properties of a protease from *Pseudomonas maltophilia* which is an EDTA-sensitive alkaline serine protease. Nakajima and co-workers (157) described an alkaline protease of *Escherichia freundii* which was sensitive to EDTA and
had a molecular weight of 45,000. The protease enzymes from *Serratia* sp. (151) resembled the subtilisins in that they have alkaline pH optima with casein as substrate; in other respects, however, they were not similar.

Protease production by Gram-negative anaerobic bacteria has not been extensively investigated. *Bacteroides amylophilus* was reported to produce protease(s), active at pH 7.0, which was neither induced nor repressed by a wide range of nutrients. The protease was synthesized by exponentially growing organisms and 20% was liberated into the growth medium. The cell-bound protease was completely accessible to the protein substrate (13).

The major function of extracellular proteinases and other hydrolytic enzymes is most reasonably a nutritional one which evolved to allow the microorganism growing in its natural environment to utilize complex non-diffusible substrates as a source of nutrients. In addition to the nutritional role, extracellular proteinases of the genus *Bacillus* are thought to be required for sporulation (37) and thus clearly have an intracellular function in a specific developmental process.

Limited proteolytic degradations are responsible for the induction of biological activities, as in the formation of biologically active enzymes from their inactive precursors. The initial step in proteolytic degradation might be the opening of one or a few exposed peptide bonds, or the splitting of a small amount of unfolded protein in equilibrium with the native protein. An example of induction of biological activity by limited proteolysis is the conversion of the extracellular zymogen of Group A streptococci to the active enzyme (53,127). The role of a protease
in natural activation of *Clostridium botulinum* neurotoxin has been reported by Bibhuti *et al.* (12). The specific toxicity of the simple protein increases during incubation of the culture. Since the conversion of progenitor toxin to the more toxic form can be accomplished with trypsin, one mechanism for the natural activation of progenitor toxin would be through the action of a suitable enzyme(s) produced by the culture. One such enzyme is a protease with trypsin-like specificity which activates progenitor toxin obtained from young cultures of the same proteolytic type B strain (38).

Catalytic processes involving specific enzymes in the membrane may be involved in secretion of exoenzymes. A protease located in the outer membrane of *E. coli* was reported to cleave a protein located in the cytoplasmic membrane, the respiratory enzyme nitrate reductase. This cleavage is accompanied by solubilization of the enzyme (140).

The importance of intracellular proteolytic activity in the physiology of the bacterial cell has been implicated and might include roles in: protein turnover leading to continued regeneration of labile proteins, increased proteolysis during cell division, proteolytic maturation of proteins, and preferential breakdown of structurally altered proteins (169). It has been shown that the autolysin of *Streptococcus faecalis* is present in an inactive form in the cell wall but is activated by a neutral proteinase; and that the active form of the autolysin is associated with recently synthesized wall (193).
In the past few years, considerable importance has been placed upon proteolytic enzymes as tools for studies of the structure of proteins and for investigation of hydrolysis products which possess biological activity. This is exemplified in the case of the structure and the biochemistry of diphtheria toxin. The toxin molecule is released from the bacterial cell as a single polypeptide chain having two non-overlapping cystine bridges. The toxin contains a protease sensitive site which is readily hydrolyzed to yield two sulfhydryl linked polypeptides. One polypeptide is responsible for binding the toxin to its target cell; the second hydrolysis product is responsible for inducing the biochemical lesion in the protein synthesizing system of the cell. A sequence containing three arginine residues is presumed to represent an exposed loop in the intact molecule since it is abnormally sensitive to proteolytic attack. Short treatment with proteases with trypsin-like specificity yielded two large peptides, an amino-terminal fragment A and a carboxyl-terminal fragment B (74), which facilitated further studies on the structure and characteristics of the toxin molecule.

*Bacteroides melaninogenicus* has been shown to possess proteolytic activity but this activity has not been characterized. An organism dependent on peptides for growth (228) might be expected to be actively proteolytic. It has been reported that strains K110 and CR2A have a limited ability to ferment free amino acids, but the organisms can more readily dissimilate peptides (228). Many strains of *B. melaninogenicus* have been observed by Sawyer et al. (185) to be proteolytic, and the organisms appear to grow well in culture media without carbohydrate supplementation, suggesting that the fermentation of proteinaceous constituents play an important role in the metabolism of *B. melaninogenicus*. Hausman and Kaufman have found caseinolytic activity associated with a particulate fraction from
the autolysate supernatant of \emph{B. melaninogenicus} (84). Gibbons reported that forty-two of forty-seven strains of \emph{B. melaninogenicus} liquified gelatin (35). On the other hand, Oliver and Wherry (163) and Cohen (32) found that their strains fermented a number of carbohydrates but did not attack gelatin.

Hydrolysis of proteinaceous substrates by \emph{B. melaninogenicus} was also reported by Weiss (230), Schwabacher et al. (187) and Pulverer (173). Burdon (26) reported that his asaccharolytic strains of \emph{B. melaninogenicus} were highly proteolytic, attacking gelatin, coagulated serum, egg albumin and milk. The protease found in these organisms is not likely to be collagenase since casein was attacked. Gelatin, the denatured form of collagen, is generally susceptible to a number of proteases which are incapable of attacking native collagen. Therefore, the collagenases constitute a class of unique proteases capable of attacking native collagen which is resistant to other proteolytic enzymes.

\emph{B. melaninogenicus} may elaborate more than one protease, as activity has been demonstrated in the washed cells as well as in the supernatant. The cellular and soluble proteases of \emph{B. melaninogenicus} which hydrolyze gelatin are also active against a number of protein substrates including azocoll, casein, azocasein, and N,N-dimethylcasein. For the azocoll assay, the rate of dye released from the dye-protein conjugate reflects the proteolytic activity in the sample. This assay is usually qualitative rather than quantitative. Casein, having many different potentially susceptible bonds, is generally used as a protein substrate for enzymes having unknown, undefined, or broad substrate specificities. The assay depends on the determination of the amounts of TCA soluble peptides liberated from the
casein substrate by the enzyme as detected by measuring absorption at 280 nm. This assay is not extremely sensitive, and is therefore not useful for measuring small amounts of proteolytic activity. Among the reasons for its relative insensitivity, the most important seems to be its high background reading. The assay also fails to measure all bond cleavages. Hydrolysis of small numbers of bonds in such an assay would be expected to result in larger peptides, proportionally greater numbers of which would, because of their size, be precipitated by trichloroacetic acid and not be distinguished from uncleaved protein. In addition, because the assay relies upon the absorbance of soluble peptides at 280 nm and such absorbance varies from one peptide to the next, equal degrees of proteolysis by different enzymes do not result in the same increment of increased absorbance. Another assay, the dimethylcasein assay, depends on the conversion of primary amino groups into dimethyl-amino groups, a change which does not affect many properties of the substrate protein but does prevent its reaction with trinitrobenzene-sulfonic acid (TNBS), a sensitive reagent for the determination of protein amino groups. The proteolytic activity is followed by determining, with TNBS, the new amino groups produced after hydrolysis. The low background values obtained with N,N-dimethylcasein results in greater sensitivity and accuracy not possible with the unmodified casein.

The use of $^{14}$C-labeled N,N-dimethylcasein as a substrate for determining total proteolytic activity offers several advantages over other methods. The assay is more sensitive than spectrophotometric procedures. The labeled substrate is stable and can be stored for a long period of time; the assay is rapid and is not affected by the presence of large concentrations of peptides or amino acids in the sample to be assayed.
Another assay is based on the solubilization of a covalently linked chromophore from a modified protein. An example of this type of substrate is azocasein. After incubation with the enzyme, the unhydrolyzed protein is precipitated and hydrolysis products containing coupled dye are quantitated spectrophotometrically. The important point is that the absorption maximum of the covalently linked chromophore is different than that of chromophores contaminating the enzyme preparation.

In 1962, in a review that has become a landmark in the field, Pollock (170) defined an extracellular enzyme as one that "exists in the medium around the cells, having originated from the cell without any alteration to cell structure greater than the maximum compatible with the cell's normal processes of growth and reproduction". Externalization of enzymes could be accomplished either by active secretion during logarithmic growth or unintentionally (170) as a result of cell lysis, aging and leakage during division.

A consideration of the possible mechanisms involved in secretion of proteins must necessarily be related to the nature of the membrane. Costerton et al. (33) summarized evidence suggesting that various protein molecules, both structural membrane proteins and enzymes, are inserted into the membrane basic phospholipid bilayer.

May and Elliott reported that a protease was secreted from B. subtilis cells apparently as it was synthesized since there was no significant intracellular accumulation. They speculated that none of the enzyme molecules were ever present in a completed form inside the cell membrane but rather that the nascent polypeptide chain was extruded through the membrane as it was synthesized to take up its tertiary structure with
enzyme activity only on the outside (147).

A specific hypothesis to explain protein excretion, the signal hypothesis, has been developed. An elongation of peptide chain on membrane-bound ribosome results in discharging the nascent chain across the membrane; the signal sequence for excretion is then removed from the polypeptide chain by proteolytic cleavage, which was reported to be in the outer membrane fraction in *E. coli* (97).

The presence of large pores through the outer membrane of *P. aeruginosa* was reported by Hancock et al. (82). The organism secretes three proteases into the medium and has been shown to possess membrane-bound peptidases, thus the larger pores would permit entry of quite large peptides into the periplasmic space, rendering them susceptible to peptidases, whereas the extracellular proteases may be involved in the initial processing of proteins in the environment (82).

The release of lipopolysaccharide-phospholipid-protein complexes from *E. coli* has been observed for growing and stationary phase cells. The outer membrane fragments were preferentially released from those regions where newly synthesized proteins are inserted into the outer membrane (164).

Membrane bound structures have been found to be associated with exoenzyme produced by *B. licheniformis*. After protoplast formation the enzyme is found associated with vesicles (183, 184).

The outer layers of the cell envelope, particularly in Gram-negative bacteria, would also pose a barrier to exoprotein secretion. Certain enzymes found outside of the cytoplasmic membrane are not released into the medium but are bound to the outer layers of the cell envelope (33, 127).
The location of enzymes in bacteria has been determined by a variety of techniques. Many enzymes now are thought to be external to the cell membrane, as judged by criteria such as availability to substrates and inhibitors, elutability by nondamaging solvents, inhibition by specific antibodies and release by osmotic shock or by such compounds as polymyxin B. Preparation of protoplasts in stabilizing media with measurement of enzymes released, indicated the location of the liberated enzyme outside the permeability barrier in the intact cell (183). The outer membrane layer contains charged moieties but it is not yet clear what forces are involved in determining if a molecule will remain bound to the cell, either in association with mucoprotein (194), with various components in the periplasmic space (30), with lipopolysaccharide or protein of the outer membrane (96) or to be released into the menstruum (127). Clearly, the properties of the enzyme such as hydrophobicity and charge will have a bearing on the location of proteins relative to the cytoplasmic membrane.

Studies on the alkaline phosphatase (APase) of *Pseudomonas aeruginosa* by Ingram et al. (96) showed that a certain percentage of the enzyme was complexed with lipopolysaccharide which was also released during secretion. Phosphatase is located in three areas: the culture filtrate, the outer cell wall surface, and the periplasmic space. The results suggest that APase may become associated with, and bound to, a cell wall fraction which contains LPS and liberation of the complex from the outer wall may be accomplished by mechanical shearing forces developed during growth.

Cell suspensions of *Micrococcus sodonensis* secrete seven to ten individual proteins including an alkaline phosphatase and a protease. The appearance of enzyme activities in the extracellular medium was found
to be dependent on the co-secretion of at least one of several polysaccharides (19). A functional membrane-bound enzyme, the galactosyl transferase system of *Salmonella typhimurium*, was reconstituted *in vitro* from purified components including lipopolysaccharide, phosphatidyl ethanolamine and enzyme protein (181).

MacGregor reported a proteolytic activity which was found in extensively washed membrane preparations (140). This membrane-bound protease was found to be responsible for the cleavage and solubilization of nitrate reductase enzyme from the cytoplasmic membrane of *E. coli* (140). Regnier and Thang (174) reported that at least 50% of the protease activity found in *E. coli* is associated with the membrane. This membrane-bound protease was found to have many characteristics in common with trypsin.

As many microorganisms are known to produce extracellular proteolytic enzymes, several studies have been carried out on the regulation of the production of extracellular proteases by Gram-positive bacteria, especially *Bacillus* strains. However, only a few detailed reports have appeared on Gram-negative bacteria (77). Among these organisms there are marked differences in the way in which environmental factors affect enzyme production (234). In general, induction, end product inhibition and catabolic repression have been implicated in the regulation of the synthesis of these enzymes. An efficient regulatory control has been described by Tanaka and Tuchi (218) for *Vibrio parahaemolyticus*. In this organism the production of a protease was induced by amino acids and was subject to catabolite repression by easily metabolizable carbon sources.
Repression of protease synthesis by amino acids has been widely reported as an example of end product repression in bacteria of the genera *Bacillus*, *Serratia* and *Arthobacter* (77), but does not appear to occur in all Gram-negative organisms that have been studied (218).

The production of microbial cellular and extracellular enzymes has been investigated extensively in batch cultures, but applications of continuous culture techniques in these studies have not been widespread (22,77). In the studies that have been reported, the production of constitutive and inducible enzymes followed one of two general patterns (Fig. 1), and it is considered that the relationship between enzyme production and growth rate depends on the characteristics of the regulatory mechanism involved (22).

For constitutive enzymes where the rate of enzyme production is a function of the product of cell concentration and growth rate, the relationship between the rate of enzyme production and dilution rate for such enzymes is linear (Fig. 1A). This has been observed for the penicillinases production by *Bacillus licheniformis* by Wouters and Buysman (239) and for several other enzymes (31,40). An example of this is seen (Fig. 2) where the content of amidase per cell of *Pseudomonas aeruginosa* rises to a peak as the growth rate (dilution rate) is increased and the content then falls as the growth rate is increased beyond this point (31).

A non-linear relationship between rate of enzyme production and dilution rate is sometimes found for inducible enzymes (Fig. 1B). Such a behaviour is particularly apparent when organisms producing such enzymes are grown under conditions where the inducer is the growth-limiting substrate (40). The advantages of continuous culture are undeniable in fields of microbial biochemistry and metabolism (23,219), and chemostat experiments
have made a valuable contribution in the elucidation of mechanisms of enzyme regulation. Moreover, the unique growth conditions provided by chemostasis have contributed to our understanding of those processes which allow microbes to adapt to changing nutritional and other environmental conditions.

The chemostat offers possibilities which are absent in any closed culture system. During growth in batch culture microbes continuously change their environment as a result of consumption of nutrients and accumulation of waste products, therefore, the morphological and metabolic properties of the cells are apt to change during the growth period (226). In the chemostat cells can be grown in steady states at any of a whole range of growth rates. In addition, it is usually possible to make any substrate growth-limiting. Thus, the nutritional status of an organism as well as its growth rate can be varied at will. Once a steady rate is reached, neither the properties of the culture nor those of the environment undergo further change. The culture then has become time-independent, and conditions are stable. Therefore, experiments with the chemostat are highly reproducible and are ideal for studying the properties of an organism as a function of growth rate.

Growth rate of a culture can be varied by changing the dilution rate, the total cell mass of the culture remaining the same. However, at extremely low growth rates, a proportion of the cells may become non-viable and the growth yield, Y, which is defined as the cell mass (m) produced by the metabolism of unit mass of the substrate, may decrease. A lower cell mass at lower dilution rates is to be expected only when growth is limited by the substrate whose metabolism supplies energy.
FIGURE 1. General patterns of enzyme production in continuous culture.

A. Constitutive enzymes whose synthesis only depends on cell concentration.
B. Inducible enzymes.

FIGURE 2. The effect of dilution rate on the synthesis of amidase by *Pseudomonas aeruginosa* growing in a chemostat under steady-state conditions.
In the case of chemically complex media, where there is an unsatisfactory vagueness about the concentration of medium constituents, it can be assumed that growth is ultimately restricted by the exhaustion of some one substance, other substances remaining in sufficient concentration not to affect the growth rate on their own account. For example, in the case of *B. melaninogenicus*, which requires a complex medium for growth due to its obligate requirement for hemin and peptides, hemin can thus be singled out and called the "limiting substrate".

In continuous cultures, the rate of bacterial growth may be regulated by controlling the rate of nutrient addition. The rate of nutrient addition is usually expressed as the dilution rate $D$, which is the volume of nutrient added hr$^{-1}$ expressed as a fraction of the volume of the vessel.

The dilution rate will determine the length of time that a bacterial cell will remain in the chemostat and in the absence of bacterial cell division

$$\frac{dx}{dt} = Dx$$

where $x$ is the number of bacteria present in the vessel. In fact, bacterial cell division is occurring as defined by

$$\frac{dx}{dt} = Kx$$

where $K$ is the growth constant. Any change in bacterial concentration would be defined by

rate of change in cell concentration = rate of growth - rate of dilution

or

$$\frac{dx}{dt} = Kx - Dx$$
A chemostat, run at one dilution rate, soon establishes steady state conditions: there is no change in bacterial numbers. This indicates that bacterial growth exactly balances the bacteria lost by dilution:

\[ Kx = Dx \text{ or } K = D \]

When the dilution rate approaches the maximum growth rate and eventually exceeds it, more bacteria are washed out than are produced by cell division and the bacterial concentration falls (87).
II. MATERIALS AND METHODS

A. Organisms

B. melaninogenicus subspecies asaccharolyticus strain K110 was obtained from Dr. P.A. Mashimo. This is a collagenolytic strain originally isolated by Macdonald and co-workers (134) from a patient with diagnosed gingivitis. B. melaninogenicus subspecies asaccharolyticus strain 2D was isolated in the laboratory from a gingival scraping taken from an individual with periodontal disease. The gingival sample was streaked on freshly poured blood agar plate and incubated at 37°C in a N₂:H₂:CO₂ (85:10:5) atmosphere. Black colonies were repeatedly subcultured on the same medium until a pure culture was obtained. The isolate was characterized according to standard procedures (94). Neither strain K110 nor 2D required vitamin K for growth.

B. Growth

1. Anaerobiosis

Liquid or agar cultures were usually incubated in anaerobic jars (Torball, Torsion Balance, Clifton, New Jersey) evacuated and flushed with H₂:CO₂ (95:5) (Canadian Liquid Air, Montreal) or in an anaerobic glove box (Coy Manufacturing, Ann Arbor, Michigan) containing an atmosphere of N₂:H₂:CO₂ (85:10:5) at a temperature of 37°C. Humidity in the glove box was controlled between 45 and 55% with desiccated silica gel. It was found that contamination problems were minimized if the humidity was kept at 45%. Oxygen levels in the chamber were
monitored every two days with a trace-oxygen analyzer (Lockwood and McLorie, Inc., Horsham, Pa.). An oxygen level of two to five ppm was considered to be acceptable. In some instances, organisms were cultured in pre-reduced media in stoppered tubes or flasks using conventional anaerobic techniques as described by Holdeman and Moore (94).

2. **Media**

   a. **Trypticase-Yeast-Hemin (TYH) medium.**

   Liquid cultures of *B. melaninogenicus* were maintained in medium containing trypticase, 17 mg/ml; yeast extract (Difco), 3 mg/ml; NaCl, 5 mg/ml; K$_2$HPO$_4$, 2.5 mg/ml and hemin, 5 μg/ml. The pH was adjusted to pH 7.0 with HCl or NaOH. The hemin solution was made up by dissolving 0.05 gm of hemin in 1 ml 1 N NaOH and 100 ml distilled water. This solution was stored at 4°C.

   b. **Basal medium.**

   The basal medium consisted of trypticase (17 g/l); yeast extract (3 g/l); K$_2$HPO$_4$ (2.5 g/l) and NaCl (5 g/l).

*B. melaninogenicus* requires hemin for growth but apparently retains enough of the compound to sustain growth through one transfer in hemin-free liquid media (146). In order to obtain cells in a hemin-deficient state it was necessary to grow them once in basal medium. Cells obtained from the basal medium would not grow when subcultured a second time in hemin-free media. These organisms will be referred to as hemin-depleted *B. melaninogenicus*. 
c. Supplemented media.

When needed, the following supplements were added separately or together to either the TYH medium or the basal medium: sodium thioglycolate, 0.05%; glucose, 0.1%; hemin (Sigma), 10, 5, 2.5, 2, 1.5, 1 and 0.5 mg/ml; succinic acid (Eastman), 0.1% or 0.25%; single L-isomers of amino acids (Sigma), 0.5% and cysteine, 0.1-0.5%. In all cases, the compounds were added to the medium before autoclaving and the pH was adjusted to 7.0 with either HCl or NaOH.

3. Continuous Cultures

The continuous culture was performed in an anaerobic chamber (Coy Manufacturing, Ann Arbor, Michigan) containing an atmosphere of \( \text{N}_2: \text{H}_2: \text{CO}_2 \) (85:10:5) at a temperature of 37°C. The chemostat consisted of a glass vessel equipped with a rubber stopper which was punctured by three stainless steel tubes (2 mm I.D.). One tube supplied gas, another growth medium, and the other carried away the effluent or overflow. The growth flask had a working volume of 100 ml. The culture was stirred vigorously by a mechanically-driven teflon-covered stirring bar. The addition of medium was controlled by a peristaltic pump equipped with variable speed control. The cultures were supposed to be in steady state after eight changes of the medium.

The dilution rate, \( D \), was determined by measuring the output of culture from the chemostat.

\[
D = \frac{\text{output}}{\text{working volume}} \text{ (hr}^{-1} \text{)}
\]
C. Protease

1. Assays for Proteolytic Activity

   a. The azocasein assay.

