Genetic studies on antibiotic resistance in 
Bordetella pertussis

Richard George Quist
The University of Montana

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GENETIC STUDIES ON ANTIBIOTIC RESISTANCE
IN BORDETELLA PERTUSSIS

by

Richard G. Quist

B.S. Syracuse University, 1962

Presented in partial fulfillment of the requirements for the degree of

Master of Science

UNIVERSITY OF MONTANA

1966

Approved by:

[Signatures]

Chairman, Board of Examiners

Dean, Graduate School

AUG 23 1966

Date
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CHAPTER I

INTRODUCTION

*Bordetella pertussis*, the etiological agent of whooping cough, was first demonstrated in 1906 by Bordet and Gengou at the Pasteur Institute. The organism was isolated in 1900 but could not be cultured on the media in use at that time. Development of the Bordet-Gengou potato extract-blood agar medium (Bordet and Gengou, 1906) provided a partial solution to the culturing problem. However, a fluid medium was needed for large scale cultivation systems used for producing cells employed in various studies and vaccine production.

From this period much of the research on *B. pertussis* was centered around developing various media and determining growth requirements of the organism. The medium of choice would be chemically defined and would support the growth of and maintain the organism in its virulent form. Hornibrook (1940) developed a synthetic medium replacing blood with starch and found that nicotinamide or nicotinic acid could be substituted for yeast hydrolysate and was a growth factor for the organism. A semi-synthetic fluid medium used for cultivating cells to be employed in the preparation of vaccine was then developed (Cohen and Wheeler, 1946). This medium also substituted starch for blood as did the semi-synthetic medium of Pollock (1945). In a partially defined liquid medium which also substituted starch for blood (Ungar et al., 1950), recently isolated virulent strains of *B. pertussis* were found to
be more exacting in their growth requirements and conditions of growth than avirulent strains.

Various media replacing blood with activated charcoal (Ensminger et al., 1934; Ungar et al., 1950; Mishulow et al., 1953; Abe, 1953; Turner, 1961; Rauch and Pickett, 1961; Holt, 1962) and glass beads or anion exchange resins (Kuwajima et al., 1958) were also developed. The substitution of starch or charcoal for blood made it apparent that blood played no nutritive role in the growth of *B. pertussis* but rather served to absorb toxic materials from the medium (Pollock, 1949; Dawson et al., 1950). Poor growth of *B. pertussis* in various media and the necessity of incorporating starch, charcoal or blood, supposedly to absorb inhibitory products, led to the identification of toxic materials produced during growth or in preparation of the media. An oleic acid-like substance (Pollock, 1949; Kuwajima et al., 1958), autoclaved cysteine, sulfides and colloidal sulfur (Rowatt, 1957; Schuhardt et al., 1952) and a peroxide-like substance (Gilder and Granick, 1948; Rowatt, 1957; Barry et al., 1956) have been implicated as growth inhibitors.

Culture conditions for *B. pertussis* vary with the medium, the optimum pH being between 6.8 and 7.2, with good aeration generally resulting in increased growth in fluid media. Solid media cultures incubated at 37°C for 48 to 96 hours have a tendency to dry and since this organism is very sensitive to drying conditions a method of keeping the agar surface moist should result in improved growth. A procedure for growing cells under a 0.4 mm fluid layer on an agar base (Holt, 1962) gave up to $1.2 \times 10^{11}$ organisms per ml and allowed reuse of the agar base up to three times. This culturing system yielded cells which
retained their normal, minute coccobacillary form and were fully anti-
genetic in terms of the mouse protection test.

Although culturing *B. pertussis* has been a problem for investi-
gators, the only essential nutrients required for growth are amino acids
and the growth factor, nicotinic acid (Jebb and Tomlinson, 1955; Turner,
1961). Nicotinic acid can replace cozymase or yeast dialysate and was
found to be required by all species of *Bordetella* (Fukimi et al., 1953;
Jebb and Tomlinson, 1955; Proom, 1955). Thus, it appeared that *B. per-
tussis* could synthesize Coenzyme I from nicotinic acid (Hornibrook,
1940; James, 1949).

Since *B. pertussis* does not utilize common sugars, the amino
acids must serve as carbon, nitrogen and energy sources. The two unde-
finite solid media, Bordet-Gengou potato extract-blood agar and charcoal
agar composed of heart infusion broth and yeast extract, apparently are
rich in the amino acids necessary for good growth of the organism.
Most fluid media contain various amino acids, glutamic acid having the
most important influence on growth. Glutamic acid is actively used by
resting cell suspensions (Jebb and Tomlinson, 1951; Abe, 1953; Meyer
and Cameron, 1957) and, of all amino acids used, served to support the
best growth in partially defined media (James, 1949; Dawson et al.,
acids which stimulate growth but are not essential are aspartic acid,
alanine and serine. In an incubation period of 12 to 15 days, virulent
and avirulent strains of *B. pertussis* completely remove glutamic acid,
aspartic acid, serine, glycine and proline from the medium while ala-
nine and threonine are not completely used (James, 1949). Rowatt (1957)
noted that starch, niacinamide and cystine or cysteine were essential for growth and that the overall yield of cells was proportional, within wide limits, to the glutamic acid concentration. Apparently, a correlation exists between the growth phase of the organism and the preference for amino acids in a partially defined medium (Vajdic et al., 1964). Cells in the exponential phase utilize glutamic acid and proline to the greatest extent, stationary phase cells use alanine, cystine, glycine and serine while cells in the decelerating phase showed a preference for histidine.