   The azocasein substrate was prepared by dissolving 2 g of azocasein in 100 ml of phosphate buffered saline (PBS) in a boiling water bath. The solution was stored at -20°C. The reaction mixture contained from 0.1-0.5 ml of either a washed cell suspension ($A_{660}=1.0$), or soluble protease, and was made up to a 1 ml volume with PBS containing 50 mM β-mercaptoethanol (SME). The reaction components were pre-incubated at 37°C for 15 min before the addition of 1 ml of azocasein substrate and incubation was then continued for 60 min at 37°C. The reaction was terminated and unhydrolyzed protein precipitated by the addition of 2 ml of 10% trichloroacetic acid (TCA). The acidified solution was mixed and incubated at room temperature for 20 min and filtered through Whatman No. 1 filter paper. Absorbance at 370 nm of the filtrates was determined. Control lacking enzyme was included in all assays.

   Azocasein hydrolysis was also measured on agar plates. One gram of agar (Difco) was dissolved in 50 ml boiling PBS pH 7.0 and mixed with 50 ml of a 2% azocasein-PBS solution. The solution was heated to 80°C and 30 ml of molten agar were poured into each plate. Three mm holes were made in the agar plates and 20 μl of the enzyme solution was placed in the holes and the plates were incubated at 37°C. Enzyme activity was demonstrated by the presence of a clear zone around the holes. The logarithm of the diameter of the zones was directly proportional
to the actual enzyme activity. BME (final concentration 50 mM) was added to the B. melaninogenicus culture supernatant or enzyme preparation. Samples containing known amounts of trypsin were included in each assay.

b. The azocoll assay.

The reaction mixture for the azocoll assay contained:
Tris-HCl buffer (0.05M, pH 7.2), 4.8 ml; BME in the same buffer (50 mM), 0.2 ml; and supernatant from sedimented culture or enzyme preparation, 0.5 ml. The reaction components were pre-incubated at 37°C for 15 min before addition of 20 mg of azocoll. Incubation was continued at the same temperature in a shaking water bath. Two milliliter samples were removed at various time intervals, chilled in ice, and filtered to remove insoluble substrate. The amount of solubilized chromophore was determined by measuring the absorbance of the filtrate at 520 nm.

c. The casein assay.

The substrate for the casein assay was prepared by dissolving 1 g of casein in 100 ml of 0.1 M phosphate buffer (pH 7.4), and heating for 15 min in a boiling water bath. The reaction mixture contained 2.5 ml casein, 1.0 ml BME (50 mM) in phosphate buffer, 0.5 ml of supernatant from a sedimented culture or a 10 times concentrated cell suspension, and phosphate buffer to 5.0 ml. The mixture was incubated at 37°C and the reaction terminated by the addition of 5.0 ml of 10% trichloroacetic acid. After 20 min at room temperature the contents of the tubes were filtered and the $A_{280}$ of the filtrates determined.
d. The dimethyl casein assay.

The dimethyl casein substrate was prepared by the method of Lin et al. (126), lyophilized and stored at -20°C.

The reaction mixture contained 0.5 ml of the enzyme and 1 ml of 0.1% dimethyl casein in PBS pH 7.0 and was incubated for 1 hr at 37°C. The reactions were terminated by immersing the samples in a boiling water bath. One milliliter of a solution of 0.1% trinitrobenzenesulfonic acid (TBS) and 1 ml of a 4% sodium bicarbonate solution, pH 8.5, were added to each sample and the mixtures were incubated in the dark for 30 min at 50°C. After incubation 1 ml of 10% sodium dodecyl sulfate (SDS) solution and 0.5 ml of a 1 N HCl solution were added to each sample, and the absorbance at 340 nm was determined relative to a blank incubated with all of the components present in the sample except active enzyme.

e. The radiochemical assay.

A $^{14}$C-labeled N,N-dimethylcasein was used as the substrate in this radiochemical assay. N,N-dimethylcasein was prepared as described by Lin et al. (126) and was labeled with $^{14}$C by addition of 0.5 mCi $[^{14}$C] formaldehyde (specific activity 44.3 mCi/mM, International Chemical and Nuclear Corp.) diluted with 3 ml 37% formaldehyde solution. The $^{14}$C-labeled N,N-dimethylcasein had a specific activity of 0.02 μCi/mg protein. The substrate used in the following procedure (43) was prepared by mixing 300 mg $[^{14}$C]N,N-dimethylcasein with 2 gm cold Hammersten quality casein in 60 ml distilled water, and dissolving the suspension by raising the solution pH to 12.0 with 1 N NaOH. The pH of the solution was
then lowered to 7.0 with 1 N HCl, and the concentration of casein was adjusted to 2% and the molarity of the Tris buffer to 0.01 M at pH 8.0. The reaction mixture contained from 0.01 to 0.1 ml protease sample in a total volume of 0.5 ml. The solution was buffered by addition of 0.01 ml of 1 M Tris-HCl, pH 8.0. One ml of precipitating reagent (8% trichloroacetic acid) was added to one of two tubes containing identical amounts of enzyme immediately before the addition of substrate. This tube served to determine the background of non-precipitable $^{14}$C. $[^4]$C casein substrate (0.5 ml) was added to all tubes, and the samples were incubated for 30 min at 37°C. At the end of incubation, 1 ml of 8% trichloroacetic acid was added to the samples containing active enzyme, and all tubes were incubated for 30 min at 37°C to ensure complete precipitation of protein. The protein precipitate was removed by filtration through Whatman No. 1 filter paper, and the filtrate was extracted with two additions of 1 ml ethyl ether. After removal of ether, the aqueous phase was left at room temperature overnight to remove all traces of ether. Ether extracted filtrate (0.4 ml) was assayed for $^{14}$C in a scintillation counter.

f. The hemoglobin assay.

Hemoglobin (Hb) was denatured in alkaline urea solution by suspending 2.0 g Hb in 50 ml distilled water, adding 36 g urea, 8 ml 1 N NaOH and diluting with distilled water to 80 ml. The mixture was allowed to stand for 30-60 min at room temperature before adding 10 ml of 1 M boric acid solution. After thorough shaking, the pH was adjusted to 7.5 with 1 N HCl and the
suspension was diluted to 100 ml with distilled water and then centrifuged at 4000 x g for 15 min. The substrate concentration in the assay was 6.7 mg Hb/ml reaction mixture; incubation temperature was 37°C and the substrate solution was equilibrated to 37°C before the assay. Five percent trichloroacetic acid (TCA) was added to precipitate the unhydrolyzed proteins and the precipitate was filtered and A₂₈₀ of the filtrate measured.

g. Determination of esterase activity.

Hydrolysis of the amino acid esters benzoylarginine ethyl ester (BAEE) and acetyltyrosine ethyl ester (ATEE) was determined by the procedure of Prestidge, Gage and Spizizen (172). Activity against tosyl arginine methyl ester (TAME) was determined by the method of Hummel as described by Walsh (229). A solution containing 10⁻³ M of each substrate was prepared in 0.1 M Tris-HCl buffer pH 7.8 containing 0.01 M CaCl₂. Assays were performed in quartz cuvettes which were held at 30°C in a thermostatted compartment. The reference solution was prepared by mixing 0.5 ml of substrate solution and 0.5 ml of the same buffer in a cuvette. One half ml of substrate solution and 0.4 ml of buffer were placed in the assay cuvette. After 5 min incubation, the absorbance at 247 nm of the two cuvettes was balanced. At zero time, 100 µl of the enzyme preparation were added to the assay cuvette, mixed thoroughly for 5 sec and the difference in absorbance was recorded for a period of about 15 min. The rate of increase of absorbance was directly proportional to the concentration of the standard enzyme. Activity was calculated from the slope of the linear
portion of the reaction curve. One unit was equal to the hydrolysis of 1 micromole of substrate per min. BAEE hydrolysis was indicated by an increase in absorbance at 254 nm. The reaction mixture contained 1.9 ml of enzyme preparation in buffer; 0.3 ml of 2 M glycine-NaOH buffer (pH 9.0); 0.8 ml of 0.68 mg of BAEE in 0.01 M Tris-HCl (pH 8.0). ATEE hydrolysis was measured as the decrease in absorbance at 237 nm, in a reaction mixture composed of 1.7 ml of enzyme preparation in buffer; 0.3 ml of 2M glycine-NaOH buffer (pH 9.0) and 1 ml of 0.5 mg of ATEE per ml in 0.01 M Tris-HCl (pH 8.0).

2. **Purification of the Protease**

Unless indicated otherwise, all steps in the purification of the enzyme were carried out at 4°C.

a. Preparation of bacterial cells for enzyme assays.

Cells intended for use in enzyme assays were harvested by centrifugation at 12,000 x g for 10 min, washed twice with PBS, resuspended and standardized to an absorbance of 1.0 at 660 nm in the assay buffer.

b. Preparation of culture supernatants for enzyme assays and column chromatography.

Cells were harvested from 48 hr cultures by centrifugation. The supernatant was concentrated to 1/10 the original volume by one of the following procedures: Amicon ultrafiltration using a PM-10 membrane; freeze-drying; or flash evaporation. Ammonium sulfate precipitation was also used to concentrate the protease activity of the culture supernatant.
c. Preparation of cell-extract for enzyme assays and protease purification.

Bacterial cultures were prepared by inoculating 4 litres of standard medium with a 2% inoculum of a 24 hr culture of \textit{B. melaninogenicus} strain 2D and incubated anaerobically at 37°C for 48 hr. Upon removal from the anaerobic chamber, cultures were centrifuged at 16,000 x g for 15 min. The cells were washed twice with 0.1 M PBS (pH 7.0) and resuspended in the same buffer. Cells were then broken by one of the following procedures.

(i) The French pressure cell

Harvested and washed cells from four litres of 48 hr cultures were resuspended in 80 ml PBS containing 10 μg each of protease free deoxyribonuclease and ribonuclease enzymes. The cells were then passed through the French press (American Instrument Co., Inc.) three times at a pressure of 20,000 psi (1 lb/in²). The broken cell suspension was centrifuged at 121,000 x g for 1 hr using the A-321 rotor of the International ultracentrifuge.

(ii) The Mini-Mill

Ten ml of a washed cell suspension \((A_{660}=5)\) were mixed with 15 gm of glass beads, placed in the Mini-Mill (Gifford-Wood, Inc.) and stirred for 15 min at 4°C. The disintegrated cells plus liquid were separated from the glass beads by filtration using a coarse sintered glass filter. The beads were washed with PBS; the
filtrate and the washings were made up to 20 ml with PBS and centrifuged at 121,000 x g for 1 hr.

(iii) Sonication

Ultrasonic disintegration was attained by the use of a Biosonik model sonicator (Bronwill Scientific, Co.) tuned to provide maximum power. The cell suspension was placed in an ice-cooled bath, and treated with ultrasound until microscopic examination revealed that the majority of cells were broken.

d. Release of cell bound HA and protease from 2D cells.

Cell bound HA and protease were released from \textit{B. melaninogenicus} strain 2D using two methods:

(i) Release of periplasmic enzymes by osmotic shock

Cells were harvested and washed with 0.01 M Tris-HCl (pH 7.3) - 0.03 M NaCl. Washed cells were suspended in 0.03 M Tris-HCl (pH 7.3) at a ratio of 1 g cells (wet weight) to 40 ml buffer. An equal volume of 1 M sucrose in 0.03 M Tris-HCl was added. The suspension was made 1 mM with respect to EDTA and mixed at 21°C for 10 min. The cells were removed by centrifugation at 0°C. The pellet of cells was resuspended in cold distilled water and incubated at 4°C for 10 min. The mixture was centrifuged and the supernatant osmotic shock fluid was assayed for HA and protease.
(ii) Release of periplasmic enzymes by treatment with Polymyxin B

Twenty ml of a washed cell suspension which had been mixed with 5 ml of 0.4 mg/ml Polymyxin B in 0.1 M PBS (pH 7.0) were incubated at 37°C for 60 min and then centrifuged at 12,000 x g for 10 min. The supernatant was assayed for HA and protease activity.

e. Ethanol precipitation.

Forty ml of cell extract were cooled to -10°C in an ethanol-dry ice bath and 60 ml of cold absolute ethanol were added over a period of 20 min with continuous gentle stirring. The temperature of the bath was maintained at -10°C. The mixture was centrifuged at 12,000 x g for 10 min and the pellet resuspended to the original volume in PBS.

f. Chromatographic procedures.

(i) Gel filtration

Proteinases, concentrated from the culture supernatant and precipitated with ethanol from the cell extract, were chromatographed on a Sephadex G-100 column (62 x 1.6 cm) equilibrated with PBS and/or PBS containing 6 M urea. The sample volume was 2-4% of the total column volume. Columns were run at 4°C.

Protease was also chromatographed on columns of Sepharose 2B and Sepharose 4B (28.3 x 1.6 cm) equilibrated with PBS or PBS containing 6 M urea and/or 0.1% SDS.
(ii) Ion-exchange chromatography

Protease, concentrated from culture supernatant or precipitated with 60% ethanol from the cell-extract was applied to DEAE and CM-Sephadex ion exchange columns equilibrated in PBS (pH 7.0), 0.05 - 0.5 M Tris-HCl (pH 8.4) and 0.1 - 0.5 M phosphate buffer (pH 7.4); and protein was eluted with a linear NaCl gradient in the different buffers. In some instances, PBS containing 0.1% SDS was used to equilibrate and elute both ion exchange columns.

(iii) Activated thiol-Sepharose-4B

Activated thiol-Sepharose-4B was swollen and additives were removed in PBS pH 7.0 (200 ml/g powder). The column (10 x 1.6 cm) was equilibrated with 0.1 M phosphate buffer, deaerated to avoid the oxidation of free thiol groups, and containing 0.1 M NaCl and 1 mM EDTA to remove trace amounts of heavy metal ions. Five ml of the dialyzed, ethanol treated sample in PBS (pH 7.0) was added to the column and eluted with the same equilibration buffer. Low flow rates were used during sample application and elution (5 ml/hr) and 1 ml fractions were collected. The lowest possible concentration of reducing agent, 10 mM L-cysteine in PBS (pH 7.0), was used to elute coupled proteins.
(iv) Organomercurial agarose

An agarose mercury column (30 ml volume) which selectively bound thiol containing molecules was prepared according to the procedure of Sluyterman and J. Wijdenes (200) by activating the Sepharose-4B with cyanogen bromide. The activated agarose was quickly washed with cold 0.1 M sodium bicarbonate at pH 9.0 and resuspended in 10 volumes of dimethyl sulfoxide (DMSO) at 0°C. Six grams of p-aminophenylmercuric acetate dissolved in 100 ml DMSO was added. After gentle stirring for 20 hr at 0°C, the suspension was warmed to 35°C, filtered and washed 4 times at 37°C with 20% DMSO to remove the free mercurial compound. The agarose was resuspended in 0.1 M ethylenediamine, adjusted to pH 8.0 and gently stirred. After storage overnight at room temperature, the agarose was washed, packed into a column and reacted with 2-nitro-5-mercaptobenzoic acid in order to eliminate all residual reactive groups of the activated agarose.

In order to test protein binding, a column of 10 ml volume was used. A 2% solution of papain in 50 mM sodium acetate pH 5.0 containing 0.1 M KCl, 0.5% butanol, 10% DMSO, 1 mM EDTA and 10 mM Na₂SO₃ (standard buffer) was passed through the column until the absorbance at 280 nm of the effluent equaled the absorbance of the sample applied to the column. The column was washed
free of unbound protein with standard buffer. The papain was eluted with standard buffer containing 0.5 mM HgCl₂.

(v) Octyl-Sepharose CL-4B

A column (1.6 x 11 cm) of Octyl-Sepharose CL-4B was equilibrated with PBS containing 1M NaCl. An ethanol precipitated sample of the cell extract containing 1M NaCl was added to the column. The hydrophobically bound proteins were eluted in a 20-50% gradient of ethylene glycol in PBS.

g. Gel electrophoresis

(i) Polyacrylamide gel (10%) electrophoresis was performed as described by Nagai et al. (156), with 0.25 M Tris, 1.92 M glycine, 0.1% SDS buffer (pH 8.3) in a vertical gel plate apparatus. Twenty to fifty µl of the protein samples (20 µg protein) were applied to the stacking gel. Samples were electrophoresed at a constant current of 40 mA with constant water cooling. The final concentration of SDS (BioRad) was 0.1% in both stacking and running gels and in the running buffer. Samples were prepared in a solubilization mixture containing 0.125 M Tris (pH 6.8), 4% SDS, 10% βME, 20% glycerol and 0.01% bromophenol blue as a marker. The samples were denatured by heating for 2 min in a boiling water bath. The gels were stained with 0.2% Coomassie brilliant blue in 30% methanol/10% acetic acid for 5-12 hr and destained in 10% acetic acid.
(ii) Polyacrylamide gel electrophoresis was also performed as above without SDS in the stacking and running gels, running buffer and samples. In this system the samples were not heated prior to electrophoresis.

(iii) Glycoproteins were detected by staining the gels with the cationic carbocyanine dye "Stains-all" (SA) (110). The gels were fixed and SDS removed in 25% isopropanol. A stock solution of SA 0.1% (w/v) in formamide was stored at 4°C in a brown bottle for a maximum of 6 weeks. Five milliliters of the stock solution were diluted with 20 ml formamide, 100 ml isopropanol and 275 ml of the tris-glycine buffer without SDS and the pH adjusted to 8.5 with 1 N NaOH. The gels were stained overnight in the dark and destained with 10% isopropanol for 18-36 hr at room temperature. The gel was checked carefully to ensure that the purple SA had not deteriorated due to SDS, pH or light. The glycoproteins stained blue and the proteins red by this procedure.

(iv) Lipoprotein electrophoresis

Lipoproteins were prestained prior to electrophoresis. Acrylamide gels of 3%, 5% and 7% were prepared in 0.18 M Tris-citrate buffer pH 9.0 without SDS. The running buffer was 0.065 M Tris- 0.018 M borate buffer pH 9.0. The tris-citrate buffered-stain was prepared by dissolving 25 mg Sudan black B in 24.4 ml
ethylene glycol and 0.625 ml of 0.5 M tris-citrate buffer (pH 9.0). The solution was incubated at 60°C for 1 hr and filtered through Whatman No. 1 paper and stored at 4°C refrigerator. Fifty µl of rabbit plasma incubated with 50 µl of the buffered stain at 37°C for 30 min was used as a lipoprotein standard.

h. Gas chromatographic analysis of carbohydrates in glycoproteins.

Neutral sugars and hexosamines were analyzed by the chromatographic procedure described by Porter (171). Neutral and amino sugars were released from glycoprotein or glycopeptides by hydrolysis with Dowex 50-X2 (H⁺) resin, followed by nitrous acid deamination of the resin bound hexosamine to neutral 2,5-anhydrohexoses. Hexoses and 2,5-anhydrohexoses were then reduced with NaBH₄, acetylated with acetic anhydride and chromatographed as the corresponding neutral alditol acetates. A Bendix-Series 2500 gas chromatograph equipped with a temperature programmer, a hydrogen flame ionization detector and a 6 ft U-shaped, 1/4 in diameter glass column was used. The column packing material consisted of 3% ECNSS-M on 100/120 mesh gas-chrom Q (Applied Science Laboratories, Inc.). The carrier gas was helium at a flow rate of 40 ml min⁻¹. Hydrogen and air flow to each detector were 50 ml min⁻¹ and 70 ml min⁻¹, respectively. Samples (2 µl) were injected at a column temperature of 150°C and eluted as the temperature was increased linearly to 200°C at a rate of 3°C per min. Two hundred fifty nanomoles of myoinositol in 50 µl was used as an internal standard.
i. Lipid analysis.

The lipids were extracted from the purified protease preparation (0.3 mg protein) by adding 3 ml of chloroform and 6 ml of methanol, mixing and incubating at room temperature for 10 min. The solution was extracted with a mixture of 3 ml of CHCl\(_3\) and 3 ml of 0.74% KCl. The lower CHCl\(_3\) layer was removed and the methanol layer was reextracted with 12 ml of H\(_2\)O-saturated CHCl\(_3\). The combined CHCl\(_3\) fractions were evaporated to dryness under a stream of N\(_2\), resuspended in 0.5 ml acetone and evaporated to dryness, the procedure was repeated and the residue dissolved in CHCl\(_3\) and stored at -20\(^\circ\)C.

Phospholipids were analyzed by the thin-layer chromatographic method described by Yavin (240) using the lipids extracted from the purified protease sample. Phosphorous assay was performed on the developed spots of phospholipids, after tracing around the spots and scraping them into acid-washed screw capped tubes.

For fatty acid analysis, the extracted lipid was fractionated by thin-layer chromatography to remove the free fatty-acids (20), and the neutral lipids were eluted from the plate and saponified with 15% KOH in methanol at 70\(^\circ\)C for 1 hr. The solution was acidified with H\(_2\)SO\(_4\) and extracted three times with equal volumes of pentane. The pooled pentane extracts were dried under nitrogen and methylated with BF\(_3\)/methanol reagent. The methylated fatty acids were extracted into pentane and then separated by gas-liquid chromatography using a column of 10% diethylene glycol succinate on 60-80 mesh chromosorb G at 160\(^\circ\)C.
The fatty acid methyl esters were identified by comparison of their retention times with those of known standards.

D. **Hemagglutination**

1. **Assay**

Cells from a 48 hr culture were harvested by centrifugation, washed twice in PBS and resuspended in PBS to give an absorbance at 660 nm of 1.0. Growth liquor obtained from the centrifugation of a 48 hr culture was also assayed. Hemagglutination was measured in microtiter plates by adding 0.025 ml of a 2.5% suspension of formalinized human red blood cells (FRBC) in PBS to 0.025 ml of a 2-fold serial dilution of the sample. The samples were diluted in PBS containing 25 mM TMEM. Results were recorded after 30 min incubation at 37°C. The HA activity was recorded as the reciprocal of the highest dilution showing complete hemagglutination (no erythrocyte pellet formation) and was considered as the HA titer. In some instances, HA was measured in test tubes (100 x 12 mm) by the addition of 0.2 ml of a 2.5% RBC suspension to 0.2 ml culture supernatant or bacterial cell suspension. The mixture was agitated gently for 10 min at room temperature. Samples possessing hemagglutinating activity usually clumped the RBC within 10 min. The extent of clumping was assessed visually and scored on a 0 to 4+ basis.

2. **Preparation of Red Blood Cells**

Hemagglutination was assayed with formalinized and non-formalinized human red blood cells (RBC). Fresh human RBC obtained from the Canadian Red Cross Blood Transfusion Service were washed three times with PBS at 4°C and used as a 2.5% suspension in the same buffer.
Formalinized human RBC were prepared by resuspending 25 ml of washed packed cells in 200 ml PBS pH 7.2. Fifty ml of formalin was placed in a dialysis tube and this was submerged in the cell suspension, and the mixture was gently agitated at 20°C. After 4 hr, the remaining formalin was transferred from the dialysis sac to the cell suspension and this mixture was stirred slowly for 18 hr. Cells were washed free of formalin with 0.9% NaCl. The cells were stored at 4°C as a 25 percent suspension in PBS containing 0.02% sodium azide.

3. Determination of the Effects of Various Reagents on HA

Equal volumes of the culture supernatant and the reagent being tested were incubated at 37°C for 30 min, and then assayed for HA activity by the microtiter method. A control using PBS instead of the culture supernatant was always assayed in parallel with the samples. In some instances, the effect of a reagent on HA was measured by serially diluting the sample in PBS containing an appropriate concentration of the reagent.

Treatment of RBC with different enzymes and reagents was done by mixing two volumes of the desired concentration of the test compound in PBS with one volume of PBS washed packed cells. The RBC suspension was incubated at 37°C for 1 hr, and the cells were washed three times and resuspended to 2.5% in PBS.

4. Adhesion and Elution of Hemagglutinin from RBC

Ten ml of formalinized RBC (25%) were mixed with 20 ml of culture supernatant and incubated at 4°C for 30 min. The RBC suspension was centrifuged and the supernatant assayed by the microtiter test for the presence of unadsorbed hemagglutinin. The RBCs with the adsorbed HA
were resuspended in 10 ml of PBS-urea (8M) and shaken for 30 min at 37°C. The RBC were removed by centrifugation and the supernatant was assayed for HA after dialysis against PBS.

E. Infectivity

Cells used for inoculation of animals were harvested from blood agar plates or from broth cultures and resuspended to 10⁹ cells/ml in phosphate-buffered saline, pH 7.0. Guinea pigs weighing 150 to 200 g were shaved on the abdomen and injected subcutaneously with 0.5 ml of either in vitro cultured cells or exudate aspirated from an infected guinea pig. The animals were observed for up to four weeks. The criteria for evaluating a positive infection were: (i) the presence of an abscess (pustular or necrotic); (ii) the transmissibility of the disease. The latter was demonstrated by injecting material aspirated from a lesion into a second animal to produce a similar pathology. Exudate was aspirated from infected guinea pigs using a sterile disposable syringe while the animal was under light ether anaesthesia. The exudate was examined for microbial contamination by plating on blood agar and incubating it anaerobically and aerobically.

The vascular permeability assay was performed following the method of Craig (36) with concentrated culture supernatant from a 48 h culture of B. melaninogenicus. Purified cholera toxin (10 μg/ml) served as a test control. The toxin was donated by the National Institute of Health (Bethesda, Maryland). Test samples of 0.1 ml were injected intracutaneously into the skin of shaved guinea pigs in duplicate. Six to nine injections were made on each guinea pig. After 20-24 hr, filtered Evans Blue dye (5% in saline) was injected intracardially, (0.1 ml/100 g body weight). The diameter of the resulting blue area around the site of inoculation was then
measured. The control was sterile medium concentrated 10 times in a Diaflo ultrafiltration apparatus.

F. Metabolic End Product Analysis

1. Preparation of Samples

Volatile and non-volatile fatty acids were analyzed by gas liquid chromatography (GLC) as described by Holdeman and Moore (94). For the analysis of volatile fatty acids, culture supernatants were acidified to pH 2 or below with 50% aqueous H₂SO₄, and the volatile fatty acids were extracted into ether. The ether was then dried with anhydrous MgSO₄ and 15 μl was injected into the chromatographic column. For the analysis of non-volatile fatty acids, 1.5 ml of the culture supernatant acidified as described above was methylated by addition of either 2 ml of methanol or 1 ml of boron trifluoride-methanol, 14% w/v (Applied Science Laboratories). The tubes were stoppered and incubated overnight at room temperature. The methylated acids were extracted into 0.5 ml chloroform and 15 μl samples were analyzed by GLC. Known standards of volatile and methylated fatty acids were prepared with each set of samples.

2. Operating conditions of the gas chromatograph

Samples were analyzed in a Bendix model 2500 gas chromatograph (Canadian Dynamics, Vancouver, B.C.) equipped with a hydrogen flame ionization detector. The carrier gas flow was set at 90 ml min⁻¹ and the oven temperature at 120°C for volatile acids and 125°C for non-volatile acids. Column packing material was prepared by mixing 10 gm of acid washed chromosorb W (60-80 mesh) (J. Manville Co.) with 1.1 g of Resoflex LAC-1-R296 dissolved in 20 ml of chloroform. The mixture was
mixed gently until the CHCl₃ had evaporated and was then packed into a 6' x 1/4" U-shaped tube.

G. Collagenase Assay

Collagenase was measured as described by Gisslow and McBride (75). Acid-soluble collagen was extracted from fresh fetal calf skin as described by Gallop and Seifer (65), except that particulate matter was removed by filtration. Lyophilized collagen was stored in stoppered flasks at -20°C. This material was solubilized in 0.01% cold acetic acid at a concentration of 2 mg/ml, and the pH adjusted to 8.5 by the addition of 1 M K₂HPO₄ and was then acetylated with acetic-¹⁴C-anhydride in benzene. The labeled mixture was then acidified with glacial acetic acid and the acetylated collagen dialyzed against cold distilled water to remove ¹⁴C-acetic acid. The ¹⁴C-collagen was lyophilized and stored at -20°C until needed. The substrate was prepared by solubilizing the lyophilized, acetylated collagen in 0.01% acetic acid at a concentration of 1 mg/ml by stirring overnight at 4°C. A typical reaction mixture for C. histolyticum collagenase and B. melaninogenicus collagenase is shown in Table 1. Enzyme, buffer and cysteine were incubated for 15 min at 20°C before addition of the substrate. At the appropriate time 0.1 ml of the reaction mixture was added to a "microfuge" tube (Beckman Instruments, Inc.), containing 50 μl of 0.04 N phosphotungstic acid and 50 μl of 2 N HCl. The samples were left at room temperature for 10 min and then centrifuged for 5 min in a Beckman-Spinco microfuge (model 152, Beckman Instruments, Inc.). One hundred μl of the supernatant was analyzed for ¹⁴C. The control sample for each assay contained buffer or uninoculated medium in place of cells.
<table>
<thead>
<tr>
<th>Components</th>
<th>B. melaninogenicus</th>
<th>C. histolyticum</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{14}$C-collagen (0.1% in 0.01% acetic acid)</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Tris-HCl Buffer (0.05 M, pH 7.2) with CaCl$_2$ (0.005 M)</td>
<td>0.1</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Cysteine (0.05 M)$^1$</td>
<td>0.1</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>Collagenase (30 units/ml)</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>Cell suspension$^2$</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

$^1$Cysteine hydrochloride was made up to 0.05 M concentration in 0.05 M Tris-HCl buffer and neutralized by adding 5 N NaOH.

$^2$Cells were resuspended in PBS pH 7.0 to give an $A_{660} = 10$. 
H. **Protein Determination**

Protein was determined by the method of Lowry et al. (132) using bovine serum albumin as a standard.

I. **Hexose Determination**

Total hexoses were measured by the anthrone assay (223) using glucose as a standard.

J. **Microdetermination of Lipids**

Lipids were quantified according to the procedures described by Pande and Parvin (165):

1. Ultramicro method for 2-12 µg lipid
2. Micro method for 20-140 µg lipid
3. Semimicro method for 170 µg to 1.33 mg lipid

An aliquot of the lipid solution to be analyzed was oxidized with acid dichromate. The reaction was followed by direct colorimetry (micro method), and by an iodometric colorimetry (ultramicro method).

In the ultramicro method, 1.0 ml of 0.034% (w/v) potassium dichromate in 97% sulfuric acid was added to 2-12 µg dried lipid sample. A control tube was also included which did not contain any lipid. The tubes were placed in a boiling water bath for 15 min and then cooled; 9.0 ml water was added to all the tubes, contents were mixed well, and 0.5 ml of these solutions was added to 4.5 ml CdI₂-starch reagent. The reagent blank was prepared by adding 0.5 ml 3.6 N sulfuric acid to 4.5 ml CdI₂-starch reagent. The color intensities were read against the reagent blank at 575 nm.

In the micro method, 2.0 ml 0.15% potassium dichromate in 96% (w/v) sulfuric acid was added to tubes containing 0 (two blank tubes required) to 140 µg solvent-free lipid. After heating and cooling as described above,
4.5 ml water was added and the solutions were re-cooled after mixing; 0.1 ml freshly prepared aqueous 20% Na$_2$SO$_3$·7 H$_2$O (w/v) was added to reduce the dichromate in one of the blanks. All tubes were read against the reduced blank at 440 nm. The unreduced blank tube serves as a control, showing the amount of dichromate present initially.

K. Microdetermination of Phosphorous

Total free and organic phosphorous was determined by the procedure of Chen et al. (29). Samples were placed in acid-alkali cleaned thick walled glass tubes and 4 drops of concentrated H$_2$SO$_4$ were added. The tubes were heated over bunsen flame until white fumes of sulphur trioxide appeared. Two drops of perchloric acid (72%) were then added and the samples heated until liquids became clear, and then cooled and volumes were adjusted to 25 ml in a volumetric flask. Standards containing up to 8 μg phosphorous and a blank containing only water (4 ml) were used. Four ml of a freshly prepared reagent containing 1 volume 6 N H$_2$SO$_4$, 2 volumes distilled water, 1 volume 2.5% ammonium molybdate and 1 volume 10% ascorbic acid were added and the tubes incubated at 37°C for 1 hr. The samples were allowed to cool to room temperature and absorbance at 820 nm against the blank was measured.

L. Glucosidase Assay

Alpha-glucosidase and β-glucosidase activities were determined with p-nitrophenyl α-D-glucoside (α-PNPG) and p-nitrophenyl β-D-glucoside (β-PNPG) (Calbiochem) as substrates, respectively. The enzyme activity was assayed in the purified protease preparation and with known glycosidic enzymes purchased from Miles Laboratories, Inc. The reaction mixture for each assay contained 0.2 ml of PBS, pH 7, enzyme (0.2 to 1.0 ml) and
distilled water to a final volume of 3.0 ml. This mixture was incubated at 37°C for 5 min prior to the addition of substrate. The reaction was initiated by the addition of 1.0 ml of 10 mM prewarmed solution of the appropriate substrate in PBS (pH 7.0). The assay was terminated by placing the tubes in an ice bath and adding 1.0 ml of 0.2 M Na₂CO₃. Appropriate controls lacking enzyme or substrate were included with each assay. The hydrolysis of substrate was monitored at 400 nm in a Perkin Elmer-Hitachi spectrophotometer model 124.

M. Lipase Assay

The lipase activity in the purified protease preparation was determined by a modification of the method of Huggins, Charles and Lapides as described by Winters (237), which is based on the amount of p-nitrophenol released during the hydrolysis of p-nitrophenyl acetate (Eastman Organic Chemicals, Rochester, N.Y.). The reaction mixture consisted of 4 ml of 0.06 M phosphate buffer, pH 7; 0.5 ml of enzyme preparation; and 1.0 ml of 4 x 10⁻³ M substrate in the same buffer. After incubation at 30°C for 1 hr, the absorbance at 410 nm was read against a reagent blank.

N. Reagents and Chemicals

The following reagents and chemicals were purchased from Sigma Chemical Company: trypsin, papain, neuraminidase Type VI, C. histolyticum collagenase, wheat germ lipase, pronase, BSA, dithiothreitol, trinitrobenzenesulfonic acid, sodium borohydride, tosyl-L-arginine methyl ester-HCl (TAME), benzoylarginine ethyl ester (BAEE), and acetyltyrosine ethyl ester (ATEE).

The following were purchased from Calbiochem: tosyl-L-phenylethyl chloromethyl ketone (TPCK), phenyl methyl sulfonyl fluoride (PMSF), azocoll and azocasein.
The following were purchased from Difco: hemoglobin, D-mannose, D-fructose, D-galactose, sucrose, L-arabinose and D-mannitol. Acrylamide and β-mercaptoethanol were purchased from Eastman.

Ficoll, Sephadex G-100, Sepharose 2B, Sepharose 4B, activated thiol-Sepharose 4B, octyl-Sepharose CL-4B, DEAE-Sephadex A-50 and CM-Sephadex C-50 were purchased from Pharmacia (Montreal).

Mixed glycosidases (T. cornutus) were purchased from Miles Laboratories, Inc. All other chemicals used were Fisher reagent grade (Fisher Scientific Company).

The results of the present investigation may be divided into three sections. In the first, the preliminary identification of B. melaninogenicus strains is reported. The second section deals with the partial characterization and purification of the soluble hemagglutinin. The last section presents the techniques used in attempt to purify and characterize the soluble and cell-bound protease(s) of B. melaninogenicus.
III. RESULTS

A. Characterization of B. melaninogenicus

The purpose of this part of the thesis is to describe experiments which were carried out to characterize B. melaninogenicus strains 2D and K110 and further to investigate strain 2D which was used in this study.

1. Fatty Acid Production

The first step in characterizing the organisms was to subdivide them on the basis of the acidic end products produced during growth. Organisms were cultured for 48 hr in TYH medium and the supernatants analyzed for volatile and non-volatile fatty acids by gas chromatography (Fig. 3 and 4). The peaks were identified by comparing their retention times with those of standards. Both K110 and 2D strains produced acetic, propionic, isovaleric, isobutyric, and butyric acids in addition to an unknown compound which was shown to be phenylacetic acid by mass spectroscopy and gas chromatography (Susan Jensen, personal communication). The results indicated that both strains correspond to the subspecies known as B. melaninogenicus ss. asaccharolyticus. The presence of phenylacetic acid in cultures of B. melaninogenicus ss. asaccharolyticus had not been noted previously and should provide a useful tool in identifying this organism in mixed infection.
Gas chromatography of volatile fatty acids produced by

*B. melaninogenicus*

Volatile fatty acids were extracted from acidified culture supernatant.

\[ \begin{align*}
  a &= \text{acetic acid;} \\
  p &= \text{propionic acid;} \\
  \text{iso } b &= \text{isobutyric acid;} \\
  b &= \text{butyric acid;} \\
  \text{iso } v &= \text{isovaleric acid}
\end{align*} \]
FIGURE 4.

Gas chromatography of non-volatile fatty acids produced by

*B. melaninogenicus*.

Methylated fatty acids were extracted from acidified culture

supernatant.

\[ \lambda = \text{lactic acid} \]

\[ \text{pa} = \text{phenylacetic acid} \]
Retention Time (min)
2. **Collagenase Activity**

*B. melaninogenicus* has been reported to possess a cell bound collagenase which is believed to be associated with pathogenicity (67,83,84). In order to determine if collagenase was associated with K110 and 2D strains, 48 hr cultures were harvested and cells assayed for collagenase by incubating them with $^{14}$C-collagen. Both K110 and 2D strains possess a cell bound collagenase. Unlike other microbial collagenases, the *B. melaninogenicus* enzyme is oxygen sensitive and is stimulated by reducing agents. No soluble collagenase was detected in the culture supernatant of either organism.

The specific activity of the cell bound collagenase was expressed as the μg of collagen solubilized per hr by 1 ml of cells (Absorbance$_{660}$ = 1.0). *B. melaninogenicus* strain K110 had a specific activity of 122 μg/hr/O.D. Strain 2D *B. melaninogenicus* had a specific activity of 140 μg/hr/O.D.

3. **Pathogenicity**

a. Infectivity.

*B. melaninogenicus* strain 2D was tested for its ability to produce an infection in the guinea pig model system. Cells from a 48 hr liquid culture were washed, resuspended in sterile PBS and injected into the groin of a 200 g guinea pig. Within 18 hr the animal developed symptoms of a rapidly spreading infection: darkening of skin and loss of hair in the thoracic area and accumulation of a large volume of fluid. Material aspirated from the animal was dark in colour, watery and foul-smelling, and when examined by phase-contrast microscopy
appeared to contain a pure culture of *B. melaninogenicus* along with red blood cells indicating that the organism had invaded the circulatory system. Culture of the exudate on blood agar plates confirmed that it contained a pure culture of *B. melaninogenicus*. Fatty acid analysis revealed that acetic, isobutyric, butyric and phenylacetic acids were present. These acids are produced by *B. melaninogenicus* in *in vitro* culture. The transmissible nature of the infection was proven by using the exudate to infect another guinea pig. Sterilizing the exudate by autoclaving resulted in a loss of infectivity. Strain 2D was thus one of the few *B. melaninogenicus* strains capable of producing an infection without the support of other organisms (139,215). The infection produced symptoms similar to those described in the literature for infections produced by CR2A (139). Strain K110 failed to produce infection when pure cultures were injected into guinea pigs but was infective in mixed culture (146). Generally, successful infections resulted when 200-250 g guinea pigs were infected instead of larger guinea pigs and when 48 hr cells were used instead of 24 hr cells.

b. Vascular permeability.

Filtrates from stationary phase cultures of *B. melaninogenicus* strains 2D and K110 were sterilized by filtration through a 0.45 μm millipore filter and concentrated 10 times by ultrafiltration through an Amicon PM-10 membrane. The concentrated culture filtrates were tested to determine if they would increase vascular permeability by injecting 0.2 ml intracutaneously
Table 2. Vascular Permeability Test

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Blueing diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholera toxin</td>
<td>10</td>
</tr>
<tr>
<td>2D</td>
<td>7</td>
</tr>
<tr>
<td>K110</td>
<td>4</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
</tr>
</tbody>
</table>

The test samples were injected intracutaneously, in a random sequence, into the shaved backs of three guinea pigs in duplicates, and six injections were made on each guinea pig.

Evans Blue (5% in saline) was injected intracardially, (0.1 ml/100 g body weight), about 20–24 hr after injection of samples. The dye was disseminated throughout the vascular system almost immediately, and the guinea pigs were sacrificed after 10 min.
into each of two guinea pigs. The control was sterile medium concentrated 10 times in the same way as the culture supernatant. After 24 hr, a blue dye was injected into the test guinea pigs by cardiac puncture. The diameter of the blue areas around the site of inoculation of the different samples represented the effect of these samples on vascular permeability in the test animals (Table 2).

It was found that *B. melaninogenicus* produced a factor which was released from the cells and which exhibited biological properties similar to *Vibrio cholerae* enterotoxin. Boiling of the concentrated culture supernatant destroyed the blueing factor suggesting that it was a heat sensitive protein.

4. Growth of Strain 2D *B. melaninogenicus*

   a. Hemin requirement.

Strain 2D of *B. melaninogenicus* has an obligate requirement for hemin. The response of 2D to varying amounts of hemin was followed. The results are shown in Fig. 5. It may be seen that growth of the bacterium was roughly proportional to the hemin concentration over a range of 0.25 to 2.5 μg hemin per ml. The organism grew in a medium free of hemin for one generation but when transferred to fresh medium lacking hemin, little if any growth occurred whereas subculture to hemin containing media always resulted in good growth.

The organism grew in the presence of a concentration of hemin as low as 0.2 μg/ml medium; concentrations of hemin higher than 20 μg/ml had no further enhancing effect on the growth of the organism. Addition of glucose to the TYH medium inhibited the growth of 2D.
FIGURE 5

Response of \textit{B. melaninogenicus} (Strain 2D) to Hemin.

A 0.1 ml inoculum of 24 hr hemin-depleted 2D culture was used to inoculate 10 ml each of media containing different concentrations of hemin. Growth was measured (A$_{660}$) after 24 hr incubation anaerobically at 37°C.
b. Growth response to amino acids.

The addition of glutamic acid to cultures of 2D in TYH medium resulted in a marked increased in growth rate and total cell yield when compared to unsupplemented medium (Table 3). The growth response of 2D was dependent on the concentration of glutamic acid over the range of 0.2 to 4 mg/ml (Fig. 6). Enhancement of growth also occurred when L-serine, L-asparagine, L-methionine and L-proline were added to TYH medium.

Addition of L-cysteine, DL-valine, L-histidine, L-tryptophan, glycine or L-arginine to TYH medium produced marked inhibition of the growth of strain 2D. L-leucine caused 66% inhibition of growth. Growth was not affected by the addition of L-lysine or L-phenylalanine to the TYH medium. The difference in inhibition by amino acids when comparing 24 and 40 hr cultures (Table 3) could be due to the different times at which the cultures reach the stationary phase of growth. Addition of L-cysteine, adjusted to pH 7.0 by NaOH, to a growing culture of 2D in TYH medium (28 hr) caused inhibition of growth (no further increase in \(A_{660}\) was detected).

Although glutamic acid or asparagine caused enhancement of growth of 2D in TYH medium they were unable to replace hemin in trypticase yeast-extract medium.