Cystine and cysteine may serve as sulfur sources for culturing B. pertussis in partially defined medium (Fukimi et al., 1953; Abe, 1953; Imamura, 1952; Proom, 1955; Jebb and Tomlinson, 1957). The sulfur-containing peptide, glutathione, was found to be a satisfactory sulfur source (Abe, 1953; Jebb and Tomlinson, 1957). A casein hydrolysate broth containing glutathione at a concentration of 0.001% was used for the production of B. pertussis vaccine (Wilson, 1965) and was included along with 0.0012% L-cystine in a similar medium (Farrell and Taylor, 1945). L-cystine and glutathione served as sulfur sources in a partially defined medium containing 19 amino acids, inorganic salts, nucleic acid derivatives and growth factors including liver coenzyme concentrate (Wilson, 1963). This medium was labeled as chemically defined, although the liver coenzyme concentrate incorporated at a concentration of 100 mg/liter is not chemically defined. The presence of coenzymes in this medium permitted serial transfers of cells, which is an important feature of a useful fluid medium.
A problem which has not been overcome in culturing \textit{B. pertussis} is the conversion of virulent, freshly isolated strains of avirulent strains having altered morphological and metabolic characteristics. This degradation process is most active in synthetic media but may occur on certain blood media. Bordet-Gengou agar incorporating sheep and human blood produces \textit{B. pertussis} cells having unchanged morphology and virulence after 39 generations (Toomey and Takacs, 1938). However, cells cultured in the presence of horse blood retained the typical morphology but lost their virulence and, in all blood media tested, the organism retained its ability to produce specific agglutinins. Phases of the degradation process were classified as states I, II, III and IV (Leslie and Gardner, 1931) leading to considerable confusion and the suggestion that virulent and avirulent groups should replace the arbitrarily defined phases (Toomey, 1938; Standfast, 1951; Burrows, 1959).

Peculiarities in nutritional requirements and the need for a suitable medium for vaccine production have stimulated studies on nutritional characteristics and metabolism of \textit{B. pertussis} while the development of antibiotic resistance has received little attention. Although the vaccine for whooping cough is used widely and several antibiotics are available for treating cases, the mortality in young children is significantly high. Treatment of any bacterial infection with antibiotics usually results in the development of resistant organisms regardless of whether the antibiotic has bacteriostatic or bactericidal activity. Streptomycin was used in the treatment of whooping cough prior to the development of chloramphenicol, tetracycline and chlor-tetracycline, with chloramphenicol becoming the antibiotic of choice.
Although drug resistance in *B. pertussis* has not been studied to any extent, drug resistance and its development has been studied extensively in various other organisms, especially *Escherichia coli*. Studies of drug resistance are no longer carried out solely for the purpose of improving public health and combating infection but rather as an aid in studying hereditary mechanisms of microorganisms. A procedure designed to show that resistance to streptomycin and penicillin in *Staphylococcus aureus* originated independently of the antibiotics by mutation and was not induced by some interaction between the antibiotic and bacteria helped to dispel the idea held by many investigators that the development of antibiotic resistance was simply an adaptation process (Demerec, 1948). Later, streptomycin resistance was shown to be a genetically recessive mutation (Lederberg, 1951). *B. pertussis* having high resistance to streptomycin was shown to exhibit traits characteristic of mutants (Alexander and Redman, 1949). These strains occurred irregularly in different independent cultures of the same population size and strain and were therefore independent of the action of streptomycin. The resistance trait was maintained through subculture in the absence of streptomycin and the rate of occurrence of resistant strains was consistent with mutation frequency. These, along with numerous other studies relating drug resistance and mutation, stimulated interest in studying the hereditary aspect of drug resistance.

Drug resistant strains of an organism can be obtained by culturing the organism in medium containing an antibiotic in sufficient concentration to permit growth only of resistant cells. Another method is to subculture the organism in medium in which the antibiotic concentration
is increased in a stepwise manner. In the latter procedure, media containing the antibiotic can be inoculated or the antibiotic can be added to a culture of actively metabolizing cells. The stepwise procedure has been used to develop strains of *B. pertussis* resistant to streptomycin (Smolens and Vogt, 1953; Bochagova, 1960), chlorotetracycline (Bochagova, 1960) and oxytetracycline (Smolens and Vogt, 1953; Bochagova, 1960). Attempts to develop chloramphenicol-resistant strains of *B. pertussis* by the stepwise procedure were not successful (Gray, 1950; Smolens and Vogt, 1953). In the most recent study on drug resistance in *B. pertussis*, a streptomycin-sensitive strain was rendered streptomycin-resistant by exposure to DNA extracted from the resistant variant of the same strain (Branefors, 1964). This work also indicated that streptomycin resistance is related to independence of starch in the growth medium, since transformed cells were also starch-independent.

Multiple drug resistance has not been reported in *B. pertussis* but has been shown to occur in *E. coli*, *Shigella*, *Vibrio comma*, *Serratia marcescens*, *Salmonella* and other organisms (Watanabe, 1963). Multiple drug resistance studies involving resistance to streptomycin, chloramphenicol, tetracycline and sulfanilamide, together or in various combinations have been stimulated by the finding that these multiple resistance factors are carried and transferred by an episome (Watanabe, 1963). Since a variety of organisms possess multiple drug resistance factors the possibility exists that multiple drug resistant strains of *B. pertussis* could be obtained either through direct mutation, or by developing resistance to each antibiotic in a stepwise manner.
Streptomycin resistance was transferred from resistant to sensitive cells in the first conclusive positive results obtained in an attempt to detect recombination in *E. coli* K-12 (Lederberg and Tatum, 1946; Tatum and Lederberg, 1947). Although streptomycin resistance was not a selected marker, its transfer during recombination was highly significant to drug resistance studies. Later, Lederberg (1950) demonstrated that growth inhibitors could be used efficiently for selective markers in recombination experiments by obtaining *E. coli* K-12 resistant to both azide and streptomycin following the plating of single resistant mixed cultures on media containing both azide and streptomycin. The infective heredity of episome-carried multiple drug resistance reviewed by Watanabe (1963) has been shown to involve a transfer of the resistance factors without the need for host chromosome transfer and is not necessarily restricted to cells of the same genus. Today, drug resistance transfer studies are centered around multiple resistance while the transfer of single integrated resistance factors is of secondary importance.