5. Hemagglutinin and Protease Activity of \textit{B. melaninogenicus}

\textit{B. melaninogenicus} strains K110 and 2D produce both a soluble and a cell bound hemagglutinin (HA). They agglutinate rapidly with
Table 3. Effect of the addition of amino acids on Growth of B. melaninogenicus ss. asaccharolyticus 2D in TYH medium

<table>
<thead>
<tr>
<th>Amino acid added</th>
<th>Concentration (mM)</th>
<th>A_660 24 h</th>
<th>A_660 40 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>0.38</td>
<td>0.98</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>34</td>
<td>0.75</td>
<td>1.3</td>
</tr>
<tr>
<td>L-serine</td>
<td>47.6</td>
<td>0.58</td>
<td>1.2</td>
</tr>
<tr>
<td>L-asparagine</td>
<td>37.8</td>
<td>0.62</td>
<td>1.1</td>
</tr>
<tr>
<td>L-methionine</td>
<td>33.5</td>
<td>0.5</td>
<td>1.1</td>
</tr>
<tr>
<td>L-proline</td>
<td>43.4</td>
<td>0.48</td>
<td>1.1</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>41.3</td>
<td>0.03</td>
<td>0.04</td>
</tr>
<tr>
<td>L-valine</td>
<td>42.7</td>
<td>0.02</td>
<td>0.06</td>
</tr>
<tr>
<td>L-tryptophan</td>
<td>24.5</td>
<td>0.04</td>
<td>0.06</td>
</tr>
<tr>
<td>L-histidine</td>
<td>32.2</td>
<td>0.04</td>
<td>0.05</td>
</tr>
<tr>
<td>Glycine</td>
<td>66.6</td>
<td>0.03</td>
<td>0.1</td>
</tr>
<tr>
<td>L-arginine</td>
<td>28.7</td>
<td>0.03</td>
<td>0.04</td>
</tr>
<tr>
<td>L-leucine</td>
<td>38.1</td>
<td>0.12</td>
<td>0.18</td>
</tr>
<tr>
<td>L-lysine</td>
<td>34.2</td>
<td>0.4</td>
<td>0.98</td>
</tr>
<tr>
<td>L-phenylalanine</td>
<td>30.3</td>
<td>0.36</td>
<td>1.0</td>
</tr>
</tbody>
</table>
FIGURE 6

Effect of glutamic acid on growth of *B. melaninogenicus*.

TYH media containing increasing concentrations of glutamic acid were inoculated with 2D and growth response was recorded at 24 hr.
Percent Increase in $A_{660}$
human red blood cells.

Both strains produce soluble and cellular protease(s) which are active against a number of substrates including azocoll, casein and azocasein. Table 4 presents the soluble and cellular HA and protease activities of both 2D and K110.

Due to greater pathogenicity of the 2D strain of B. melaninogenicus and its production of greater amounts of HA and protease, this organism was chosen for detailed studies of the soluble hemagglutinin and soluble and cellular proteases.

6. Effect of Washing 2D Cells on the HA and Protease

Cells from a 4 day old culture were harvested by centrifugation and resuspended in 1/10 of the original volume of PBS-ßME (50 mM). The cells were collected by centrifugation and the process repeated six times. Cells and supernatant from each washing were assayed for hemagglutinin and protease. HA can be sequentially eluted from the bacterial cells by washing with phosphate buffered saline in the presence of a reducing agent (Table 5). In the meantime the HA titer of the cells remained the same. HA could not be eluted from cells harvested from a 24 hr culture. Tris buffer containing 25 mM ßME or cysteine could not replace the PBS in releasing the cell-bound HA. The presence of reducing agent in the washing buffer resulted in better elution of the HA from 2D cells than using only buffer. Anaerobic elution of 2D HA using PBS in the anaerobic chamber gave the same results.

When the HA eluted from 2D cells was compared to the soluble HA in culture supernatants, no differences were noticed
Table 4. Hemagglutinin and Protease of 2D and K110

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hemagglutinin</th>
<th>Protease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soluble</td>
<td>Cell bound</td>
</tr>
<tr>
<td></td>
<td>Titer/ml</td>
<td>Titer/ml/1 A_{660}</td>
</tr>
<tr>
<td>2D</td>
<td>1280</td>
<td>20480</td>
</tr>
<tr>
<td>K110</td>
<td>640</td>
<td>5120</td>
</tr>
</tbody>
</table>

The HA activity was assayed by the microtiter method using washed cells resuspended in PBS (A_{660} = 1.0) and the supernatant from a 48 hr culture of 2D and K110. Formalinized human RBC were used for the HA assay. The protease was assayed in both cells and culture supernatant using casein as substrate.
Table 5. Effect of washing 2D cells on the HA

<table>
<thead>
<tr>
<th>Sample</th>
<th>HA titer of Supernatant</th>
<th>HA titer of Pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth liquid</td>
<td>64</td>
<td>256</td>
</tr>
<tr>
<td>First wash</td>
<td>64</td>
<td>256</td>
</tr>
<tr>
<td>Second wash</td>
<td>64</td>
<td>256</td>
</tr>
<tr>
<td>Third wash</td>
<td>32</td>
<td>256</td>
</tr>
<tr>
<td>Fourth wash</td>
<td>32</td>
<td>256</td>
</tr>
<tr>
<td>Fifth wash</td>
<td>4</td>
<td>256</td>
</tr>
<tr>
<td>Sixth wash</td>
<td>16</td>
<td>256</td>
</tr>
</tbody>
</table>

A ten-times-concentrated suspension of four days old 2D culture was washed six times with 25 mM ßME in PBS, and the washing and the pellet were assayed for HA by the microtiter plate.
between the two in respect to optimum pH, stability and the effect of inhibitors on the hemagglutinin. Centrifugation of the eluted HA at 100,000 x g resulted in the recovery of most of the HA in the pellet, which implies that the release of HA from the cells by washing might be due to stripping off of bits of the outer membrane of the 2D cells. Protease was not eluted from 2D cells by either procedure suggesting that the protease enzyme may be more tightly bound to the cells than is the cellular HA.

7. Release of Periplasmic Enzymes from 2D Cells

Since the cellular HA and protease of *B. melaninogenicus* are accessible to RBC and high molecular weight substrates respectively, it is probable that both are localized external to the cytoplasmic membrane. An attempt was made to determine if the HA and protease were located in the periplasmic space, by osmotic shock treatment, and by treatment with the membrane-disrupting antibiotic polymyxin B. Only 10-18% of the cellular protease was released by either procedure, therefore the enzyme cannot be considered as periplasmic. The HA seemed to be less tightly bound to the 2D cells than the protease, correlating with the results obtained from elution of the HA and protease by successive washings of the cells. The release of the HA from the cells by polymyxin B treatment was related to the time of exposure to the antibiotic (Table 6). Most of the cells treated with the antibiotic were intact and no lysis occurred as judged by microscopic examination. There was a rough correlation between the appearance of soluble HA and loss of activity from the cells. There was no direct effect by Polymyxin B on the HA in the microtitre assay.
Table 6. Release of the HA from 2D cells by treatment with Polymyxin B

<table>
<thead>
<tr>
<th>Time of exposure min</th>
<th>HA titer of pellet</th>
<th>HA titer of supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>512</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>512</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>256</td>
<td>4</td>
</tr>
<tr>
<td>15</td>
<td>128</td>
<td>8</td>
</tr>
<tr>
<td>30</td>
<td>64</td>
<td>16</td>
</tr>
<tr>
<td>60</td>
<td>64</td>
<td>64</td>
</tr>
</tbody>
</table>

A 400 ml culture of 2D B. melaninogenicus was harvested, washed and resuspended in 20 ml PBS with 25 mM βME. 5 ml of cell suspension was mixed with 5 ml of 0.4 mg/ml Polymyxin B and incubated at 37°C. Samples were taken after 1, 5, 15, 30 and 60 min, kept in ice and then centrifuged. The pellet and supernatant were assayed for HA by the microtiter method.
B. Soluble Hemagglutinin of *B. melaninogenicus*

*B. melaninogenicus* possesses a cell-bound and a soluble hemagglutinin(s) which is thought to be cell associated hemagglutinin which has been released from the cell surface. Studies were carried out to provide evidence to support this assumption and at the same time to define the adherence properties of *B. melaninogenicus* which might contribute to the establishment of the organism in the gingival crevice.

1. Adherence of *B. melaninogenicus* 2D Cells to Formalinized

*Human RBC*

Cultures of 2D cells were tested for adherence to RBC by harvesting the cells, resuspending them in PBS (A_{660} = 1.0) and mixing with packed FRBC for 30 min at room temperature with shaking. Hemagglutination was determined by the standard test tube assay and the samples were observed microscopically for *B. melaninogenicus* bound to RBC. By following FRBC adherence versus culture age of *B. melaninogenicus* 2D, it was found that the adhesion to RBC occurred at 24 hr (Table 7). Washing the bacteria-RBC aggregates 4 times with PBS did not dislodge the microorganisms.

2. Determination of Optimal Conditions for the Hemagglutinin Assay

The effect of pH on the HA activity of *B. melaninogenicus* strain 2D is shown in Fig. 7. Optimum activity occurred over a pH range of 7.0 to 7.5, but good activity could be demonstrated in the range of pH 6.0 to 8.0. Hemagglutination activity decreased rapidly below pH 6.0 and above pH 8.0.

Red blood cells from a number of animals and from humans were
Table 7. Influence of culture age on the adherence of 2D to FRBC.

<table>
<thead>
<tr>
<th>Culture age hrs.</th>
<th>Adherence with FRBC checked by the test tube method</th>
<th>% of 2D cells not adhering to FRBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>16</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>24</td>
<td>+++</td>
<td>50</td>
</tr>
<tr>
<td>36</td>
<td>+++</td>
<td>40</td>
</tr>
<tr>
<td>48</td>
<td>++++</td>
<td>20</td>
</tr>
<tr>
<td>60</td>
<td>++++</td>
<td>30</td>
</tr>
</tbody>
</table>

The extent of adherence was assessed visually by the test-tube HA assay, and expressed as the percent of non-adhering cells to FRBC after microscopic examination.
FIGURE 7. Effect of pH on HA of *B. melaninogenicus*

Supernatant of 48 hr culture was tested for HA by the microtiter plate using 0.2 M each of Na acetate buffer at pH 5.0-6.0; phosphate buffer at pH 6.0-7.5 and tris buffer at pH 7.5-9.0.
tested in the HA assay using the microtiter plate. The results are shown in Table 8. Rabbit and human RBC showed the strongest activity and guinea pig the least, but all cell types were capable of hemagglutinating with *B. melaninogenicus*.

Formalinized RBC, prepared as mentioned in the Materials and Methods, were as effective in the HA assay as were fresh RBC. The formalinized RBC were more stable than were non-formalinized RBC. Furthermore, the cells retained the microscopic appearance and shape of fresh normal RBC. For the quantitative studies of the adsorption or elution of soluble hemagglutinin, formalinized cells can be used advantageously in place of normal cells. Formalinized cells were found to pack more quickly and firmly than normal cells upon centrifugation so that greater accuracy was obtained in preparing suspensions. A stock suspension of the modified cells may be prepared and used over a period of months with assured constancy of concentration and reactivity.

The microtiter assay was also performed using a range of RBC concentrations as shown in Fig. 8. It was determined that the optimal RBC concentration necessary to observe HA was in the range of 2.5 to 1.25%.

The effect of incubation temperature on the hemagglutination assay is shown in Fig. 9. From these results, 37°C was chosen as the most satisfactory incubation temperature for the HA assay using an incubation period of 30 min. Hemagglutination occurred at 4°C, but was very slow.
Table 8. Effect of the source of red blood cells on HA activity.

<table>
<thead>
<tr>
<th>Source of RBC</th>
<th>HA titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea pig</td>
<td>8</td>
</tr>
<tr>
<td>Rabbit</td>
<td>32</td>
</tr>
<tr>
<td>Frog</td>
<td>16</td>
</tr>
<tr>
<td>Human</td>
<td>64</td>
</tr>
<tr>
<td>Formalized human</td>
<td>64</td>
</tr>
<tr>
<td>Formalized rabbit</td>
<td>64</td>
</tr>
</tbody>
</table>

The microtiter plate method was used to assay the HA in a 72 hr culture supernatant with 2.5% washed RBC of different sources.
FIGURE 8  Optimal erythrocyte concentrations for microtiter hemagglutination assay.

Assay conditions: RBC suspended in PBS (pH 7.0) containing 50 mM $\beta$ME, culture supernatant from a 48 hr culture. The microtiter plates were incubated for 30 min at 37°C. The concentrations of RBCs were expressed as percentage of packed cells.
FIGURE 9. Effects of incubation temperature on HA.

Assay conditions: 1.25% (vol/vol) RBC suspended in PBS (pH 7.0) containing 50 mM βME, culture supernatant from a 48 hr culture. The microtiter plates were incubated for 30 min at various temperatures.
3. **Relationship of HA to Culture Age**

The appearance of cellular and soluble hemagglutinins was followed as a function of the age of the culture. The organisms were grown at 37°C under anaerobic conditions. As shown in Fig. 10, the production of cell-bound HA paralleled the growth curve of the organism. The soluble HA titer increased during the period between 34-48 hr and correlated with the increased infectivity of the organism. It also continued to increase after growth had ceased, presumably due to liberation of cellular HA as a result of cell lysis.

4. **Effects of RBC Modification on HA**

The results of the treatment of RBC with various enzymes are presented in Table 9. Treatment with trypsin and α-chymotrypsin appears to have some inhibitory effect. However, since the inhibitory effects represent only a two-fold dilution, this is probably not significant.

Treatment of the RBC with neuraminidase caused enhancement of the HA activity, which may have been due to the unmasking of the active receptor or binding site for the HA on the RBC. Pronase, subtilisin and galactosidase treatments of RBC caused inhibition of the HA activity which may have been due to alteration of the receptor structure for the HA on the RBC.

5. **Modification of the HA**

In order to obtain information concerning the nature of the component(s) necessary for HA, the culture supernatant was treated with several reagents and salts prior to the HA assay. A number of salts had no effect on the HA, which might suggest
FIGURE 10. Relationship of HA to culture age.

Cells grown on TYH medium were harvested, washed and resuspended in PBS ($A_{666} = 1.0$). Culture supernatant was assayed without concentration. The HA was assayed by the microtiter method, and the results were expressed as the number of wells of positive HA.
Table 9. Effect of treatment of RBC on their ability to hemagglutinate with soluble HA.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Units/mg</th>
<th>HA titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>1 mg/ml</td>
<td>65</td>
<td>16</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>32</td>
</tr>
<tr>
<td>α-chymotrypsin</td>
<td>1 mg/ml</td>
<td>65</td>
<td>16</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>32</td>
</tr>
<tr>
<td>Pronase</td>
<td>1 mg/ml</td>
<td>49</td>
<td>4</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>32</td>
</tr>
<tr>
<td>Subtilisin</td>
<td>1 mg/ml</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>32</td>
</tr>
<tr>
<td>DNase</td>
<td>1 mg/ml</td>
<td>1200</td>
<td>32</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>32</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>250 µg/ml</td>
<td>1.0</td>
<td>64</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>β-galactosidase</td>
<td>250 µg/ml</td>
<td>4.0</td>
<td>4</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>Dextranase</td>
<td>250 µg/ml</td>
<td>4.0</td>
<td>32</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>32</td>
</tr>
</tbody>
</table>

Red blood cells were exposed to the different enzymes for 30 min at 37°C in 0.1 M acetate buffer (pH 4.0) in case of neuraminidase, β-galactosidase and dextranase, and in PBS (pH 7.0) for the other reagents in the presence of 25 mM using 48 hr culture supernatant of 2D B. melaninogenicus.
that agglutination of the HA and RBC is not an ionic interaction. EDTA had no effect on the HA activity. Pretreatments with reagents which normally interact with proteins (trypsin and formalin) inhibited the HA reaction. Treatment with sodium periodate also inhibited the HA reaction. These results give some indication that protein and/or carbohydrate moieties as well as disulfide bonds may be necessary for HA to occur. However, these conclusions are very tentative because of the crude and relatively non-specific nature of these treatments.

6. **Effect of Carbohydrates on HA**

As mentioned in the Literature Survey, several bacterial HA reactions are inhibited by incubation with specific carbohydrates, suggesting that these compounds may be receptors. The role of carbohydrates in the *B. melaninogenicus* HA reaction was assessed by pre-incubating the culture supernatant for 30 min with various carbohydrates. Glucose, lactose, fructose, rhamnose, ribose, cellobiose, fucose and sucrose at a concentration of 5% had no effect on the HA reaction. Galactose inhibited the HA titer by 75%.

7. **Stability of the Soluble HA**

The HA activity was stable at 4°C for 3 days in the absence of a reducing agent. At 37°C and 20°C, the activity was stable for 48 hr in the absence of a reducing agent.

Heating at 57°C for 30 min did not inhibit the HA activity, but heating at 70°C-80°C for 10 min or 100°C for 5 min completely destroyed hemagglutination. No change in activity occurred after freezing and thawing in the absence of BME.
8. **Oxygen Sensitivity of the Soluble HA**

The effect of oxygen on HA was demonstrated in an experiment in which the supernatant from a 48 hr culture of 2D was subjected to vigorous aeration for 30 min prior to the assay (Table 10). The HA activity was constantly inhibited by 2 titers upon aeration, but activity could be restored by the addition of β-mercaptoethanol (25 mM). A definite increase in the HA titer occurred when a reducing agent such as βME or cysteine was included in the HA assay, although the effect in this case was not as pronounced because the supernatant contained endogenous reducing activity. When the culture supernatant was collected and assayed in the anaerobic chamber, reducing agents had no beneficial effect on the HA activity.

9. **Effects of Sulfhydryl Modifiers on HA**

Since reducing conditions are required for the HA activity, reagents that bind to sulfhydryl compounds were tested for inhibition of the HA (Table 11). HgCl₂ inhibited HA and inhibition could be reversed by adding βME. Iodoacetic acid and iodoacetamide, which alkylate sulfhydryl groups, irreversibly inhibited the soluble HA.

10. **Ultracentrifugation of Soluble HA**

Ultracentrifugation of the supernatant at 141,000 x g for 1 hr resulted in the sedimentation of 83% of the HA activity. A small amount remained in the supernatant (Table 12). The majority of the HA in the culture supernatant seemed to be of high molecular weight and might be particle-bound; it is not known whether the non sedimenting HA represented a different HA or the HA in a different form.
Table 10. Effect of aeration on soluble HA

<table>
<thead>
<tr>
<th>Aeration</th>
<th>3ME*</th>
<th>HA titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>16</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>64</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>64</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>8</td>
</tr>
</tbody>
</table>

*3ME was added immediately before the assay.
Table 11. Effect of sulfhydryl modifiers on HA

<table>
<thead>
<tr>
<th>*Inhibitors</th>
<th>Concentration (mM)</th>
<th>HA titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>Control + βME</td>
<td>0</td>
<td>64</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>HgCl₂+βME</td>
<td>5</td>
<td>32</td>
</tr>
<tr>
<td>Iodoacetic acid + βME</td>
<td>25</td>
<td>2</td>
</tr>
<tr>
<td>Iodoacetamide + βME</td>
<td>25</td>
<td>2</td>
</tr>
</tbody>
</table>

*Forty-eight hr culture supernatant was incubated for 30 min at room temperature with the reagent in Tris-HCl buffer, pH 8.2 before assaying it for HA. βME was used at a concentration of 25 mM.*
Table 12. Ultracentrifugation of soluble HA

<table>
<thead>
<tr>
<th>HA</th>
<th>HA titer</th>
<th>% of total HA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity prior to</td>
<td>2448</td>
<td>100</td>
</tr>
<tr>
<td>centrifugation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pellet</td>
<td>2034</td>
<td>83.0</td>
</tr>
<tr>
<td>Supernatant</td>
<td>388</td>
<td>15.8</td>
</tr>
</tbody>
</table>

The HA was assayed by the microtiter plate method and was expressed as HA titer per total volume of the sample.
11. Partial Purification of soluble HA

a. Concentration of the HA.

Because the HA is extracellular and therefore dilute, it was necessary to concentrate it as a first step in purification procedures. A number of procedures were tested to determine the most effective method. The HA in the supernatant could be concentrated by freeze-drying, by flash evaporation and by \((\text{NH}_4)_2\text{SO}_4\) precipitation. It could not be concentrated by the use of Ficoll or by ultrafiltration through Amicon PM-10 or XM-50 membranes due to adsorption of the HA to the dialysis tubing and to the ultrafiltration membranes.

b. Chromatography.

i) Affinity adsorption

By definition HA binds to RBCs. It seemed therefore possible to devise an affinity chromatography procedure which would take advantage of this characteristic. Formalinized RBCs were mixed for 30 min at 4°C with supernatant containing HA, and washed as described in Materials and Methods.

Hemagglutinin was eluted most satisfactorily with 8 M urea containing 25 mM \(\beta\)ME. A number of salts including \(\text{NaCl, NH}_4\text{Cl, MgCl}_2, \text{MgSO}_4, \text{CaCl}_2, \text{KCl}\) and \(\text{LiCl}\) were not effective in releasing HA. A variety of salt concentrations (up to 6 M) and pH conditions were tested but none proved to be effective.

Galactose, glucose and cellubiose did not elute the HA. Acetaldehyde, triton X-100, \(\text{H}_2\text{O}\)-saturated
butanol as well as guanidine HCl were not effective in eluting the HA from RBCs. The characteristics of the urea eluate are shown in Table 13. Some bacteria have been shown to bind to neuraminic acid residues on RBC (209). This does not appear to be the case with the *B. melaninogenicus* HA, as the removal of neuraminic acid residues with neuraminidase increased the ability of RBC to bind HA, and elution of HA adsorbed to neuraminidase-treated RBCs with 8 M urea was found less effective than elution of adsorbed HA from untreated RBCs which might be due to a tighter binding of soluble HA to neuraminidase-treated RBC.

This affinity adsorption technique resulted in an 11.0 fold-purification and 50% recovery of the soluble HA of the supernatant (Table 14). A second treatment of the RBC with 8 M urea yielded more of the HA, but there was no increase in the specific activity of the eluted HA. This might be due to elution of protein constituents from the RBC together with the HA. A control of RBC treated with PBS instead of culture supernatant was also eluted with 8 M urea.

ii) Gel filtration on Sephadex G-100

The HA eluted from RBC was lyophilized, resuspended in PBS and applied to a Sephadex G-100 column and eluted with PBS. The results are shown in Fig. 11 and Table 14. Even though the HA was excluded from the beads
### Table 13. Characteristics of the HA eluted from RBC with Urea

<table>
<thead>
<tr>
<th>HA in the supernatant</th>
<th>Adsorbed HA % to FRBC</th>
<th>Urea eluted HA %</th>
<th>Protein content µg/ml</th>
<th>Carbohydrate content µg/ml</th>
<th>Ultracentrifugation of Urea eluted HA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pellet %</td>
</tr>
<tr>
<td>81920</td>
<td>80600</td>
<td>98.4</td>
<td>40960</td>
<td>50.8</td>
<td>5600</td>
</tr>
</tbody>
</table>

The HA eluted from RBC with urea was centrifuged at 141,000xg for 1 hr. The HA was assayed by the microtiter plate method and is expressed as the titer produced in total volume of samples.
TABLE 14. Analysis of the HA Purification Procedures

<table>
<thead>
<tr>
<th>Methods of Purification</th>
<th>Total HA titer</th>
<th>Total Protein mg</th>
<th>HA titer/mg protein</th>
<th>Purification factor</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Culture filtrate</td>
<td>1024</td>
<td>6.8</td>
<td>150</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2. Elution from RBC</td>
<td>512</td>
<td>0.31</td>
<td>1652</td>
<td>11</td>
<td>50</td>
</tr>
<tr>
<td>3. Sephadex G-100</td>
<td>512</td>
<td>0.06</td>
<td>8533</td>
<td>57.2</td>
<td>50</td>
</tr>
</tbody>
</table>

The HA was determined by the microtiter plate and the protein was measured by the Lowry method (132).
FIGURE 11. Sephadex G-100 gel filtration of the soluble HA.

A 59 X 1.6 cm column was used at 5.6 ml/hr flow rate. The void volume of the column was determined with Blue Dextran 2000, and a sample of 0.06 mg protein of HA eluted from RBC was applied to the column and 3.2 ml fractions were collected. The optical density was followed at 280 nm for protein determination and the HA was assayed by the microtiter method.
and appeared in the void volume of the column, the recovery was satisfactory and good purification was achieved.

The adsorption and elution of the soluble HA from RBC followed by gel filtration through Sephadex G-100 resulted in a 57.2 fold-purification and 50% recovery. The partially purified HA had the same characteristics as the HA of the culture supernatant with regard to pH optima, stability and inhibition by HgCl₂. The presence of reducing agents enhanced the HA titer of the partially purified preparation.

c. Binding to Millipore filters

It was noted that HA activity was lost when the culture supernatant was passed through a Millipore filter; it seemed possible that some type of binding was occurring and this could be used advantageously in purification.

HA adsorbs to Millipore filters and can be eluted with 8 M urea. All the reagents used in the attempts to elute the HA from RBCs were tested in the Millipore filter system and were found to have no effect. It was also found that the Millipore filter had a finite capacity for binding HA, so it was likely a binding interaction rather than just adsorption or pore-size binding. The speculations that the binding phenomena of HA to Millipore filter would be advantageous in the purification of soluble HA were correct; 30.4 fold purification was accomplished, but the recovery of the HA was relatively low.
C. Protease of *B. melaninogenicus*

Actively growing cultures of *B. melaninogenicus*-2D produce protease(s) which are bound to the cell as well as being free in the growth media. When measured by the ability to hydrolyze casein, 80% of the proteolytic activity was found to be cell associated. It is not known whether the cell-free and cell-bound enzymes are the same or whether they are different gene products.

1. **Protease Assays**

The cellular and soluble protease(s) of *B. melaninogenicus* are active against a number of protein substrates including azocoll, casein, azocasein and N,N-dimethylcasein. For the azocoll assay, the amount of dye released, which reflects the proteolytic activity in the sample, is determined by measuring the absorbance at 520 nm. This assay is usually qualitative rather than quantitative.

The casein assay depends on the determination of the amounts of TCA soluble peptides liberated from the casein substrate by the enzyme. The assay depends on measuring the absorbance at OD$_{280}$, and in most cases, the cell-extract or culture filtrate used in this study contained large amounts of materials that absorb at 280 nm. The assay was, therefore, affected by the presence of large concentrations of peptides or amino acids in the sample to be assayed.

The dimethylcasein assay was also used. The proteolytic activity was followed by determining with TNBS the production of new amino groups upon hydrolysis. Low blank values were obtained with this assay, however the preparation of the substrate and the assay procedure itself were time consuming and not reproducible.
C-labeled N,N-dimethylcasein was also used as a substrate for determining total proteolytic activity and the assay was more sensitive than the spectrophotometric procedures. Since the azocasein assay also gave reliable results but was less expensive, this assay was used throughout the experimental study. The azocasein assay is based on the solubilization of a covalently linked chromophore from a modified protein. The absorption maximum of this substrate was 370 nm. The maximum absorbance of the culture supernatant and cell-extract was found to occur at a range of wavelength between 220-320 nm and little or no absorbance occurred at 370 nm. Therefore, there was no background, the assay was reproducible, quick and reliable; and the substrate was easy to prepare. The azocasein assay proved to be the easiest to perform. Control values were obtained by assay without enzyme and were subtracted from experimental values. The absorbance of the control never exceeded 0.1. The enzyme preparations had no absorbance at this wavelength. Azocasein units were defined as the change in absorbance at 370 nm per 60 min per ml at 37°C in the presence of PBS (pH 7.0).

The time course of release of azo-dye from the azocasein by B. melaninogenicus intracellular protease is shown in Fig. 12. The rate of release of the chromophore was linear during the early part of the reaction.

The effect of substrate concentration on the release of dye from azocasein was also followed (Fig. 13). A 2% solution of azocasein was adequate to ensure maximum enzyme activity.

The relationship between enzyme concentration and hydrolysis
FIGURE 12. Hydrolysis of azocasein by *E. melaninogenicus* protease

Each reaction mixture contained: cell-extract, 2 mg protein/0.25 ml; 50 mM BME in PBS, 0.75 ml; 2% azocasein, 1 ml. Incubating temperature was 37°C.
FIGURE 13. Effect of azocasein concentration.

Each reaction mixture contained: cell-extract, 2 mg protein; 50 mM BME in PBS, 0.75 ml; concentrations of azocasein in 1 ml PBS as indicated. Reaction mixtures were incubated at 37°C for 60 min.
of the substrate is described in Fig. 14. As can be seen, there is a linear relationship between the enzyme concentration and proteolytic activity. The assay is sensitive, and by increasing incubation times, small amounts of enzyme could be measured.

Azocasein was incorporated into agar to assay various preparations of protease, as mentioned in the Materials and Methods. The azocasein agar assay was useful for assaying protease in electrophoretic polyacrylamide gels.

The cellular and extracellular proteases of *B. melaninogenicus* were unable to hydrolyze hemoglobin and bovine serum albumin (BSA) at least to the extent that it was possible to detect hydrolysis products spectrophotometrically (1-3.2 mg of cell-extract and culture supernatant were incubated for 1 hr with these substrates at 37°C).

2. **Relationship of Protease to Culture Age**

The appearance of cellular and soluble proteases was followed as a function of the age of the culture. The organisms were grown at 37°C under anaerobic conditions. As shown in Fig. 15, the production of the cellular and soluble proteases paralleled the growth curve of the organism until 48 hr when a further increase of the soluble protease was noted and continued as the cells began to lyse.

3. **Cell-bound Protease of *B. melaninogenicus***

The relatively weak proteolytic activity in culture supernatant and failure to concentrate the soluble protease without decrease in activity indicated that it would be wise to proceed with studies of the cell-bound protease.

The cellular protease of *B. melaninogenicus* is localized
FIGURE 14. Effect of enzyme concentration on the azocasein assay.

Each reaction mixture contained: cell-extract, as indicated and 50 mM BME in PBS up to 1 ml; 2% azocasein, 1 ml. Tubes were incubated for 1 hour at 37°C.
Cell Extract - mg protein
FIGURE 15. Relationship of protease to culture age.

Cells grown on TYH medium were harvested, washed and resuspended in PBS ($A_{660} = 1.0$). Culture supernatant was assayed without concentration. Casein was used as the protease substrate.
in the cell envelope, probably near the cell surface since it is accessible to high molecular weight substrates. Cell-bound protease could be liberated by disintegration of a suspension of bacterial cells in buffer.

In this study, attempts were made to separate the cell-bound proteolytic activity from contaminating cellular material and to study the role of the protease in infections by \textit{B. melaninogenicus}.

a. Effect of passaging 2D cells in guinea pigs on protease production.

A 10 ml sample of a 48 hr culture of 2D was harvested and resuspended aseptically in 1 ml of sterile PBS. A 200 g guinea pig was injected in the groin with 0.5 ml of cell suspension. After 24 hr the guinea pig had a very pronounced infection. A large amount of fluid had collected in the thoracic area. The animal was anaesthetized and exudate withdrawn aseptically and placed in a sterile tube. A 0.5 ml sample of the exudate was transferred to a second guinea pig, and 0.2 ml samples of exudate were used to inoculate two tubes of TYH medium. Purity of the exudate was checked on blood agar. A 24 hr old culture of 2D in TYH medium inoculated with the laboratory non-passaged strain of 2D, as well as one tube of TYH medium inoculated with the exudate after passage in the guinea pig was harvested, washed in PBS, resuspended in PBS containing BME 50 mM (\(A_{660} = 1.0\)) and assayed for protease activity using azocasein as substrate.
After 24 hr, the second guinea pig was also infected and 0.5 ml of exudate was transferred to another guinea pig. The exudate appeared to be more infective after passage. The procedure was then repeated as mentioned above. An increase in protease production was associated with animal passage of the 2D containing exudate when assayed \textit{in vitro} by the azocasein substrate (Table 15).

In order to investigate the relationship of the cell-bound protease to the growth of \textit{B. melaninogenicus}, protease production was followed during growth of the organism in different media and under different growth conditions.

b. Effect of hemin concentration on growth and protease production.

\textit{B. melaninogenicus} has an obligate requirement for hemin and the growth rate is dependent on the amount of hemin present in the media (Fig. 5). The effect of different concentrations of hemin on the growth and protease production of 2D was determined (Fig. 16). Growth and protease production were related to the hemin concentration up to a level of 2.5 $\mu$g hemin/ml medium; increasing the concentration of hemin beyond 2.5 $\mu$g/ml had no additional effect on growth or proteolytic activity.

c. Protease production in the presence of succinate.

Protease activity was followed during growth of 2D in 0.1% succinate-trypticase medium and was compared to the protease produced by 2D grown on TYH medium. Figure 17 represents the growth curve of 2D cells on hemin and on succinate medium, as well as the respective protease activities at different culture ages.
Table 15. Effect of Passaging 2D on Protease Activity

Exudates from the passage of the organism in the first, second and third were inoculated into TYH medium and cultures were incubated anaerobically for 24 hr, checked for purity, harvested, washed, resuspended in PBS ($A_{660} = 1.0$), and assayed for protease by the azocasein assay. A 24 hr culture of 2D was used as a control in each case.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protease Units/ml/1 $A_{660}$</th>
<th>% increase in Protease activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) 2 D cells</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>2) *E_1</td>
<td>28.6</td>
<td>15</td>
</tr>
<tr>
<td>3) *E_2</td>
<td>33.4</td>
<td>40</td>
</tr>
<tr>
<td>4) *E_3</td>
<td>38.1</td>
<td>60</td>
</tr>
</tbody>
</table>

* $E_1$, $E_2$ and $E_3$ refers to cells passaged 1, 2 and 3 times respectively.
FIGURE 16. Effect of hemin concentration on growth and protease production of 2D.

The $A_{660}$ of 24 hr cultures of 2D in different concentrations of hemin in the medium was measured and then cells were harvested, washed and resuspended to $A_{660} = 1.0$ in PBS and assayed for protease by the azocasein assay.
FIGURE 17. Protease production in hemin and succinate media.

Media: Trypticase-yeast medium containing 0.1% succinate as well as TYH medium (as in Materials and Methods) were inoculated with a hemin depleted 48 hr culture, $A_{660} = 0.8$ (inoculum was 1% of the total media volume). At different culture ages, the $A_{660}$ of the culture was measured, and the cells were harvested, washed and resuspended in PBS ($A_{660} = 1.0$) and assayed for protease activity using azocasein as substrate.

$A_{660}$: , Hemin; , 0.1% succinate.
Protease: , Hemin; , 0.1% succinate.
There was 60% greater protease activity in hemin medium than in succinate medium where the growth rate was slower.

When 2D was inoculated into trypticase medium containing hemin (10 μg/ml) and succinate (0.1%), the growth of the organism and its protease production were the same as when the organism was grown on hemin medium (10 μg/ml) without succinate. Therefore, succinate had no effect on the growth rate or the protease production of 2D cells when hemin was present in the medium.

d. Effect of amino acids on protease production.

The addition of amino acids to TYH medium affected the growth rate and final yields of 2D B. melaninogenicus as well as protease production (Table 16). The amino acids were added to the TYH medium (final concentration 0.5%) and growth of 2D was followed. Cultures were harvested during the period of most rapid growth, washed and resuspended to A₆₆₀ = 1.0 in PBS and protease activity assayed using the azocasein substrate.

Amino acids did not have any effect on the protease assay. A 31-75% increase in protease production per cell occurred when the amino acids L-asparagine, glutamic acid, L-serine, L-proline and L-methionine were included in the growth medium. This correlates with the growth enhancing properties of these compounds (Table 3). The amino acids L-lysine and L-phenylalanine, which had no effect on the growth of the organism, did not affect the protease production.
Table 16. Effect of additions of amino acids to TYH medium on the proteolytic activity of *B. melaninogenicus*

<table>
<thead>
<tr>
<th>Amino acid added</th>
<th>Concentration (mM)</th>
<th>$A_{660}$</th>
<th>Protease Units/1 $A_{660}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>0.38</td>
<td>16</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>34</td>
<td>0.75</td>
<td>28</td>
</tr>
<tr>
<td>L-asparagine</td>
<td>37.8</td>
<td>0.62</td>
<td>24</td>
</tr>
<tr>
<td>L-serine</td>
<td>47.6</td>
<td>0.58</td>
<td>22.5</td>
</tr>
<tr>
<td>L-proline</td>
<td>43.4</td>
<td>0.5</td>
<td>24</td>
</tr>
<tr>
<td>L-methionine</td>
<td>33.5</td>
<td>0.54</td>
<td>21</td>
</tr>
<tr>
<td>L-lysine</td>
<td>34.2</td>
<td>0.36</td>
<td>17</td>
</tr>
<tr>
<td>L-phenylalanine</td>
<td>30.3</td>
<td>0.34</td>
<td>14</td>
</tr>
<tr>
<td>L-leucine</td>
<td>38.1</td>
<td>0.12</td>
<td>5.8</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>41.3</td>
<td>0.04</td>
<td>2.6</td>
</tr>
<tr>
<td>L-histidine</td>
<td>32.2</td>
<td>0.08</td>
<td>3.7</td>
</tr>
</tbody>
</table>
The finding obtained in batch cultures that under different nutritional conditions, the organism differed not only in its growth rate but also in protease production, suggested that it might be valuable to explore the relationship between growth rate and protease activity. This was accomplished by growing *B. melaninogenicus* in continuous culture at different dilution rates.

e. Growth rate and protease production.

A chemostat was designed for use in the anaerobic chamber (see Materials and Methods) for the continuous culture of 2D, and protease activity was assayed at different dilution rates under steady state conditions of growth. As shown in Fig. 18, the amount of protease per cell increased as the growth rate increased up to a dilution rate of 0.15 hr⁻¹, at this point there was a decrease in enzyme activity.

f. Production of the protease at different concentrations of hemin.

It was found that growth and protease production by 2D were roughly proportional to hemin concentration in batch cultures. To determine whether the hemin had a direct effect on the protease, or whether it was acting indirectly by influencing the growth rate, the organism was grown in continuous culture in different levels of hemin but at the same growth rate.

The protease was measured at steady states of growth by harvesting the culture and resuspending it after washing in PBS to an $A_{660} = 1.0$ in PBS. Results are shown in Table 17. It
FIGURE 18. Effect of dilution rate (D) on protease production by B. melaninogenicus

When a steady state had been achieved cells were harvested, washed and resuspended in PBS ($A_{660} = 1.0$) and assayed for protease by incubation for 1 hr at $37^\circ C$ with the azocasein substrate.
Table 17. Effect of hemin concentration on protease production.

<table>
<thead>
<tr>
<th>Hemin concentration (µg/ml medium)</th>
<th>$A_{660}$ at final steady rate</th>
<th>Protease Units/$A_{660}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.1</td>
<td>7.8</td>
</tr>
<tr>
<td>1.5</td>
<td>1.2</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>1.25</td>
<td>7.8</td>
</tr>
<tr>
<td>2.5</td>
<td>1.2</td>
<td>8.2</td>
</tr>
<tr>
<td>5</td>
<td>1.3</td>
<td>8.7</td>
</tr>
<tr>
<td>10</td>
<td>1.3</td>
<td>7.9</td>
</tr>
<tr>
<td>20</td>
<td>1.2</td>
<td>8.2</td>
</tr>
</tbody>
</table>
can be seen that different hemin concentrations had no direct
effect on the protease activity in continuous culture.

In the continuous culture where the growth rate of 2D was
controlled using the chemostat and the cells were at steady
state of growth in TYH medium, changing the medium to TYH
containing 0.5% L-cysteine at the same dilution rate resulted in
decreased cell yields (measured by $A_{660}$) and a parallel decrease
in protease activity per cell.

g. Preliminary characterization of the cellular protease,
of *B. melaninogenicus*.

1) Effect of reducing agents on the cellular protease

The data in Table 18 show the effect of
different reducing agents on the activity of the protease
in the cell-extract of *B. melaninogenicus* 2D. A concen-
tration of at least 10 mM reducing agent was required for
maximum proteolytic activity. Increasing the concen-
tration of reducing agent resulted in only a slight
increase in activity. A preparation of 50 mM
concentration of BME or cysteine was chosen to be
routinely used in the assays for protease.

Freshly prepared culture extract was not
as dependent on the presence of exogeneous reducing
agent, presumably because the extract contained endogenous
reducing systems. After a period of time the endogenous
sources became oxidized and it was necessary to supply redu-
ucing activity to the system. Activity against azocoll was lost
Table 18. Effect of reducing agents on the cellular protease

<table>
<thead>
<tr>
<th>Reducing agent</th>
<th>Protease Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>6.8</td>
</tr>
<tr>
<td>cysteine</td>
<td>19.2</td>
</tr>
<tr>
<td>dithiothreitol</td>
<td>18</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>17.6</td>
</tr>
<tr>
<td>thioglycolate</td>
<td>13.4</td>
</tr>
</tbody>
</table>

0.5 ml samples of cell extract containing 3.2 mg protein were assayed in the presence of 50 mM concentration of each of the reducing agents using azocoll as substrate.
when the protease preparation was aerated for 20 min, but was restored by addition of reducing agent.

The increased activity of the protease in the presence of reducing agents indicated that the cellular protease of *B. melaninogenicus* could be classified as a sulfhydryl protease. The latter are characterized primarily by their sensitivity to thiol reagents and generally contain cysteine moieties as essential active site components.

ii) Stability and thermolability of the cellular protease.

The thermolability of the protease in the cell extract was determined as the percent of activity lost after incubating the enzyme in PBS at the indicated temperature (Table 19). The protease was partially or completely inactivated at temperatures of 40°C and above.

The protease activity was stable at 4°C in the absence of βME for 12 hr, after this there was a gradual loss of activity. Incubation of the protease at 21°C and 37°C for 4 hr in the presence of βME resulted in 20% loss of activity. The enzyme was more stable at room temperature than at 37°C for 4 hr in the absence of reducing agents. Freezing and thawing of the cell-extract at −70°C resulted in 8-10% loss of activity in the absence of βME and 14-25% loss of proteolytic activity.
Table 19. Thermolability of the proteolytic activity in the cell-extract.

<table>
<thead>
<tr>
<th>Temperature, °C</th>
<th>Time, minutes</th>
<th>activity lost %</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>40</td>
<td>30</td>
<td>11</td>
</tr>
<tr>
<td>50</td>
<td>15</td>
<td>19</td>
</tr>
<tr>
<td>50</td>
<td>30</td>
<td>31</td>
</tr>
<tr>
<td>60</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>60</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td>60</td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>100</td>
<td>1</td>
<td>80</td>
</tr>
<tr>
<td>100</td>
<td>5</td>
<td>100</td>
</tr>
</tbody>
</table>

The protease was assayed by the azocasein assay at 37°C for 1 hr.
in the presence of βME. Therefore, protease preparation were kept free of reducing agent until they were assayed.

The results suggested that the protease was subject to autodigestion as inactivation of the enzyme was more rapid when reducing agents were present.

h. Purification of the cellular protease of *B. melaninogenicus*.

i) Preparation of cell-extract

As the cell-bound protease of 2D could be liberated by disintegration of a suspension of bacterial cells in buffer, a series of experiments was conducted to determine the most effective method for liberating the enzyme.