The development of antibiotic resistance or dependence in an organism may be accompanied by alterations in metabolism or composition of cellular components. An unidentified compound present in the cell walls of streptomycin-dependent strains of *Staphylococcus aureus*, *Bacillus cereus* and *Bacillus megaterium* has been reported (Lawrence and Scruggs, 1966). Recent evidence indicates that streptomycin is bound to the 30s ribosome in streptomycin-dependent *E. coli* (Spotts, 1966), and that growth in the absence of the antibiotic results in a change in the ribosomes, which alters but does not destroy their activity in
protein synthesis. Altered growth requirements which accompanied chloramphenicol resistance in *S. aureus* were a decreased requirement for niacin, thiamin and arginine (Ramsey and Padron, 1954). Altered nutritional requirements related to dihydrostreptomycin resistance have been used to facilitate the isolation of auxotrophic mutants of *Pseudomonas* (Ishida et al., 1966). The only evidence for alteration of metabolism in drug resistant strains of *B. pertussis* is that streptomycin-resistant strains do not require starch for growth and serial transfer in fluid medium (Branefors, 1964).

The frequency of spontaneous mutation to streptomycin resistance in *B. pertussis* is approximately $1 \times 10^{-10}$ (Alexander and Redman, 1949). This low spontaneous mutation rate along with problems generally encountered in growing the organism suggest that a means of increasing the mutation frequency is necessary to facilitate the study of streptomycin resistance and subsequent metabolic alterations in this organism. Of the various chemical and physical agents available for inducing mutations in bacteria, ultraviolet irradiation has been used most frequently, either alone or in conjunction with chemical agents.

The association of the maximum mutagenic efficiency and the maximum ultraviolet absorbance of DNA at a wavelength of 2600 Angstroms led to the suggestion that the basic effect of UV on a bacterial cell is the immediate and specific inhibition of DNA synthesis, all other effects being secondary (Kelner et al., 1955). Numerous studies were undertaken and the relationship between DNA and UV irradiation was firmly established. An excellent and comprehensive review on effects of UV irradiation on nucleic acids and their derivatives was published.
by Shugar (1960). Recently, investigations have been carried out to show a specific effect of UV irradiation on the DNA molecule, centering around the formation of Thymine dimers. UV irradiation of DNA in solution results in irreversible hydrogen bond breakage and the formation of formamide and heat stable linkages between two complementary DNA strands (Marmur and Grossman, 1961). This led to the suggestion that cross links may have been due to the formation of dimers of thymine. Other studies led to the isolation of a product identified as a thymine dimer from thymine irradiated in frozen solution (Beukers and Berends, 1960). Evidence that UV-induced thymine dimers in DNA cause biological damage was gained through studies on the loss of transforming activity by UV-irradiated transforming DNA and by investigations centered around the photoreactivation phenomenon. The formation of thymine dimers between adjacent thymines probably interferes with normal base pairing during DNA replication and the occurrence of this interference could provide a relationship between UV-induced mutation rates and the number of adjacent thymines within the locus being investigated (Bollum and Setlow, 1963).

Ultraviolet irradiation has been used alone or in conjunction with various chemicals to induce mutations in bacteria. Auxotrophic mutants of *E. megaterium* have been obtained by growing UV-irradiated cells in the presence of 8-azaguanine (Wachsman and Mangalo, 1962) and auxotrophic mutants of *Pseudomonas* were isolated by culturing UV-irradiated survivors in the presence of dihydrostreptomycin and determining the nutritional requirements of survivors (Ishida *et al.*, 1966). Auxotrophic mutants of *E. coli* have been recovered from UV-irradiated
cultures (Lederberg, 1952), and UV irradiation alone was used to study mutation to streptomycin resistance and prototrophy in *Neisseria meningitidis* and *E. coli* (Lie, 1965). In the last experiment, UV irradiation had a strong mutagenic effect in *E. coli* in the two loci studied but had a very low mutagenic effect in meningococci. However, the mutagenic effect was not investigated at a survival of less than 1.2 percent. To our knowledge, the inactivating and mutagenic effect of UV irradiation on *B. pertussis* has not been investigated.
CHAPTER II

STATEMENT OF PROBLEM

This investigation was undertaken primarily to develop strains of *Bordetella pertussis* having genetic markers which could be used in future studies for elucidating hereditary mechanisms of the organism.

Experiments were designed to investigate various procedures including ultraviolet irradiation for developing stable antibiotic resistant strains and to test the possibility of using antibiotic resistance development to facilitate selection of strains having altered growth requirements.
CHAPTER III

MATERIALS AND METHODS

I. GENERAL METHODS AND MATERIALS

(1) Organisms employed:

Six laboratory strains of *Bordetella pertussis* were used throughout this work.

Strains 2 and 4, originally designated 5375L45 and 5373L-2, respectively, were donated by Dr. Munoz of the Rocky Mountain Laboratory, Hamilton, Montana. Strain 11, originally number 3747, was donated by Dr. Andersen of the Staten Serum Institute. Strain 16, originally number 68-72, was obtained from Dr. Laughner at UCLA. Strain 23, originally number 2, was donated by Dr. Ulrich of the Mayo Clinic, and strain 63, originally number 99, was obtained from Ohio State University.