There was an increase in the total cellular protease activity in the broken cell suspension after cell disintegration. It is possible that breakage of cells might have unmasked protease from protein complexes and/or solubilized enzyme from membrane structures. Some of the enzyme remained bound to the mechanically ruptured cell envelopes.

The extent of casein hydrolytic activity released varied with the technique used to rupture the cells (Table 20). Breakage of the cells in the French Pressure cell proved to be the most effective method since 80% of the cellular protease was liberated in the cell-extract.
Table 20. Comparison of methods for liberating protease

<table>
<thead>
<tr>
<th>Method</th>
<th>% of cellular protease found in the cell-extract</th>
<th>Protease per mg/protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) French Pressure cell</td>
<td>80</td>
<td>3.6</td>
</tr>
<tr>
<td>2) Mini-Mill</td>
<td>40</td>
<td>1.9</td>
</tr>
<tr>
<td>3) Sonication</td>
<td>25</td>
<td>0.8</td>
</tr>
</tbody>
</table>

The cells were obtained from early stationary phase cultures of *B. melaninogenicus* grown in THY medium and were washed and suspended in PBS.
Following breakage the cell-extract was dialyzed against PBS overnight at 4°C. This resulted in a 1.8 fold purification and only a 6% loss of the protease activity (Table 22). The dialyzed cell-extract was centrifuged at 121,000 x g for 1 hr to sediment any residual particulate fragments. Ninety-two percent of the protease remained in the supernatant with a two-fold increase in the specific activity. Therefore, dialysis and ultracentrifugation were used as preliminary steps in the purification of the \textit{B. melaninogenicus} protease from cell-extract.

ii) Ethanol precipitation.

The protease was precipitated from the dialyzed and centrifuged cell-extract by 60% ethanol at -10°C. Under appropriate conditions, the proteolytic activity was not destroyed and the precipitation was almost quantitative. Conditions were determined such that the protease to be separated had a low solubility when most other components of the system had high solubilities. The precipitation was carried out at a low temperature to prevent protein denaturation. Special precautions were needed to insure that the temperature was held at -10°C at every stage during the process. A brief rise of temperature to a few degrees above 0°C for a few minutes had undesirable effects on the stability of the sample. Constant slow stirring was essential in
order to prevent any element of the protein solution from attaining, even temporarily, an unduly high ethanol concentration which might denature the protein. The necessary precautions were fundamentally simple but they had to be strictly maintained and not relaxed in any step in the process.

The effects of different concentrations of ethanol on the precipitation of protease at different temperatures are shown in Table 21. A concentration of 60% ethanol at -10°C for 20 min. proved to be most satisfactory, giving a high recovery and a significant increase in specific activity. Other conditions resulted in higher recovery of the protease but the enzyme had lower specific activity.

This technique provided a simple and effective step for the purification of the protease from the cell-extract. The ethanol precipitated sample was then resuspended in PBS and centrifuged at 121,000 x g for 1 hr and the pellet was discarded. The supernatant was dialyzed overnight against PBS (Table 22).

   iii) Sephadex G-100 in 6M urea.

The dialyzed and centrifuged protease precipitated from the cell-extract by ethanol was applied to a column of Sephadex G-100 (1.6 x 62 cm) equilibrated with 6M urea in PBS. The flow rate was 5 ml/h and 2 ml fractions were collected. All of the protease was
Table 21. Ethanol precipitation of protease from cell-extract.

<table>
<thead>
<tr>
<th>Ethanol concentration</th>
<th>Time</th>
<th>% Recovery</th>
<th>Temperature °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-5</td>
<td>-10</td>
</tr>
<tr>
<td>30%</td>
<td>5</td>
<td>-</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>-</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>-</td>
<td>45</td>
</tr>
<tr>
<td>40%</td>
<td>5</td>
<td>39</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>46</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>48</td>
<td>66</td>
</tr>
<tr>
<td>50%</td>
<td>5</td>
<td>89</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>79</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>92</td>
<td>94</td>
</tr>
<tr>
<td>60%</td>
<td>5</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>98</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>96</td>
<td>100</td>
</tr>
<tr>
<td>70%</td>
<td>5</td>
<td>90</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>96</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>102</td>
<td>102</td>
</tr>
</tbody>
</table>
Table 22. Purification of protease from *B. melaninogenicus* - 2D

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Activity Units</th>
<th>Specific Activity Units/mg Protein</th>
<th>% Recovery</th>
<th>Purification Factor$^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. cell-extract</td>
<td>2080</td>
<td>2.8</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>2. Dialysis$^1$</td>
<td>1955</td>
<td>5.04</td>
<td>94</td>
<td>1.8</td>
</tr>
<tr>
<td>3. Centrifugation$^2$</td>
<td>1799.2</td>
<td>8.06</td>
<td>86.5</td>
<td>2.9</td>
</tr>
<tr>
<td>4. Ethanal precipitation</td>
<td>1540</td>
<td>19.4</td>
<td>74</td>
<td>6.9</td>
</tr>
<tr>
<td>5. Ultracentrifugation of ethanol precipitate</td>
<td>1354.5</td>
<td>38.7</td>
<td>65.12</td>
<td>13.8</td>
</tr>
<tr>
<td>6. Dialysis$^3$</td>
<td>1330</td>
<td>62</td>
<td>64</td>
<td>22.1</td>
</tr>
<tr>
<td>7. Sephadex G-100 in 6 M Urea</td>
<td>2662.5</td>
<td>434</td>
<td>128</td>
<td>154.8</td>
</tr>
<tr>
<td>8. Sepharose 2-B in 6 M Urea A</td>
<td>125.0</td>
<td>1302</td>
<td>6</td>
<td>464.4</td>
</tr>
<tr>
<td></td>
<td>B 3203</td>
<td>2083.2</td>
<td>154</td>
<td>774</td>
</tr>
</tbody>
</table>

$^1$Dialysis against 10 volumes of PBS overnight.

$^2$Centrifugation at 121,000 x g for 1 hr

$^3$Dialysis against 10 volumes of PBS (pH 7.0) overnight.

$^4$Purification factor is the fold increase in the enzyme specific activity
excluded from the column and eluted as a single peak at
the void volume, indicating it had a large molecular
weight (Fig. 19). The eluted protease represented 220%
of the starting material. The increase in activity could
be due to removal of inhibitors or components that were
binding to the active sites of the protease, or to cont-
aminating proteins which competed as substrates. The
pooled, dialyzed, concentrated protease was purified
7-fold in this step. Electrophoretic analysis of the
protease indicated that there were a number of proteins
present in this fraction (see Section h-vi)

iv) Sepharose-2B in 6 M urea.

The protease-containing eluate from a
Sephadex G-100 column was dialyzed against PBS overnight,
concentrated by freeze-drying, made 6M with respect to
urea and fractionated by chromatography on a column of
Sepharose-2B equilibrated with 6 M urea in PBS (Fig. 20).
The flow rate was adjusted to 6.2 ml/hr and 2.3 ml fractions
were collected. When the column was eluted with the same
buffer, the active protease emerged in 2 fractions
differing in specific activity. When these fractions
(A and B) were separately chromatographed on the same
column, each emerged as a single peak at the original
elution volume, one in the void volume (A) and the other
included in the column (B). The fractions were pooled
separately and dialyzed against PBS overnight and
FIGURE 19. Gel filtration of the ethanol precipitated protease.

A 6 ml sample of ethanol precipitated protease (20.4 mg protein) containing 6 M urea was applied to the Sephadex G-100 column and the protease was eluted in PBS-6M urea (pH 7.0). The void volume of the column was determined with Blue Dextran, and absorbance at 280 nm was recorded. The fractions were assayed for protease by the azocasein assay.
The pooled, dialysed, concentrated protease from Sephadex G-100 (3 ml of total 2.2 mg protein) was eluted from a Sepharose-2B column (1.6X 28.3 cm) equilibrated with 6M urea in PBS (pH 7.0). The absorbance at 280 nm was measured and protease activity was assayed using azocasein as substrate.
concentrated by freeze-drying to their original volume. At this stage, it was not possible to decide whether fractions A and B represented the same or different compounds. Both components were found to consist of the same proteins when examined by SDS-polyacrylamide gel electrophoresis in tris-glycine-SDS buffer which separates protein subunits on the basis of molecular weight. Both lacked electrophoretic mobility in polyacrylamide gels without SDS due to their size and not due to charge (Section h-vi).

No difference in pH profile and response to inhibitors between fractions A and B were found which indicates that they are the same.

A summary of the enzyme purification scheme is given in Table 22. The protease was purified 464-fold in fraction A, and 774-fold in B, with a combined recovery of 160%. The enzyme preparation obtained after the Sepharose-2B step will hereafter be referred to as purified protease.

Since the protease was eluted from the Sepharose-2B column as two components having different, but still large molecular weights, which did not migrate in polyacrylamide gel electrophoresis; it seemed possible that the protease activity might have been associated with a component of the cell wall. This would result in the association of proteolytic activity with random sized
and charged fragments when the cells are disintegrated. Difference in chromatographic mobilities between fractions A and B might have been due to random fragments from cell wall being associated with the purified protease. Denaturation of both fractions by boiling in SDS might dissociate these fragments resulting in two similar components, which might represent a polymeric form of the enzyme. Since fraction A contained very little enzyme, detailed studies on the purified protease were performed on fraction B.

v) Other chromatographic procedures.

**Activated (SH) thiol-Sepharose 4B**

Sepharose-4B containing thiol as functional groups was used in an attempt to selectively bind SH-containing proteins. The thiol proteins covalently bind to the immobilized thiol and can be eluted by reduction of the S-S-bond with an appropriate reducing agent.

A 20 ml column of activated thiol-Sepharose 4B was used in an attempt to immobilize the protease present in the ethanol precipitate. Proteins not bound to the Sepharose were eluted with deaerated PBS containing 1 mM EDTA. Forty-one percent of the protease added to the column did not bind; it had the same specific activity as the starting material. Approximately 89% of the bound protease was eluted with 20 mM cysteine-HCl in PBS (pH 7.0) with a two-fold increase in
specific activity (Fig. 21). The procedure was not as effective as gel filtration and was therefore abandoned. Both protease fractions behaved in a similar respect with regard to inhibitors, gel electrophoresis, etc. suggesting that their different binding properties were due to saturation of the column or masking of thiol group in some of the fractions.

**Sepharose mercury (Hg) chromatography**

Chromatography on Sepharose containing immobilized Hg was attempted. The ethanol precipitated sample was applied to a 30 ml column of Hg-Sepharose and 44.7% of the protease did not bind. There was no purification of this fraction. Eighty-two percent of the bound protease was recovered with a 4-fold increase in specific activity (Fig. 22). Polyacrylamide gel electrophoresis in SDS revealed that both peaks containing protease were composed of similar proteins (Section h-vi).

The binding of the protease to both the activated thiol-Sepharose and the Sepharose mercury columns gave additional indication regarding the nature of the protease as a sulfhydryl enzyme. The proportion of the protease that did not bind to either column might have
5.8 ml sample of ethanol precipitate (8.8 mg protein) was applied to a 1.6 x 10 cm column. Unbound proteins were eluted with deaerated PBS containing 1 mM EDTA. The bound protease was eluted with 20 mM cysteine-HCl in PBS (pH 7.0).

Fractions of 1 ml were collected at a flow rate of 2.8 ml/hr. Elution of protein was monitored by measuring absorption at 280 nm. Enzymatic activity was determined by the azocasein substrate.
FIGURE 22. Sepharose mercury chromatography

The column was equilibrated with 50 mM acetate buffer pH 5.5. The ethanol precipitated enzyme was applied in 50 ml (170 mg protein), and the column was rinsed with acetate buffer (50 mM pH 5.5); fractions of 2 ml were collected at a rate of 3 ml/hr. Bound protein was then eluted with 10 mM cysteine in acetate buffer.
50 mM Acetate buffer (pH 5.5)

10 mM Cysteine in acetate buffer (pH 5.5)

Fraction Number
been due to oxidation or blocking of their cysteine moieties.

In attempts to separate contaminating proteins from the protease complex, the following procedures were applied without success as judged by gel electrophoresis and by specific activity: Gel-filtration through G-100 and Sepharose 2B and 4B in various buffers and in the presence of SDS and/or urea at different concentrations, selective heat denaturation and \((\text{NH}_4)_2\text{SO}_4\) precipitation. Ion exchange chromatography on CM-Sephadex C-50 and DEAE-Sephadex A-50 under different conditions of buffers, pH and in the presence of SDS (0.1-0.2%) and/or urea (2M-8M), were also tried in an effort to break the protease complex in the cell-extract and the ethanol precipitate to minimum functional components without success, since the protease activity was poorly recovered from a large number of fractions without significant purification.

Hydrophobic interaction chromatography was used to bind the protease non-covalently to an inert support in phosphate buffer containing 1 M NaCl. Bound proteins were eluted in a 20-50% gradient of ethylene glycol in PBS. A poor recovery resulted from this column with no significant purification.
vi) Gel electrophoresis.

Polyacrylamide gel electrophoresis performed as described by Nagai et al. (156) in a 10% gel in tris-glycine-SDS buffer, pH 8.3, demonstrated only three major bands and one minor band in the purified protease obtained from chromatography on Sepharose 2B (Fig. 23).

Figure 24 represents the polyacrylamide gel electrophoresis in Tris-glycine SDS buffer of the different protease preparations obtained at different steps of the purification scheme presented in Table 22. The gel revealed the presence of a total of fifteen major bands in the cell-extract and ten major bands in the centrifuged and dialyzed ethanol precipitated sample. The protease sample obtained from gel filtration through Sephadex G-100 possessed seven major bands while the purified protease possessed only four bands, thereby indicating that the purification procedure eliminated most of the protein fractions present in the cell-extract.

The polyacrylamide gel electrophoresis in Tris-glycine-SDS buffer of the crude as well as the partially purified enzyme preparations throughout the different chromatographic and various purification procedures are represented in Fig. 25. The samples obtained from the various purification procedures revealed the removal of some major bands as compared to
FIGURE 23. Polyacrylamide gel electrophoresis in Tris-glycine-SDS buffer of the purified protease.

A and B represent Fractions A and B eluted from the Sepharose-2B column with 6 M urea.
FIGURE 24. Polyacrylamide gel electrophoresis in Tris-glycine-SDS buffer of protease fractions obtained during various steps in the purification process.

$G_{el_1}$, cell-extract; $G_{el_{II}}$, dialysed and centrifuged cell extract; $G_{el_{III}}$, ethanol-precipitated sample; $G_{el_{IV}}$, centrifuged ethanol-treated sample; $G_{el_{V}}$, dialysed and centrifuged ethanol-precipitated sample; $G_{el_{VI}}$, protease sample obtained after gel filtration through Sephadex G-100; $G_{el_{VII}}$, purified protease preparation obtained after fractionation on Sepharose-2B. Two mg protein of samples were solubilized in a solubilization mixture containing 4% SDS, 20% glycerol, 0.125 M Tris (pH 6.8) and 0.01% bromophenol blue; the samples were then boiled for 2 min in a boiling water bath. Ten percent ME was added and boiling was continued for another minute.
FIGURE 25. Polyacrylamide gel electrophoresis of protease fractions obtained from the different purification procedures.

Gel_I ethanol precipitated sample; Gel_{II} Fraction 1 from thiol-Sepharose chromatography; Gel_{III} Fraction 2 from the thiol-Sepharose column; Gel_{IV} protease eluted from Octyl-Sepharose CL-4B column; Gel_{V} Fraction 1 from the mercury Sepharose column; Gel_{VI} Fraction 2 from the mercury Sepharose column; Gel_{VII} protease fraction eluted from Sephadex G-100 with 0.1% SDS in PBS; Gel_{VIII} protease fraction obtained from Sephadex G-100 eluted with 0.1% SDS and 4 M urea in PBS; Gel_{IX} protease sample eluted from Sephadex G-100 with 6 M urea; Gel_{X} crude cell-extract; Gel_{XI} purified protease preparation. Fraction 1 refers to protease which did not bind to the column, Fraction 2 refers to protease which bound to the column and was eluted with reducing agent. 20 μl samples, each containing 20 μg protein, were applied to the gel after boiling for 3 min in 4% SDS, 20% glycerol, 0.125 M Tris buffer (pH 6.8), 0.01% bromophenol blue and 10% BME.
the cell-extract (Gel x), however none of these procedures yielded a purification comparable to that obtained (Gel XI) by the purification scheme presented in Table 22.

The samples were electrophoresed towards both the anode and cathode in the absence of SDS without denaturation. The samples were dissolved in a solubilization mixture without SDS and βME. The SDS was also omitted from the running buffer, the stacking and running gels. Twenty μg protein of each sample were carefully layered through the Tris-glycine buffer onto the top of the upper gel. In the crude and partially purified enzyme preparations, not all proteins migrated from the spots as evidenced by the stain at these locations (Fig. 26). Many bands are separated by ever increasing distance suggesting an exponential decrease in molecular size and thus a loss of similar size subunits. No migration of samples occurred when the gel was electrophoresed in the other direction. The purified protease preparation revealed the presence of one major band that did not migrate in the polyacrylamide gel. The use of 7.5% and 5% polyacrylamide gels did not improve the electrophoretic mobility of the purified protease. Therefore, it seemed possible that the purified protease might still be bound in a large molecular weight complex.
FIGURE 26. Polyacrylamide gel electrophoresis in Tris-glycine buffer without SDS.

The gel depicts the electrophoretic properties of the proteins present in the different protease preparations obtained during purification. Gel$_{I}$, cell-extract; Gel$_{II}$, dialysed cell-extract; Gel$_{III}$, dialysed cell-extract after centrifugation; Gel$_{IV}$, ethanol-precipitated fraction; Gel$_{V}$, centrifuged ethanol precipitated fraction; Gel$_{VI}$, dialysed and centrifuged ethanol precipitated fraction; Gel$_{VII}$, protease fraction eluted from Sephadex G-100 with 6 M urea. Gel$_{VIII}$, Fraction A of the purified protease preparation; Gel$_{IX}$, Fraction B of the purified protease preparation; Gel$_{X}$, 30 µg protein of the Fraction B; Gel$_{XI}$, 40 µg protein of Fraction B; Gel$_{XII}$, 50 µg protein of Fraction B. The samples were solubilized in 0.01% bromophenol blue contained in 20% glycerol and 0.125 M Tris buffer (pH 6.8). There were applied to the gel without boiling and in the absence of SDS and βME. SDS was also omitted from the running buffer, the stacking and running gels.
Polyacrylamide gel electrophoresis in the absence of SDS with lipoprotein prestained samples revealed that the purified protease contained lipoprotein staining material which was unable to penetrate the gel, whereas a standard lipoprotein, as represented by rabbit plasma, did migrate (Fig. 27).

The purified protease is a complex of a number of proteins as judged by its inability to enter the polyacrylamide gel unless it has been boiled in SDS.

Detection of glycoproteins in SDS polyacrylamide gels was accomplished (110) by using a cationic carbocyanine dye "Stains-all" (SA) using a tris-acetate-SDS buffer system (pH 7.4). This procedure was capable of reliably detecting the proteins and, at the same time, differentiating the glycoproteins.

A sample of the purified protease (20 μg protein) was layered on each of two gels and subjected to electrophoresis. One gel was fixed and stained for proteins with 0.2% Coomassie blue, and the other was fixed and stained with SA. Protein staining revealed the presence of four (3+1) protein bands. In the single staining procedure with "Stains-all", where the glyco-proteins stained blue, and the proteins red, the purified protease was detected as four bands which all stained as glycoproteins. These results are represented diagrammatically in Fig. 28. It can be concluded that all four
FIGURE 27. Polyacrylamide electrophoresis of fractions A and B stained for lipids.

Gel I, Fraction A of the purified protease; Gel II, Fraction B of the purified protease; Gel III and Gel IV Standard glycoprotein samples of rabbit plasma. The samples were solubilized in 0.1% Sudan black B in ethylene glycol prestain buffered by 0.03 M Tris-citrate buffer (pH 9.0). The buffered prestain was first heated at 60°C for one hour before filtration through Whatman No. 1 paper. The samples were applied to the gel in a concentration of 20 μg protein each and electrophoresed in the absence of SDS.
FIGURE 28. Diagram of glycoprotein and protein bands on slab gels following electrophoresis of purified protease.

Comparison of staining patterns of purified protease separated by SDS-polyacrylamide gel electrophoresis. Coomassie blue (CB) and "Stains-all" (SA) staining are compared in 10% Tris-acetate buffered (pH 7.4) polyacrylamide gel electrophoresis system containing 0.1% SDS.
major bands which represent the purified protease preparations are glycoproteins.

i. Characterization of the purified protease.

   i) Chemical composition of the purified protease

   Table 23 indicates the chemical composition of the purified protease. The protein content of the purified protease preparation was determined by the Lowry method (132). Duplicate samples of the purified protease preparation were analyzed for hexose by the orcinol-sulfuric acid method (223), with an equimolar solution of glucose as a standard. The lipid content of the purified preparation was determined by the micro-method of Pande and Parvin (165) which detects lipids in the concentration range of 20-140 μg. Total free and organic phosphorous was determined by the micro-determination procedure described by Chen et al. (29).

   The chemical analysis of the purified protease preparation demonstrated the presence of carbohydrate, lipid and protein as major constituents. No phosphate was detected in total sample and in the lipid extract from the enzyme preparation.

   ii) Gas-liquid chromatography

   Gas chromatographic analysis (171) of neutral sugars and hexosamines present in the purified protease revealed the presence of glucose and
Table 23. Chemical Composition of the Purified Protease Preparation

<table>
<thead>
<tr>
<th>Final Protease Preparation</th>
<th>mg protein mg dry weight</th>
<th>mg carbohydrates /mg dry weight</th>
<th>mg lipids dry weight</th>
<th>Phosphates in total Sample and lipid extracts.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction A</td>
<td>2.5</td>
<td>0.25</td>
<td>0.35</td>
<td>0</td>
</tr>
<tr>
<td>Fraction B</td>
<td>3.1</td>
<td>0.24</td>
<td>0.79</td>
<td>0</td>
</tr>
</tbody>
</table>
glucosamine in addition to two unidentified sugars.