(2) Culture media:

The charcoal agar of Rauch and Pickett (1961) was used as the solid medium throughout these experiments. This medium had the following composition:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Source</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart infusion broth</td>
<td>Difco</td>
<td>25.0 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>Difco</td>
<td>3.5 g</td>
</tr>
<tr>
<td>Soluble starch</td>
<td>Fisher</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>Difco</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Powdered charcoal</td>
<td>Fisher</td>
<td>4.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td></td>
<td>1 liter</td>
</tr>
<tr>
<td>pH adjusted to 7.2 with 2N NaOH</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Medium CM-211, a modification of the fluid medium of Cohen and Wheeler (1966), was used to obtain cells for UV irradiation, some antibiotic testing and antibiotic resistance development. This medium had the following composition:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Source</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casamino acids</td>
<td>Difco</td>
<td>14.0 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>Difco</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Niacin</td>
<td>Nutritional Biochem.</td>
<td>0.02 g</td>
</tr>
<tr>
<td>Glutathione (reduced)</td>
<td>Sigma</td>
<td>0.01 g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>Baker, Reagent</td>
<td>0.5 g</td>
</tr>
<tr>
<td>MgCl$_2$.6H$_2$O, 25% soln.</td>
<td>Merck, Reagent</td>
<td>0.4 ml</td>
</tr>
<tr>
<td>CaCl$_2$, 1% soln.</td>
<td>Baker, Reagent</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>FeSO$_4$.7H$_2$O, 1% soln.</td>
<td>Fisher, Reagent</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>CuSO$_4$.5H$_2$O, 0.1% soln.</td>
<td>Mallinckrodt, Reagent</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>B-nicotinamide adenine-dinucleotide, Grade III, yeast</td>
<td>Sigma</td>
<td>7.8 mg</td>
</tr>
<tr>
<td>Distilled water to 1 liter</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The starch was dissolved in boiling distilled water, added to the medium and pH was adjusted to 6.8 with 2.0 N NaOH. B-NAD, which was found to improve growth upon serial transfer in this medium, was suspended in 20 ml of 0.05 M phosphate buffer at pH 7.0. This solution was filter-sterilized using a Millipore filter disc having a pore size of 0.25 microns, and stored at 4°C for a maximum of one week. Prior to inoculation, 0.5 ml of this solution was added to 25 ml of the CM-211 medium. The medium was dispensed in 25 and 50 ml volumes in 250 ml erlenmeyer flasks and all fluid medium cultures were incubated on a reciprocal shaker at 37°C.

The ability of the partially defined medium of Wilson (1963) to support growth and serial transfer of typical strains 23, 63 and 4 was determined in preliminary experiments. This medium had the following composition:
### Inorganic salts

<table>
<thead>
<tr>
<th>Salt</th>
<th>Supplier</th>
<th>mg/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>Fisher, Reagent</td>
<td>2500</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>Baker, Reagent</td>
<td>500</td>
</tr>
<tr>
<td>KCl</td>
<td>Fisher, Reagent</td>
<td>200</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>Merck, Reagent</td>
<td>100</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Baker, Reagent</td>
<td>20</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>Fisher, Reagent</td>
<td>10</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>Mallinckrodt, Reagent</td>
<td>5</td>
</tr>
</tbody>
</table>

### Amino acids

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Supplier</th>
<th>mg/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-alanine</td>
<td>Nutritional Biochem.</td>
<td>100</td>
</tr>
<tr>
<td>L-arginine</td>
<td>Nutritional Biochem.</td>
<td>40</td>
</tr>
<tr>
<td>DL-aspartic acid</td>
<td>Sigma</td>
<td>130</td>
</tr>
<tr>
<td>L-cystine</td>
<td>Fisher</td>
<td>10</td>
</tr>
<tr>
<td>L-glutamic acid</td>
<td>Nutritional Biochem.</td>
<td>155</td>
</tr>
<tr>
<td>Glycine</td>
<td>Nutritional Biochem.</td>
<td>50</td>
</tr>
<tr>
<td>DL-histidine</td>
<td>Sigma</td>
<td>50</td>
</tr>
<tr>
<td>L-hydroxyproline</td>
<td>Sigma</td>
<td>20</td>
</tr>
<tr>
<td>DL-isoleucine</td>
<td>Sigma</td>
<td>120</td>
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<tr>
<td>DL-leucine</td>
<td>Nutritional Biochem.</td>
<td>210</td>
</tr>
<tr>
<td>L-lysine</td>
<td>Nutritional Biochem.</td>
<td>70</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>Nutritional Biochem.</td>
<td>60</td>
</tr>
<tr>
<td>DL-phenylalanine</td>
<td>Sigma</td>
<td>100</td>
</tr>
<tr>
<td>L-proline</td>
<td>Nutritional Biochem.</td>
<td>60</td>
</tr>
<tr>
<td>DL-serine</td>
<td>Nutritional Biochem.</td>
<td>130</td>
</tr>
<tr>
<td>DL-threonine</td>
<td>Nutritional Biochem.</td>
<td>80</td>
</tr>
<tr>
<td>DL-tryptophane</td>
<td>Nutritional Biochem.</td>
<td>30</td>
</tr>
<tr>
<td>L-tyrosine</td>
<td>Nutritional Biochem.</td>
<td>40</td>
</tr>
<tr>
<td>DL-valine</td>
<td>Sigma</td>
<td>120</td>
</tr>
</tbody>
</table>

### Nucleic acid derivatives

<table>
<thead>
<tr>
<th>Nucleic Acid</th>
<th>Supplier</th>
<th>mg/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>Nutritional Biochem.</td>
<td>10</td>
</tr>
<tr>
<td>Guanine</td>
<td>Nutritional Biochem.</td>
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</tr>
<tr>
<td>Hypoxanthine</td>
<td>Sigma</td>
<td>0.3</td>
</tr>
<tr>
<td>Thymine</td>
<td>Sigma</td>
<td>0.3</td>
</tr>
<tr>
<td>Uracil</td>
<td>Nutritional Biochem.</td>
<td>0.3</td>
</tr>
<tr>
<td>Xanthine</td>
<td>Sigma</td>
<td>0.3</td>
</tr>
<tr>
<td>2-deoxy-D-ribose</td>
<td>Sigma</td>
<td>0.5</td>
</tr>
</tbody>
</table>