The fatty acid composition of the lipid extracts of the purified protease was also studied. The lipids were extracted from the purified protease preparation into chloroform and methanol. No phospholipids were detected using the two-directional thin-layer chromatographic method described by Yavin (240). Gas chromatographic analysis of the neutral lipids in fraction A and B are presented in Table 24. The predominant fatty acids in fraction A were linoleic, stearic, palmitoleic and arachidonic. In fraction B, palmitic, stearic and oleic acid predominated. In addition, a number of compounds which were not identified but which may be cyclic or odd chain fatty acids (142) were also found.

iii) Stability of the purified protease

The purified enzyme was found to be less stable than the crude protease. Eight hour incubation at room temperature resulted in about 40% loss of activity, while incubation at 4°C for 2 days resulted in 70% loss. Boiling the enzyme for 1 min resulted in loss of 100% of the proteolytic activity. Freezing at -20°C and thawing at room temperature resulted in 24-35% loss of activity of the purified protease preparation. Five to ten percent of the proteolytic activity was lost when the preparation was stored at -70°C for two weeks.
Table 24. Gas-liquid chromatographic analysis of fatty acids in the purified enzyme preparation.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Percentages of total fatty acid measured</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fraction A</td>
</tr>
<tr>
<td>C-16</td>
<td>2</td>
</tr>
<tr>
<td>C-16:1</td>
<td>8</td>
</tr>
<tr>
<td>C-18</td>
<td>15</td>
</tr>
<tr>
<td>C-18:1</td>
<td>2</td>
</tr>
<tr>
<td>C-18:2</td>
<td>17</td>
</tr>
<tr>
<td>C-20:4</td>
<td>12</td>
</tr>
</tbody>
</table>

The percentage of composition of fatty acids was determined by comparing the area in $\text{mm}^2$ of each peak to that of the total sample.
Longer storage periods resulted in progressively greater losses.

iv) pH optimum of the purified protease

The effect of pH on the activity of the purified protease was determined by the azocasein method in various buffers of 0.1 M ionic strength. As shown in Fig. 29, the purified protease was active over a wide pH range with a pH optimum of 7.0.

The effect of different buffers on the proteolysis as measured in the azocasein assay indicated that activity in Tris buffer was reduced 25% compared to PBS, HEPES (N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid), EPPS (N-2-hydroxyethylpiperazine propane sulfonic acid), MOPS (morpholinopropane sulfonic acid) and glycine buffer. Therefore, in cases where the phosphate ions had to be excluded from the purified protease preparation, EPPS buffer was used instead of PBS.

v) Modification of protease activity

The results of a number of experiments which were undertaken to show the effects of various reagents on the activity of purified protease are shown in Table 25. The substrate for all the reactions was azocasein. The type of inhibitor frequently provides an insight into the active functional group of the enzyme. The purified protease was inhibited by HgCl₂ and alkylating agents such as iodoacetic acid and iodoacetamide (Fig. 30,31). The
FIGURE 29. Effect of pH on the purified protease activity.

Symbols: (○) 0.1 M phosphate buffer, (△) 0.1 M Tris-HCl buffer
(○) 0.1 M glycine-NaOH buffer

Purified protease preparation (0.09 mg protein) was incubated with 0.75 ml of the appropriate buffer containing 50 mM βME for 15 min at 37°C; 1 ml of azocasein (2%) was added to each tube, and after one hr incubation, 2 ml of 10% TCA were added and precipitated azocasein was filtered and $A_{370}$ of the filtrate was measured. The pH of the reaction mixtures was checked and found to be as indicated.
Table 25. Modification of the purified protease

Purified enzyme (0.09 mg protein/ml) concentrated and dialysed overnight against PBS (pH 7.2) was incubated at room temperature with the inhibitor for 30 min. PMSF and TPCK were dissolved in 1-propanol and controls containing only 1-propanol were run. CaCl$_2$, iodoacetic acid and iodoacetamide were dissolved in Tris-HCl buffer pH 7.2. HgCl$_2$, EDTA, urea, SDS, guanidine HCl, lithium chloride and NaCl were dissolved in phosphate buffer pH 7.0. Inhibition of activity was expressed relative to the control incubated in the same buffer without reagent.
Table 25. Modification of the purified protease

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>% Inhibition of Protease Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mercuric chloride</td>
<td>0.01 mM</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>3 mM</td>
<td>100</td>
</tr>
<tr>
<td>Iodoacetic acid</td>
<td>10 mM</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>50 mM</td>
<td>55</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>10 mM</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>50 mM</td>
<td>82</td>
</tr>
<tr>
<td>PMSF</td>
<td>10 mM</td>
<td>0</td>
</tr>
<tr>
<td>TPCK</td>
<td>10 mM</td>
<td>0</td>
</tr>
<tr>
<td>EDTA</td>
<td>10 mM</td>
<td>0</td>
</tr>
<tr>
<td>Urea</td>
<td>8 mM</td>
<td>0</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1%</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.3%</td>
<td>65</td>
</tr>
<tr>
<td>Guanidine HCl</td>
<td>0.5 M</td>
<td>20.5</td>
</tr>
<tr>
<td></td>
<td>4 M</td>
<td>66.7</td>
</tr>
<tr>
<td>Lithium chloride</td>
<td>0.5 M</td>
<td>38.5</td>
</tr>
<tr>
<td></td>
<td>3 M</td>
<td>74.4</td>
</tr>
<tr>
<td>Acetone</td>
<td>100%</td>
<td>80</td>
</tr>
<tr>
<td>Butanol</td>
<td>100%</td>
<td>100</td>
</tr>
<tr>
<td>Phenol</td>
<td>100%</td>
<td>100</td>
</tr>
<tr>
<td>Chloroform</td>
<td>100%</td>
<td>100</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>0.5%</td>
<td>100</td>
</tr>
<tr>
<td>Ethylene glycol</td>
<td>20%</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>50%</td>
<td>15</td>
</tr>
<tr>
<td>DMSO</td>
<td>5%</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10%</td>
<td>5</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>1 M</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>3 M</td>
<td>50</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>0.1 M</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.5 M</td>
<td>0</td>
</tr>
</tbody>
</table>
FIGURE 30. HgCl$_2$ inhibition of the purified protease

Enzyme (0.09 mg protein) was incubated at 37°C in the anaerobic chamber with indicated concentrations of HgCl$_2$ in PBS pH 7.0 for 30 min and then assayed with azocasein as substrate.
FIGURE 31. Inhibition of the purified protease by iodoacetamide.

Enzyme preparation (0.09 mg protein) was incubated for 30 min at room temperature with Tris-HCl buffer (pH 8.2) containing the appropriate concentration of the reagent with 50 mM βME. One ml of 2% azocasein was used as a substrate and the reaction mixture was incubated for 1 hr at 37°C.
metal chelating agent EDTA and the serine inhibitors had no inhibitory effect on the protease. On the basis of these findings, the protease of *B. melaninogenicus* could be classified as a sulfhydryl enzyme. Guanidine HCl, which generally uncouples polar bonds and denatures proteins caused up to 66.7% inhibition of the proteolytic activity. The inhibitory effect might also be due to influence of ionic strength on the enzymatic activity. Lithium chloride at 3 M concentration inhibited 74.4% of the proteolytic activity (Fig. 32). Phenol and chloroform extraction as well as treatment with butanol, Triton X-100 and acetone caused almost complete inhibition of the protease but this was probably due to protein denaturation. However, the polar organic solvents dimethyl sulfoxide (DMSO) and ethylene glycol had little inhibitory effect on the enzyme activity. Urea (8M) and 0.1% SDS did not inhibit the protease; but 8 M urea had a stimulatory effect. The reason for this could be that urea caused denaturation of the protein complexes that could mask some of the active sites of the enzyme. It is also possible that high concentrations of urea might modify the substrate by making it more accessible to the protease enzyme.

The influence of ionic strength on the enzymatic activity is illustrated in Fig. 32. The presence of NaCl at concentrations above 0.5 M decreased
FIGURE 32. Effect of guanidine hydrochloride, lithium chloride and NaCl on the purified protease.

Enzyme preparation (0.09 mg protein) was incubated with 0.75 ml of the appropriate concentration of the reagent in phosphate buffer (pH 7.0) containing 50 mM βME for 30 min at 37°C; 1 ml azocasein (2%) was added and the reaction mixture was incubated for 1 hr.
the enzyme activity.

The purified protease was very sensitive to HgCl₂ since low concentration of the inhibitor caused complete loss of activity. The inhibition could be reversed by reducing agents. As shown in Fig. 30, the percent inhibition of proteolytic activity was proportional to the concentration of the HgCl₂.

The function of the carbohydrate and lipid components in the purified protease was investigated by testing the effect of mixed glycosidases on the activity of the purified protease, and by extracting the lipid from the purified protease preparation with phenol, chloroform or butanol.

Extraction of the lipids from the purified protease resulted in complete loss of activity, but this effect might have been due to denaturation of the protein by the organic solvents used in the extraction. Restoration of the proteolytic activity with the dialyzed extracted lipid dialyzed against PBS was unsuccessful.

Incubation of the purified protease with mixed glycosidases for 10 hr at 37°C caused 43% inhibition of the protease activity, suggesting that the carbohydrate part of the protease might be essential or required for activity.
vi) Substrate specificity of the purified protease

The purified protease was active against azocasein, casein, N,N-dimethylcasein and azocoll as substrates. No activity could be demonstrated against hemoglobin, bovine serum albumin, or $^{14}$C-collagen. The purified protease did not hydrolyze the synthetic substrates TAME, tosyl arginine methyl ester; BAEE, benzoylarginine ethyl ester or ATEE, acetyl tyrosine ethyl ester.

An identical specificity was exhibited by the enzyme from the cell-extract and the preparation obtained after ethanol precipitation as well as other preparations obtained through the purification procedure, suggesting that a single proteolytic enzyme was produced by the organism.

The purified preparation had no lipase, α or β-glycosidase, collagenase or hemagglutinating activity when assayed as described in Methods.

vii) Pathological activity of the purified protease

A concentrated purified preparation of the protease containing 2 mg protein/ml was dialyzed against PBS and injected subcutaneously into the groin of a 200 g guinea pig using 0.5 ml. The animal was observed for up to 4 weeks for the presence of abscess. The purified protease had no biological activity by itself. Adding the protease preparation to a cell suspension of 2D that caused
a typical infection in the guinea pig model system resulted in a slight increase in the necrosis of the lesion when compared to the necrotic lesions caused by 2D cells without addition of the protease preparation. Whether the purified protease was the factor responsible for this more pronounced infection, or experimental variations which are common in these kind of experiments, could not be verified.

When the purified protease was tested for vascular permeability following the method of Craig (36), no blueing effect was observed. The purified protease did not enhance the blueing effect produced by the concentrated culture supernatant of 2D.

4. Soluble Protease of *B. melaninogenicus*

Studies were carried out to investigate whether the cell-free and cell-bound protease(s) are the same or different entities by comparison of their pH optima, effects of inhibitors and properties during chromatography.

a. Demonstration of an extracellular protease.

Proteolytic activity against azocoll in culture supernatants of *B. melaninogenicus* strain 2D was detected early in the exponential growth phase (Fig. 15), and was found to increase in proportion with increasing cell numbers until mid- to late stationary phase when a further increase in activity was noted which continued as the cells began to lyse.
b. Preliminary characterization of the extracellular protease.

i) Oxygen sensitivity

The soluble protease in the culture supernatant was inactivated by oxygen. A definite increase in the rate of dye released from azocoll occurred when 0.01 M cysteine or 25 mM BME was included in the reaction mixture. Activity against azocoll was lost when the supernatant was aerated for 20 min, but was restored with the reducing agent.

ii. Stability of the protease

The stability of the protease in concentrated culture supernatants was examined by storing the enzyme at -70°C, -20°C, 4°C, 20°C and 37°C with and without reducing agent and assaying periodically for protease activity. Soluble protease was found to be more stable in the absence of the reducing agent when stored or incubated at any of the above stated temperatures. This may be due to increased rates of autodigestion in the presence of the reducing agents needed for proteolytic activity. Storage of the protease at -70°C was found to be more effective than at -20°C where some of the activity was lost even in the absence of BME.

The protease was stable when incubated at room temperature only for a period of 3 to 4 hr. Incubation at 37°C resulted in a greater loss of the
protease activity, especially in the presence of βME. It was found to be stable for 8 hr at 4°C. Boiling the culture supernatant for 5 min resulted in complete loss of protease activity.

iii. Effect of pH on protease activity

Protease activity was measured over a pH range of 4.0 to 9.0. Acetate buffers were used from pH 4.0 to 5.5, phosphate buffers from pH 6.0 to 7.5 and Tris-HCl buffers from pH 7.0 to 9.0 (Fig. 33). The optimum pH for protease activity against azocasein was between 7.0 and 7.5. Tris-HCl buffer caused a slight inhibition of the proteolytic activity (15-20%) when used in the assay mixture as compared to the phosphate buffer.

iv) Inhibition of soluble protease by various reagents

Table 26 summarizes the effect of a number of inhibitors of proteolytic enzymes on the soluble protease of *B. melaninogenicus*. The soluble protease was completely inhibited by mercuric chloride, the inhibition could be reversed by addition of the Hg binding reagent βME. The sulfhydryl reagents iodoacetic acid and iodoacetamide inhibited the protease activity. Sulfhydryl groups are apparently required for proteolytic activity. Phenyl methyl sulfonyl fluoride (PMSF) and Tosyl-phenyl-ethyl-chloromethyl ketone (TPCK), which inhibit
FIGURE 33. pH optimum of the soluble protease.

The effect of pH on the soluble protease activity was determined by the azocasein method in various buffers of 0.1 M ionic strength. (●) acetate buffer, (△) phosphate buffer, (○) Tris buffer. Dialyzed culture supernatant (1.5 mg) was incubated with the substrate for 1 hr at 37°C.
Absorbance (370 nm)
Table 26. Inhibition of soluble protease

Protease was incubated at 37°C for 30 min with each of the compounds before measuring proteolytic activity against casein. The protease activity of unmodified culture supernatant was assayed in the presence and absence of EME in same buffers and the results are expressed as % inhibition of protease activity of the control reactions. PMSF and TPCK were dissolved in 1-propanol; HgCl$_2$ was dissolved in PBS (pH 7.0) and EDTA and alkylating agents in 0.1 M Tris-HCl (pH 8.0).

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Concentration (nM)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>HgCl$_2$</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>HgCl$_2$+EME</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>75</td>
</tr>
<tr>
<td>PMSF</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>TPCK</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>EDTA</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>Iodoacetic acid</td>
<td>1</td>
<td>24.2</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>40.5</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>1</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>80.7</td>
</tr>
</tbody>
</table>
proteases requiring serine at the active site, had no effect on the soluble protease. Incubation with the metal chelator EDTA inhibited the extracellular protease by 5-60%.

c. Partial purification of the soluble protease.

i) Dialysis of culture supernatant

The dialysis of culture supernatant overnight at 4°C against PBS resulted in 2.1 fold-purification most probably due to loss of dialyzable peptides; and 6% loss of proteolytic activity occurred (Table 27).

ii) Concentration of culture supernatant

Due to the observation that the soluble protease was unstable, a variety of methods for concentrating the protease were tried in order to find the method which would lead to the least inactivation. Freeze-drying was found to be the most effective concentrating procedure provided that the glassware was coated with Silane (Bio-Rad). Another concentration procedure that proved effective was to remove water by blowing air over the supernatant contained in dialysis tubing. Evaporation phenomena kept the temperature of the enzyme solution low and resulted in less inactivation of the protease. Ammonium sulfate precipitation and ultrafiltration (Amicon PM-30 and XM-50 membranes) were not effective procedures for concentrating the enzyme.
Table 27. Partial purification of *B. melaninogenicus* extracellular protease

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total Protease Units</th>
<th>Protease specific activity units/mg protein</th>
<th>Yield</th>
<th>Purification Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate</td>
<td>72</td>
<td>2.8</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Dialysis&lt;sup&gt;1&lt;/sup&gt;</td>
<td>67.7</td>
<td>5.8</td>
<td>94</td>
<td>2.1</td>
</tr>
<tr>
<td>Freeze-drying</td>
<td>51.0</td>
<td>3.4</td>
<td>70.84</td>
<td>1.2</td>
</tr>
<tr>
<td>Sephadex G-100 gel filtration</td>
<td>32.8</td>
<td>35.4</td>
<td>45.6</td>
<td>12.6</td>
</tr>
</tbody>
</table>

<sup>1</sup> Dialysis was against 10 volumes of PBS for overnight.
iii) Purification procedures

Culture supernatant of 2D was applied to a DEAE-Sephadex A-50 column packed and washed with 0.05 M NaCl in phosphate buffer. The protease was bound to the DEAE-Sephadex and only 20% of the activity was recovered. This was eluted with a 0.05-2 N NaCl gradient. Several peaks of protein were eluted with the salt gradient, but none possessed significant proteolytic activity. No substantial purification was achieved. Further attempts at fractionation of the concentrated supernatant resulted in very low recovery.

When a sample of concentrated 48 hr culture supernatant was chromatographed on a CM-Sephadex C-50 and Sephadex G-200 columns, activity against azocoll was detected in many fractions, suggesting that the protease activity might be associated with another constituent of the supernatant. Generally, the protease activity appeared to be binding nonspecifically to the columns with considerable variation among the elution patterns of different samples. The reason for this is not known although it may have been due to proteolytic action on some of the protein components of the supernatant. It is also possible that the protease may have been associated with a component of the cell wall, which would result in the association of the enzyme with random sized and charged fragments.
iv) Gel filtration on Sephadex G-100

A Sephadex G-100 column packed, equilibrated and eluted with PBS was used to fractionate the soluble protease. The protease activity in the culture supernatant was eluted from the Sephadex G-100 column as a single peak immediately after the void volume, as shown in Fig. 34. This indicated an approximate molecular weight of 100,000 or more. 64.4% of the proteolytic activity applied to the column was recovered, suggesting the possibility that some of the protease was either inactivated or still on the column. The purification steps resulted in an approximately 12.6 fold increase in specific activity over the starting material with a total 46% recovery (Table 27).

Gel electrophoresis of the partially purified protease revealed many proteins and further attempts to purify the protease were unsuccessful. The partially purified protease was shown to be a sulfhydryl enzyme having the same characteristics as the crude protease in the culture supernatant.

It was determined that the extracellular protease was large, possibly membrane or particle bound. Therefore, there would be no advantage in attempting to purify it any further, as similar material could more conveniently be prepared from exponential phase cells.
FIGURE 34. Sephadex G-100 gel filtration of the soluble protease.

A 59 x 1.6 cm column was used at 5.5 ml/hr flow rate. The void volume of the column was determined with Blue Dextran 2000, and a sample of 18.2 mg protein of dialyzed culture supernatant was applied to the column and 1.8 ml fractions were collected. The optical density was followed at 280 nm for protein determination. The protease was assayed by the azocoll assay.
Protease units/ml

Fraction Number

A280

Protease

V₀
The results indicate that the soluble protease was similar to the cell-bound enzyme with respect to pH optima, response to inhibitors, substrate specificity and properties during chromatography. Therefore, it can be concluded that only one major protease was produced by the strain 2D of _B. melaninogenicus_ and that the extracellular protease was probably due to lysis or leakage from the cells.
IV. DISCUSSION

*B. melaninogenicus* has been implicated in the pathogenesis of mixed anaerobic infections in man \((4,25,57,231)\) and in the development of experimental anaerobic infections \((137,205)\). It is known to possess attributes which would implicate it in human periodontal disease \((138,139)\). Therefore, the organism has attracted the interest of a number of investigators throughout the years. However, detailed investigations concerning the biochemical and pathogenic capacities of the organism have been hampered by difficulties in growing many strains in pure culture and by inadequate taxonomy \((26,54)\). These facts have been responsible, at least in part, for the conflicting results in the literature.

A study of the proteolytic and hemagglutinating activity of the organisms might provide insight into the nature of their pathogenicity and into their function and behaviour as members of the indigenous flora of the human gingival crevice. Studies in our laboratory tend to validate the recent proposal \((98)\) that existing subspecies of *B. melaninogenicus* can be classified into at least two species. Our classification is based on a number of new taxonomic criteria, which taken together with the standard criteria, emphasize the unique characterization of *B. melaninogenicus* ss. *asaccharolyticus*. These criteria are pathogenicity, HA activity, collagenase and the production of phenylacetic acid. Phenylacetic acid may have some importance in clinical diagnosis since it has been found in exudate from animals \((S. Jensen, personal communication)\). Both organisms characterized for this study, produced butyric acid, phenylacetic
acid, hemagglutinin, high level of protease collagenase and were infective. Therefore, they can be classified as *B. melaninogenicus* ss. *asaccharolyticus*.

As expected, strain 2D required hemin for growth. The organism can grow on as low concentration of hemin as 0.25 μg hemin/ml medium and the growth is roughly proportional to the hemin concentration up to a concentration of 2.5 μg hemin/ml medium. Higher concentrations up to 20 μg/ml medium had neither an inhibitory nor an enhancing effect on growth.

The growth rate of 2D was influenced by free amino acids. L-serine, glutamic acid, L-methionine, L-proline and L-asparagine enhanced growth (Table 3). Glutamic acid had the most pronounced effect, increasing growth at 24 hr by 45%. Growth was inhibited almost completely by L-cysteine, DL-valine, L-histidine, L-tryptophan, glycine or L-arginine. L-leucine inhibited growth by 66% while L-lysine and L-phenylalanine had no effect.