### Other constituents

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Supplier</th>
<th>mg/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-glutamine</td>
<td>Sigma</td>
<td>100</td>
</tr>
<tr>
<td>Glutathione (reduced)</td>
<td>Sigma</td>
<td>25</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>Sigma</td>
<td>5</td>
</tr>
<tr>
<td>Niacin</td>
<td>Nutritional Biochem.</td>
<td>1</td>
</tr>
<tr>
<td>Adenosine triphosphate</td>
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<td>10</td>
</tr>
<tr>
<td>Liver coenzyme concentrate</td>
<td>Sigma</td>
<td>100</td>
</tr>
<tr>
<td>Tris (hydroxymethyl)-aminomethane</td>
<td>Fisher</td>
<td>6075</td>
</tr>
<tr>
<td>Powdered charcoal, Norit A</td>
<td>Fisher</td>
<td>200</td>
</tr>
<tr>
<td>Distilled water</td>
<td></td>
<td>1 liter</td>
</tr>
</tbody>
</table>
The partially defined medium was modified in this work in order to have a chemically defined medium for the determination of altered NAD requirements of various strains. This chemically defined medium had the following composition:

<table>
<thead>
<tr>
<th>Inorganic salts</th>
<th>mg/liter</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>2500</td>
<td>Fisher, Reagent</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>2200</td>
<td>Baker, Reagent</td>
</tr>
<tr>
<td>KCl</td>
<td>200</td>
<td>Fisher, Reagent</td>
</tr>
<tr>
<td>MgCl₂.6H₂O</td>
<td>100</td>
<td>Merck, Reagent</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>20</td>
<td>Baker, Reagent</td>
</tr>
<tr>
<td>FeSO₄.7H₂O</td>
<td>10</td>
<td>Fisher, Reagent</td>
</tr>
<tr>
<td>CuSO₄.5H₂O</td>
<td>5</td>
<td>Mallinckrodt, Reagent</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Amino acids</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>L-glutamic acid</td>
<td>155</td>
<td>Nutritional Biochem.</td>
</tr>
<tr>
<td>L-cystine</td>
<td>10</td>
<td>Fisher</td>
</tr>
<tr>
<td>DL-aspartic acid</td>
<td>130</td>
<td>Sigma</td>
</tr>
<tr>
<td>DL-serine</td>
<td>130</td>
<td>Nutritional Biochem.</td>
</tr>
<tr>
<td>Glycine</td>
<td>50</td>
<td>Nutritional Biochem.</td>
</tr>
<tr>
<td>DL-alanine</td>
<td>100</td>
<td>Nutritional Biochem.</td>
</tr>
<tr>
<td>DL-proline</td>
<td>120</td>
<td>Nutritional Biochem.</td>
</tr>
<tr>
<td>DL-threonine</td>
<td>80</td>
<td>Nutritional Biochem.</td>
</tr>
<tr>
<td>DL-histidine</td>
<td>50</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nucleic acid derivatives</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Guanine</td>
<td>0.3</td>
<td>Nutritional Biochem.</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>0.3</td>
<td>Sigma</td>
</tr>
<tr>
<td>Xanthine</td>
<td>0.3</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other constituents</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>L-glutamine</td>
<td>100</td>
<td>Sigma</td>
</tr>
<tr>
<td>Glutathione (reduced)</td>
<td>25</td>
<td>Sigma</td>
</tr>
<tr>
<td>ATP</td>
<td>10</td>
<td>Nutritional Biochem.</td>
</tr>
<tr>
<td>B-NAD, Grade III, yeast</td>
<td>7.8</td>
<td>Sigma</td>
</tr>
<tr>
<td>Tris</td>
<td>6075</td>
<td>Fisher</td>
</tr>
<tr>
<td>Powdered charcoal, Norit A</td>
<td>200</td>
<td>Fisher</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1 liter</td>
<td></td>
</tr>
</tbody>
</table>

The chemically defined medium was prepared by dissolving the amino acids in 500 ml of distilled water by heating to 80°C in a water bath. The solution was cooled and glutathione and L-glutamine were added. A 15 ml volume of a solution containing 0.002% each of xanthine, guanine...
and hypoxanthine was added next. Five ml of a 0.1% solution of CuSO₄ were added along with 200 ml of distilled water containing the dissolved inorganic salts. Tris, ATP and NAD were then added, the volume was brought to 1 liter and the pH was adjusted to 7.1 with 10 N HCl. The medium was filter-sterilized using a Millipore filter disc having a pore size of 0.25 microns.

(3) Antibiotics used:

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Stock soln. conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptomycin (Sm)</td>
<td>Sigma 10 and 50 mg/ml</td>
</tr>
<tr>
<td>Chloramphenicol (Cm)</td>
<td>Parke Davis 1 and 10 mg/ml</td>
</tr>
<tr>
<td>Tetracycline (Tc)</td>
<td>Lederle 6 mg/ml</td>
</tr>
<tr>
<td>Chlortetracycline (Cl)</td>
<td>Lederle 6 mg/ml</td>
</tr>
</tbody>
</table>

The antibiotics were dissolved in normal saline and filter-sterilized using a Millipore filter disc having a pore size of 0.25 microns. Streptomycin and chloramphenicol stock solutions were stored at 4°C for a maximum of 2 weeks while tetracycline and chlortetracycline solutions were prepared immediately before use.

(4) Stock cultures:

Cultures of the original strains were obtained from lyophilized stock cultures by transferring cells to charcoal agar plates. Once purity was established the cultures were maintained on charcoal agar slants in 20 x 125 mm screw-cap culture tubes. Incubation time was 24-48 hours after which the parent strains were refrigerated 3 weeks before transfer.