Our results correlate with the work done by Miles and Wong (149) on the inhibition of growth of an asaccharolytic strain of *B. melaninogenicus* by valine, tryptophan, glycine, histidine and arginine as well as the enhancement of growth by asparagine. These workers also reported that L-phenylalanine and L-lysine had no effect on the growth rate, a conclusion which was also confirmed by our studies. However, serine and methionine, which were reported to inhibit the growth of the asaccharolytic *B. melaninogenicus*, were found to enhance the growth of strain 2D of *B. melaninogenicus*. Glutamic acid which was found to cause the maximum growth enhancement of 2D was reported by Miles and Wong as having no effect; also, the amino acids L-serine, L-cysteine and L-leucine were found to inhibit growth of 2D in our studies, while having no effect on the growth of the asaccharolytic strain studied by Miles (149).
The ability of amino acids to influence growth indicates that they were probably assimilated by the microorganism. This contradicts the conclusions of Wahren et al. (228) who reported that the organisms were unable to assimilate these compounds. Proof that the amino acids were actually consumed is lacking and should be investigated by following incorporation or metabolism of labeled amino acids. Glutamic acid was unable to replace hemin as a growth factor, although it seems possible that glutamic acid could be deaminated and decarboxylated to form succinic acid which was reported as a possible replacement for hemin (146).

The reasons why some of the amino acids influence growth is not known. Interestingly, the amino acids that inhibited growth of B. melaninogenicus in this study are of different chemical groups. For example, inhibition was caused by the heterocyclic amino acids L-histidine and L-tryptophan, by the aliphatic amino acids glycine and valine, and by L-cysteine, a sulfur-containing compound. Therefore, a common inhibitory mechanism is unlikely. Also, it is unlikely that competitive inhibition of uptake of required amino acids is occurring, because the growth studies were done in the presence of trypticase, which has few free amino acids. It is possible that competitive inhibition between amino acids and peptide transport may exist, since it is known that peptides are used by B. melaninogenicus (228).

B. melaninogenicus strain 2D was shown to be one of the few asaccharolytic isolates which is infective as a monoculture and is thus one of the more virulent strains of this species. The infection is severe, develops rapidly and leads to death within 48 hrs. Death is probably due to septic shock resulting from massive bacteremia and infection of the peritoneal
cavity. The infection can be transmitted directly from one animal to another providing that it is truly an infection and not induction of a hypersensitive response.

Pathogenicity of *B. melaninogenicus* can be related to a number of factors. The results reported here have shown that strain 2D possesses a cholera-like toxin when tested by the vascular permeability assay. It seems highly unlikely that the toxic material is a lipopolysaccharide-like endotoxin since heating destroyed its activity. The rapidly spreading infection might also be due to collagenase and/or protease activity. The determination of whether collagenase or protease activity is essential for a successful infection will require the isolation of a mutant of an infective strain differing from the parent strain only in the absence of collagenase or protease activity.

*B. melaninogenicus* strain 2D possesses HA activity as evidenced by its ability to cause clumping of RBC and to bind strongly to RBC surfaces. Culture supernatant contains HA activity which is thought to be due to cell associated HA which has been released from the cell surface. The evidence to support this assumption is as follows:

(a) Both hemagglutinin activities are sensitive to oxidation and activity can be restored by addition of reducing agents (Table 10).

(b) Both HA are sensitive to Hg and iodoacetic acid (Table 11).

(c) Both HA are insensitive to the same salts, EDTA and carbohydrates, however, both were sensitive to galactose.

(d) Both HA are inactivated by heating.

(e) HA can be removed from cells by mild procedures such as washing with buffer (Table 5).
Electron microscopic studies have shown that the soluble HA is present in a membranous structure (Thanks to Dr. Paul Osmanski and Susan Jensen).

Soluble HA is a large molecule as evidenced by gel filtration and sedimentation at 100,000 x g (Fig. 11 and Table 12).

An increase in soluble HA is found in old cultures (Fig. 10).

Hemagglutinin activity was found associated with the cells as well as in the culture supernatants of strain 2D. The soluble HA was chosen for study since it is free of cells and therefore, likely to be purified more easily, and also to provide evidence to support the assumption that the soluble HA is the same as that which is cell associated.

The requirement of reducing substances for hemagglutination activity correlates well with the highly reduced state of the natural environment of the organism. As pointed out, the cell-free HA was of high molecular weight (Fig. 1 and Table 12) which is probably due to the association of HA with fragments of the outer membrane. Therefore, the observed effects of inhibitors and enzymes on hemagglutinating activity might be either the result of changing the properties of the associated membrane particle, or a direct effect on the HA itself.

The soluble HA of B. melaninogenicus was insensitive to carbohydrates with the exception of galactose. Galactose was also found to inhibit the hemagglutinin of the oral strains of Fusobacterium nucleatum (153). Therefore, it appears that the cell receptor for the B. melaninogenicus HA moiety contains D-galactose. D-mannose has been shown to inhibit hemagglutination of E. coli (160), which was attributed to the presence
of a mannose-specific lectin-like protein on the E. coli cell surface. The soluble HA of 2D was sensitive to heat and to treatment with pronase indicating that proteinaceous substances are involved. Insensitivity of the soluble HA to different salts might suggest that agglutination of the HA and RBC does not occur through an ionic interaction.

In order to obtain some information concerning the nature of the component(s) necessary for HA, the effect of treatment of RBC on their ability to hemagglutinate with soluble HA was determined (Table 9). Pronase and galactosidase treatments of RBC cause inhibition of the HA activity which might indicate that protein and/or carbohydrate moieties are necessary for HA to occur. Treatment of the RBC with neuraminidase caused enhancement of the HA activity. Elution of HA adsorbed to neuraminidase-treated RBCs with 8 M urea was less effective than eluting adsorbed HA from untreated RBCs. This might be due to a tighter binding of soluble HA to neuraminidase-treated RBC. The fact that neuraminic acid is often linked to galactose (238) suggest that removal of neuraminic acids may unmask and then create many new receptors or binding sites for the HA on the RBC. Pretreatment of RBC with neuraminidase was reported to result in an increased binding of the HA preparations of the oral bacterium F. nucleatum to the RBC (153).

An affinity adsorption system using formalinized RBC was developed and used to accomplish some purification of the soluble HA in culture supernatant, followed by gel filtration on Sephadex G-100. Recovery of 50% of the HA was accomplished with a 52-fold purification. However, polyacrylamide gel electrophoresis of the partially purified HA revealed a heterogenous preparation. If the HA is associated with the outer
membrane, it may exist in a large complex, and thus be associated with many proteins. Further attempts to dissociate the HA activity from the particulate fragments resulted in great loss of activity; therefore, it is possible that the HA may only be active in this complex form. Reducing conditions were required for activity of the partially purified HA. Reagents known to react with sulfhydryl groups such as iodoacetic acid, iodoacetamide and HgCl$_2$ partly inhibited the crude HA as well as the partially purified HA in culture supernatant. Therefore, it can be concluded that sulfhydryl groups are an integral part of the hemagglutination activity.

*B. melaninogenicus* produced both cell-bound and cell-free proteolytic activity which amounted to approximately 80% and 20% respectively of the total activity during exponential growth. The proportion of cell-free activity increased in stationary phase cultures. The cell-bound protease of *B. melaninogenicus* resembles the proteinases in *Streptococcus lactis* (221) and in *Bacteroides amylophilus* H18 (15). All three proteases are localized in the cell envelope, probably near the cell wall surface since they are accessible to high molecular weight substrates.

Both cellular and soluble proteases of *B. melaninogenicus* were active against a number of protein substrates including azocoll, casein, azocasein and N,N-dimethylcasein. The azocasein assay was found to be most satisfactory mainly because of low background. The assay was most effective, reproducible and easiest to perform.

Protease production by Gram-negative anaerobic bacteria has been little investigated; the study of the proteolytic activity of *B. melaninogenicus* thus seemed to be of general interest.
In order to investigate the relationship of the cell-bound protease to the growth of *B. melaninogenicus*, protease production was followed during growth of the organism in different media and under different conditions. It was found that the protease production correlated with the growth rate of the organism.

The evidence supporting this assumption was as follows:

(a) Increasing the growth rate of a continuous culture of the organism resulted in an increase in protease production by the organism (Fig. 18).

(b) Amino acids that stimulated growth stimulated protease production and this was not related to a direct effect on the activity of the enzyme (Table 16).

(c) When the growth rate was limited by hemin concentration protease synthesis was slowed (Fig. 16). Under conditions where hemin was not growth limiting, further increasing the hemin concentration did not have an effect on protease production (Table 17).

(d) There was a 60% decrease in protease activity in succinate medium compared to TYH medium with a simultaneous decrease in the growth rate due to the replacement of hemin by succinate (Fig. 17). The succinate had no direct effect on the protease activity.

The reason why protease synthesis would be dependent upon the growth rate is not known, but these findings correlate with findings reported for enzyme production in *Pseudomonas aeruginosa* (31) and *Vibrio* SA1 (234). In the former amidase synthesis and in the latter extracellular protease production were related to the growth rate of the organisms in chemostat studies. It is most probable that this reflects a complex control system.
Studies with material aspirated from guinea pig infections have shown that 2D elaborates the sulfhydryl protease in the infection as well as in vitro. An increase in protease production was associated with animal passage of the 2D containing exudate when assayed in vitro (Table 15), which might indicate a role of the protease in the infective process.

An extensive study was made of the biochemical properties of the cell-bound protease, isolated from mechanically disrupted cells. Microbial proteases are often classified by pH optimum and inhibitor sensitivity rather than by the most readily hydrolyzed substrate (102). In addition, specific inhibitors of proteolytic activity frequently provide insight into the nature of the functional groups on the enzyme. The proteolytic activity of B. melaninogenicus appeared to be due to sulfhydryl protease(s), since the cellular activity was completely inhibited by Hg$^{2+}$. Sensitivity to oxidation, restoration of action with reducing agents and the inhibitory effect of the alkylating agents iodoacetic acid and iodoacetamide supported this assumption. The metal chelating agent EDTA had no effect on the proteolytic activity. The observation that the protease was not inhibited by the serine inhibitors, nor was it activated by divalent ions, and was active at neutral pH, strongly suggests that it is not an acidic, serine or metalloenzyme type of protease. On the basis of these findings the cellular protease of B. melaninogenicus should be classified as a sulfhydryl enzyme.

Generally, the existence of an active thiol group is primarily a characteristic of the neutral proteases. The cellular enzyme of 2D had a broad spectrum of activity between pH 5.5 and 10.5, with a sharp peak of casein hydrolytic activity at pH 7.0 and a second plateau of activity between pH 8.0 and 9.0. The pH optima of the cellular protease correlate
with the neutral or slightly alkaline pH of its natural environment in the gingival crevice.

The cellular protease of *B. melaninogenicus* was found to be subject to autodigestion, particularly in the presence of reducing agents. In addition, it was not generally very stable which hampered its purification. However, the fractionation scheme presented (Table 22) gave a good recovery and was easily reproducible provided that certain precautions were followed. Among these precautions are the exclusion of reducing agent during all steps in the purification, the ultimate care in keeping the temperature below 4°C and appropriate conditions of cooling and stirring during the precipitation of the proteolytic activity by ethanol.

The purification of the cellular protease was accomplished by dialysis of the cell-extract, ultracentrifugation at 121,000 x g for 1 hr, precipitation with 60% ethanol at -10°C followed by ultracentrifugation at 100,000 x g and dialysis; gel filtration through Sephadex G-100 in 6 M urea; and gel filtration through Sepharose-2B in PBS containing 6 M urea. The specific activity of the purified preparation was increased 774 times over that of the crude preparation, and a 160% final recovery was obtained (Table 22).

The fact that an increase in the recovery of the proteolytic activity was found after chromatography of the ethanol precipitated protease suggested that the purification steps might have unmasked the protease from protein complexes, removed some inhibitors or components that were binding to active sites or removed endogenous substrate.

Polyacrylamide gel electrophoresis showed the purified protease to consist of four electrophoretically distinct bands as compared to 15 major bands
in the crude enzyme in cell-extract (Fig. 23,24). Each of the four bands was firmly bound to carbohydrate moiety as indicated by the glycoprotein stain. When the purified protease preparation was subjected to polyacrylamide gel electrophoresis without denaturation and in the absence of SDS, it revealed the presence of only one band and that band did not migrate into the gel (Fig. 26). Therefore, it seems likely that the purified protease still might have been bound in a large molecular weight complex, as was also indicated by its exclusion from G-100 Sephadex in 6 M urea. It is similar, in this respect, to the cellular protease of *Bacteroides amylophilus* released by cell disintegration which also did not penetrate polyacrylamide gels. (14). It also resembles the penicillinase of *Bacillus licheniformis* which on solubilization by deoxycholate and urea was particulate as judged by its poor mobility in starch gel electrophoresis (116).

The purified protease could represent a polymeric form of enzyme subunits bound to a cell wall component. The fact that the purification procedure used in this study always resulted in fractionation of the crude protease into four major electrophoretically distinct bands, suggests that the components of the complex purified protease were firmly bound together and not coincidentally associated through the purification steps. It also implies that the purified protease is the "minimum biologically active unit", which is present as a complex of a number of proteins unable to enter the polyacrylamide gel unless it has been denatured in SDS. Any further attempts to break down the purified protease into a less complex unit resulted in loss of the proteolytic activity. It was reported that some basic properties of certain enzymes such as the dimerization of subunits might be dependent on the retention of the enzyme in association with the cell wall (30).
More definite conclusions could be drawn if one was able to define which of the four bands found upon gel-electrophoresis contain(s) the proteolytic activity. Feasible approaches to this problem could be the use of mutants deficient in proteolytic activity, or chemically cross-linking a radioactively labeled substrate to the enzyme.

In the final step of purification, the active protease emerged in two fractions differing in both specific activity and chromatographic mobility. Evidence was obtained that both fractions represent the same protease. The influence of pH on proteolytic activity was identical. No difference was found in the response to inhibitors between the two fractions. Both were stained for lipids, had 4 distinct electrophoretic bands of glycoproteins and lacked electrophoretic mobility in the absence of SDS. The difference between the 2 fractions is in their lipid content (Table 23) which could be a reason behind the difference in their chromatographic mobility.

It can be deduced that the cellular protease released by disintegration of the cells remained firmly bound to cellular components from which it could not be completely liberated. In this respect, it resembled the cell-bound protease of *Bacteroides amylophilus* H18 which, when liberated by sonic disruption of cells harvested during exponential phase, was particle-bound and could not be easily purified (14). Also, the penicillinase of *Bacillus licheniformis*, when liberated by lysozyme treatment, appeared to be bound to membrane fragments (116).

As it was assumed that the protease activity might have been associated with a component of the cell wall, this would result in the association of proteolytic activity with random sized and charged fragments when the cells were disintegrated. This is supported by the results obtained from
ion exchange chromatography of the crude protease under different conditions of buffers, pH and in the presence of denaturing agents which resulted in poor recovery of the protease from a large number of fractions. This assumption can also account for the variations in protease liberated into the cell-extract that was found between batches of 48 hr cultures of bacteria disintegrated at different times without major changes in the proportion of unbroken cells.

The purified protease of \textit{B. melaninogenicus} was active against a number of substrates including azocoll, azocasein, casein and N,-N-dimethyl casein, and had no glycosidase, lipase, collagenase or hemagglutinating activities.

The purified protease was proven to be similar to the crude enzyme in cell-extract with respect to oxygen sensitivity, reversible inhibition by HgCl$_2$, and irreversible inactivation by the alkylating agents iodoacetamide and iodoacetic acids. EDTA and cations (Ca$^{2+}$) had no effect on the purified protease. The pH optimum was found to be at pH 7.0 with higher activity at alkaline pH values than at acid pH. The serine inhibitors PMSF and TPCK had no effect on the purified protease, therefore, the cellular protease of strain 2D of \textit{B. melaninogenicus} appears to be a sulfhydryl enzyme. The protease had certain stability aspects in common with papain enzyme, which is a typical sulfhydryl enzyme that has been extensively studied. Resistance to the organic solvent dimethylsulfoxide (DMSO) and to 8 M urea, and sensitivity to guanidine hydrochloride are similar in both enzymes (88,190). The increase in proteolytic activity caused by high concentrations of urea in the assay mixture could be explained by the possibility of that urea modifies the substrate or makes it more accessible to the enzyme. It might also be due to the unmasking of active sites which
were blocked by other components of the enzyme complex.

The results have shown that the characteristics exhibited by the crude enzyme in the cell-extract were identical with those of the purified enzyme which strongly suggests that one cellular protease was produced by *B. melaninogenicus* which was liberated from the cells by disintegration.

Chemical analysis of the purified protease preparation demonstrated the presence of carbohydrate, lipid and protein. The function of the carbohydrate and lipid is not known. The characterization of the carbohydrate and lipid moieties of the purified protease revealed the presence of components which were previously reported to be found in isolated lipopolysaccharide from the outer membrane complex of *B. melaninogenicus* ss. *asaccharolyticus* (142). Glucose, galactose and glucosamine were reported as the predominant sugars in the LPS preparations. The predominance of palmitic, palmitoleic and stearic acid was also reported as well as the presence of two unknown fatty acids that were assumed to be cyclic or odd chain fatty acids (142).

Attempts were made to determine if the protease of *B. melaninogenicus* was located in the periplasmic space by osmotic shock and treatment with polymyxin B. Only 10-12% of the protease activity was liberated, and the protease could not, therefore, be classified as periplasmic.

Ingram *et al.* found that some of the alkaline phosphatase of *Pseudomonas aeruginosa* was located exterior to the outer tripartite layer and was complexed with lipopolysaccharide which was also released during secretion (96). They hypothesized that mechanical shearing forces associated with growth lead to a stripping of LPS-alkaline phosphatase aggregates on the external wall surface.
Various enzymes found outside of the cytoplasmic membrane were not released into the medium, but were bound to the outer membrane of the cell envelope which contains charged moieties. A molecule might remain bound to the cell either in association with mucopeptide (194), with various components of the periplasmic space (30) or with lipopolysaccharide of the outer membrane (96), depending on the nature of the enzyme such as the amount of hydrophobocity and number of charged groups.

The secretion of a protease enzyme by *Micrococcus sodonensis* was found to be dependent on the co-secretion of at least one of several polysaccharides, also elaborated by these cells (19). Regnier and Thang (174) reported that at least 50% of the protease activity found in *E. coli* is associated with the membrane.

There are very few reports on extracellular enzymes of Gram-negative anaerobic organisms. Blackburn reported that a protease was liberated into the growth medium by exponentially growing cultures of *Bacteroides amylophilus* strain H18 (13).

In this study, evidence was obtained suggesting that the extracellular protease of *B. melaninogenicus* is the cell-bound protease which was probably liberated from the cells by release of outer membrane during cell growth or cell lysis. The optimum pH for the extracellular protease of 2D with azo-casein as substrate was found to be between 7.0 and 7.5 which is similar to that of the cell associated protease. The extracellular protease of *B. melaninogenicus* was also classified as a sulfhydryl enzyme due to its dependence on reducing agents and sensitivity to SH-inactivating agents. It was also found that the extracellular protease of 2D was unstable and subject to autodigestion. From the studies on the purification of the
cell-free protease of 2D, it was concluded that the extracellular protease of *B. melaninogenicus* was of high molecular weight and presumably membrane or particle bound.

The metal chelating agent EDTA caused 60% inhibition of the extracellular protease of *B. melaninogenicus* but did not affect the cell-bound protease. The difference might be due to differences in the complex in which the enzymes are bound. Generally, cations can be required for activity or for protection of the enzyme against autodigestion. A proteolytic enzyme isolated from *Clostridium botulinum* type B which was active only when in the reduced state was reported to be inactivated by EDTA (11).

The small proportion of protease located in the culture supernatant during logarithmic growth and the increase in proportion only during stationary phase made it likely that the extracellular enzyme was liberated because of release of outer membrane during cell growth or autolysis of only a few bacterial cells.

Generally, the purification of extracellular enzymes from bacteria involves some special problems. The enzyme concentration in the growth medium is usually low and large quantities of salts and extraneous compounds must be removed. Several bacterial proteinases have been partly purified but only a few of these have been isolated in a pure state and characterized in some detail.

The protease of the dialyzed and concentrated culture supernatant of 2D was partially purified by gel filtration through Sephadex G-100. The protease was eluted as a single peak at the void volume of the column with a final recovery of only 46%. Gel electrophoresis of the partially purified protease revealed many protein bands; and further attempts to
disaggregate and separate the protease in a purified form were unsuccessful.

The possible role of proteolytic activity in cell division has been suggested by various studies. Kogoma and Nishi (111) had found an increase at division followed by a decrease of an intracellular proteinase in synchronously dividing cells of *Escherichia coli*. Burdett and Murray (24) presented electron microscopic evidence of localized hydrolytic activity at the site of septum formation in *E. coli*. It has been shown that the autolysin of *Streptococcus faecalis* is present in an inactive form in the cell wall but is activated by a neutral proteinase; and that the active form of the autolysin is associated with recently synthesized wall (193).

The major function of extracellular proteinases and other hydrolytic enzymes, is most reasonably a nutritional one which evolved to allow the microorganism growing in its natural environment to utilize complex substrates as sources of nutrients.

The results presented in this study suggest that the protease of strain 2D of *B. melaninogenicus* can be classified as a sulfhydryl enzyme. The observation that the specificity exhibited by the crude enzyme in the cell-extract was identical with that of the soluble enzyme strongly suggests that one cellular protease was produced by *B. melaninogenicus* which was liberated from the cells by release of outer membrane during cell growth.
V. LITERATURE CITED