(5) Determination of antibiotic resistance and sensitivity:

For determination of antibiotic resistance, stock antibiotic solution was added to freshly prepared charcoal agar which had been
cooled to 45°C. Seven ml portions were added to sterile screw-cap culture tubes and slanted. A 24-48 hour slant of the strain to be tested, growing on charcoal agar without antibiotic was washed with 0.5 ml of CM-211 medium, and 0.1 ml of this cell suspension was transferred to the antibiotic slant. All slants were incubated in a nearly horizontal position with the space between the bottom of the cap and the neck of the tube supported by a glass rod having a 1 cm outside diameter. This position for incubation is critical in obtaining uniform growth and reproducible results as will be discussed later. Incubation time was 72-96 hours at 37°C.

The minimum antibiotic concentrations used for resistance and sensitivity determinations were 40 ug/ml for streptomycin, 80 ug/ml for chloramphenicol and tetracycline and 120 ug/ml for chlortetracycline. Growth of the parent strains 63, 23 and 4 did not occur at these concentrations in CM-211 medium or on charcoal agar during an incubation period of 96 hours. Since some antibiotics are not completely stable in solution at 37°C over a 96-hour period, this factor was taken into consideration in the concentration selection.

(6) Determination of starch requirement in CM-211 medium:

Organisms to be tested were grown in CM-211 medium with starch for 24 hours. Ten ml of this culture were centrifuged and washed two times with phosphate buffer. The pellet was resuspended in 1 ml of CM-211 medium without starch and 0.5 ml was transferred to 25 ml of CM-211 medium without starch. The cultures were incubated at 37°C for 24-48 hours. If growth was apparent at this time, 1.0 ml was transferred to fresh CM-211 medium without starch and incubated 48 hours to
determine whether or not the strain required starch for growth following serial transfer in this medium.

(7) Determination of NAD requirement in chemically defined medium:

Charcoal agar slants of the organism to be tested were incubated 24 hours and washed with 2 ml of phosphate buffer. The cell suspension was added to 5 ml of sterile buffer in a screw-cap centrifuge tube, centrifuged and washed two times. The cell pellet was resuspended in 0.5 ml buffer and 0.1 ml was transferred to 20 ml of chemically defined medium in 125 ml erlenmeyer flasks. Chemically defined medium with and without NAD was used. After 72 hours incubation, growth was recorded and 0.5 ml of each culture showing growth was transferred to 20 ml of fresh medium with and without NAD, respectively, to determine the NAD requirement for growth after serial transfer in the chemically defined medium. These cultures were also incubated 72 hours.

II. EXPERIMENTAL METHODS

(1) Ultraviolet irradiation inactivation of B. pertussis:

(a) Preparation of cells:

A loopful of cells grown for 24 hours on a charcoal agar slant was transferred to 25 ml of CM-211 medium and incubated 24 hours. One ml of this culture was transferred to 50 ml of fresh CM-211 medium, and after 18 hours incubation 40 ml were centrifuged for 20 minutes at 3000 RPM. The cell pellet was resuspended in 20 ml of phosphate buffer, vigorously agitated on a tube mixer and centrifuged. The washed pellet was resuspended in 5 ml of buffer and the optical density was adjusted to 0.20 at a wavelength of 575 mu with a Coleman Jr. Spectrophotometer.
(b) Irradiation procedure:

A 15 ml portion of the cell suspension was irradiated in a 100 ml beaker containing a 2.5 cm magnetic stirring bar used to keep the suspension homogeneous throughout the irradiation treatment. The ultraviolet light source was a new 15-watt General Electric germicidal lamp placed in an enclosure to exclude visible light. The distance from the center of the light source to the surface of the cell suspension was 44 cm. The UV source was allowed to equilibrate for 30 minutes prior to cell treatment.

(c) Sampling and incubation:

Samples of the cell suspension were removed prior to irradiation at 2.5, 5, 10, 15 and 20 minutes. Dilutions were made in phosphate buffer and, at the higher irradiation doses, samples were plated directly. A 0.2 ml sample of strains 4, 23 and 63 and 0.1 ml of strains 2, 11 and 16 were plated on charcoal agar which had been freshly prepared and poured no more than 1 hour before use. Throughout the irradiation treatment and incubation period, precautions were taken to prevent photoreactivation.

Plates were incubated in a 46 x 26 cm chromatography jar containing 1 liter of distilled water and covered with a glass top sealed with grease. The covered jar containing water was placed in a 37°C walk-in incubator 12 hours prior to plate incubation to insure a moist atmosphere and the correct temperature for incubation. All plates were incubated 96 hours under these conditions. Plates inoculated with strains 2, 11 and 16 were incubated 6 hours at 37°C with no humidity control, before incubation in the chromatography jar. Colony counts were made.
on plates containing between 30 and 300 colonies with a Quebec colony counter. The surviving fraction was determined by averaging the results of three determinations.

(2) **Selection for streptomycin resistant strains:**

(a) Spontaneous mutants:

Cell suspensions of strains 23, 63 and 4, grown in CM-211 medium or on charcoal agar slants were plated on charcoal agar containing 80-200 ug/ml streptomycin or on Bordet-Gengou agar containing human, sheep and defibrinated rabbit blood plus 80-200 ug/ml streptomycin. Heavy cell suspensions were also inoculated into CM-211 medium containing 80-200 ug/ml streptomycin. No attempt was made to obtain resistant strains by adding streptomycin to actively metabolizing cells, since it is well established that high level streptomycin resistance is due to a mutation and not an adaptation of the cell to the antibiotic.

(b) UV-induced streptomycin-resistant mutants:

Cell suspensions were prepared and irradiated as described previously in the inactivation studies. Following irradiation exposure times of 15 to 20 minutes, 5 ml of the cell suspension were transferred to 50 ml of CM-211 medium without streptomycin and incubated until the OD$_{575}$ was approximately 0.5. One ml portions of this culture were transferred to 25 ml of CM-211 medium containing 80-200 ug/ml streptomycin and incubated for a maximum of 96 hours. One ml of the culture growing in the presence of streptomycin was transferred to fresh CM-211 medium containing 200 ug/ml streptomycin, incubated 24-48 hours and a dilution of this culture was plated on charcoal agar. A single colony was transferred to a charcoal agar slant, tested for resistance and
maintained as a streptomycin-resistant strain. Each streptomycin-resistant strain developed by allowing full phenotypic expression of irradiated cells in CM-211 medium was obtained from a different cell suspension. This procedure insured that each strain resulted from a streptomycin resistance mutation and was not produced by the division of a single resistant cell in CM-211 medium without streptomycin.

(3) Relationship between irradiation exposure and development of streptomycin resistance:

Cell suspensions of strains 63, 41 and 23 were prepared for irradiation as previously described and irradiated 20, 15 and 10 minutes, respectively. At zero time and every 5 minutes thereafter, samples were removed, diluted and plated on charcoal agar. All plates were incubated for 96 hours under the special conditions described previously. A total of 60 colonies of each strain from each period of irradiation were transferred to charcoal agar slants, incubated for 48 hours and washed with 0.5 ml of CM-211 medium. A 0.1 ml portion of this cell suspension was transferred to a charcoal agar slant containing 80 ug/ml streptomycin and incubated 72 hours. Tubes showing growth were washed and 0.1 ml was transferred to a slant without streptomycin and incubated for 48 hours. Cells from this slant were used as the inoculum for slants containing 80 ug/ml chloramphenicol, 80 ug/ml tetracycline and 120 ug/ml chlortetracycline separately to determine resistance to these antibiotics in addition to streptomycin. Some of the streptomycin-resistant strains were transferred to CM-211 medium containing 200 ug/ml to provide a further check on their resistance.
Development of chloramphenicol-resistant strains:

Strains 63CmR and 4CmR were developed by irradiating a cell sus­
pension for 2 minutes using the procedure described previously. A 5 ml sample of the irradiated cell suspension was transferred to 100 ml of CM-211 medium in a 500 ml erlenmeyer flask. Following 48 hours incuba­
tion, 5 ml were transferred to 25 ml of CM-211 medium and incubated 12 hours. At this time 40 ug/ml chloramphenicol was added and the culture was incubated for 24 hours. One ml was then transferred from each of the 24-hour cultures to 25 ml of fresh CM-211 medium and 40 ug/ml chlor­
amphenicol was added following 12 and 18 hours incubation, giving a total concentration of 80 ug/ml chloramphenicol. Cells were grown for a total of 24 hours and the 1 ml transfer was repeated one more time. Dilutions of these cultures were plated on charcoal agar, a colony was transferred to a slant, tested for resistance and maintained as a strain resistant to at least 80 ug/ml chloramphenicol in Cm-211 medium or on charcoal agar. Strains 63CmRl and 4CmRl were developed using the same procedure except that cells were not irradiated.

Strains 23CmR and 23CmRl were developed by increasing the chlor­
amphenicol concentration in 10 ug/ml increments to a total of 80 ug/ml in CM-211 medium. Chloramphenicol was added to 18-hour cultures grow­
ing in CM-211 medium and after 6 hours incubation, 10 ml were centrifuged. The pellet was resuspended in 0.5 ml of CM-211 medium and 0.1 ml was transferred to a charcoal agar slant and incubated 24-48 hours. This transfer to charcoal agar after incubation in the presence of chloram­
phenicol was necessary since cells were inactivated by the antibiotic to such an extent that they would not grow in CM-211 medium upon serial
transfer. The slant was washed with 2 ml of CM-211 medium, and the cell suspension was transferred to 25 ml of fresh CM-211 medium. Following 12 hours incubation, 20 ug/ml chloramphenicol was added and the procedure repeated until growth was obtained at a chloramphenicol concentration of 80 ug/ml. CM-211 medium containing 80 ug/ml chloramphenicol was then inoculated to determine the resistance of the culture. A single colony was selected from a charcoal agar plate, tested on agar containing 80 ug/ml chloramphenicol and maintained as the resistant strain.

(5) Development of tetracycline-resistant strains:

Tetracycline-resistant strains 4TcR, 63TcR and 23TcR were developed by adding increasing concentrations of tetracycline to actively metabolizing cells in a procedure similar to that used for development of the chloramphenicol-resistant strains 23CmR and 23CmR1. Tetracycline concentrations were 10, 20, 40, 60 and 80 ug/ml. Single colonies were selected, tested for resistance and maintained as resistant strains.

(6) Selection for starch-independent strains:

The initial inoculum for starch-free CM-211 medium consisted of 10 ml of a 24-hour culture growing in 25 ml of CM-211 medium with starch. Following 24 hours incubation, 10 ml were again transferred to 25 ml of CM-211 without starch, incubated 24 hours and 5 ml were transferred to CM-211 medium without starch. Following 24-48 hours incubation, a charcoal agar slant was inoculated from this culture and 1 ml was used as the inoculum to determine if starch was required for growth and serial transfer in CM-211 medium minus starch.
(7) Transfer of resistance:

Cell suspensions of chloramphenicol-resistant, streptomycin-sensitive strain 63CmR and streptomycin-resistant, chloramphenicol-sensitive strain 63R were incubated together for various times in an attempt to obtain doubly resistant strains through transfer of resistance factors. Strain 63CmR was grown in 50 ml of CM-211 medium containing 80 ug/ml chloramphenicol, and 63R was grown in 50 ml of CM-211 medium containing 200 ug/ml streptomycin. The 18-hour cultures were centrifuged, washed once with buffer and resuspended in 5 ml of CM-211 medium. The cell suspensions were mixed, added to 15 ml of fresh CM-211 medium in a 250 ml erlenmeyer flask and incubated without shaking at 37°C. Following incubation for 2, 4 and 6 hours, the mixed cell suspensions were centrifuged and resuspended in 2 ml of CM-211 medium. Charcoal agar plates containing 80 ug/ml chloramphenicol and 200 ug/ml streptomycin were inoculated with 0.2 ml samples and incubated 48-96 hours in a sealed chromatography jar. Colonies which appeared were transferred to slants and tested for streptomycin and chloramphenicol resistance together and separately on charcoal agar and in CM-211 medium.
CHAPTER IV

RESULTS

(1) Growth in chemically defined medium:

The chemically defined medium supported the growth and serial transfer of the typical strains 23, 4 and 63. Growth was evident after an incubation period of 24 hours and reached a maximum at 72 hours with the OD$_{575}$ being approximately 0.15. These strains showed very limited growth when transferred from charcoal agar to the defined medium minus NAD and no growth was observed when serial transfers were made in the absence of NAD. The atypical strains 2, 11 and 16 showed abundant growth following a 24-hour incubation period in the defined medium with and without NAD.

The three typical strains also grew on a chemically defined agar medium prepared by adding a sterile solution of Oxoid lonagar #2 to the fluid medium. Colonies having a diameter of 1 mm appeared on these plates after an incubation period of 5-7 days. This chemically defined agar medium was not used for the determination of the NAD requirement of various strains of B. pertussis because of the extended incubation time required and difficulties involved in preparation of the medium.

Overall growth of the typical strains in the partially defined medium of Wilson (1963) was better than in the chemically defined medium, with the maximum OD$_{575}$ at 72 hours being approximately 0.2. Growth was not determined in this medium in the absence of niacin or liver coenzyme concentrate.
(2) Characteristics of atypical strains 11, 16 and 2:

Three strains which differed to some degree from fresh clinical isolates were tested for their antibiotic resistance, their ability to grow in CM-211 medium without starch and in the chemically defined medium without NAD. These strains had been transferred frequently in the laboratory and gave abundant, off-white growth on charcoal agar in an incubation period of 18-24 hours. Gram stained cells appeared considerably elongated as compared to the typical strains. The results of determinations of antibiotic resistance and growth requirements are given in Tables I and II.

The atypical strains were resistant to streptomycin and chloramphenicol but sensitive to tetracycline and chlortetracycline. As a comparison, the typical strains used throughout this work were sensitive to these antibiotics in CM-211 medium and on charcoal agar. The atypical strains also differed from the typical strains in that they did not require starch for growth and serial transfer in CM-211 medium and did not require NAD for rapid and abundant growth and serial transfer in the chemically defined medium.

Table I. Antibiotic resistance of strains 11, 16 and 2.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Streptomycin (80 ug/ml)</th>
<th>Chloramphenicol (80 ug/ml)</th>
<th>Tetracycline (80 ug/ml)</th>
<th>Chlortetracycline (120 ug/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ Resistant
- Sensitive
Table II. Growth requirements of strains 11, 16 and 2.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Starch Required</th>
<th>NAD Required</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(3) Ultraviolet inactivation:

Cell suspensions of the 6 strains used in these studies were irradiated for a total of 20 minutes to determine the extent of inactivation as a preliminary study in determining the mutagenic effect of UV irradiation. The inactivation curves of the typical strains 63, 4 and 23 (Figure 1) show that strain 23 was the most sensitive to UV irradiation since sufficient survivors were not recovered beyond the 10 minute exposure to give 30 colonies per plate when 0.2 ml of the undiluted cell suspension was plated. The curves of strains 63 and 4 show a marked change in slope at 5 minutes exposure while the curve of strain 23 does not change markedly as evidenced by failure to recover survivors after a 15-minute exposure.

Inactivation curves of the atypical strains 11, 16 and 2 (Figures 2, 3 and 4) were quite similar to one another although slight differences in recovered survivors are evident at the 2.5 and 5-minute exposure times. These strains appear to be much more resistant to the inactivating effects of UV irradiation than typical strains since a greater fraction of survivors was recovered from the atypical strains after 20 minutes.
Figure 1. Ultraviolet inactivation of strains 63, 4 and 23.
Figure 2. Ultraviolet inactivation of strain 11.

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Figure 3. Ultraviolet inactivation of strain 16.

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Figure 4. Ultraviolet inactivation of strain 2.
exposure than from the typical strains following 5 minutes exposure. The possibility exists that this marked difference is the result of differences in the ability of survivors to form visible colonies.

(4) Streptomycin resistance:

(a) Development of streptomycin-resistant strains:

A fairly extensive program of screening strains 63, 4 and 23 for streptomycin-resistant cells present in the culture or resulting from spontaneous mutations appearing in the presence of streptomycin met with limited success. A total of three streptomycin-resistant strains were obtained by spreading 0.2-1.0 ml of a cell suspension on charcoal agar plates containing from 80-200 ug/ml streptomycin. Strains 4R, 23R and 23R1 were obtained in this manner and no streptomycin-resistant strains of 63 were recovered. Attempts to recover streptomycin-resistant strains in CM-211 medium containing streptomycin were not successful.

All other streptomycin-resistant strains originally isolated were obtained by screening UV-irradiated cell suspensions for resistant cells. Strains 4R1, 4R2, 4R3, 4R4, 23R, 23R1, 23R2, 23R3, 23R4, 63R, 63R1 and 63R2 were isolated from UV-irradiated cell suspensions. A 5 ml sample of these suspensions was transferred to 50 ml of CM-211 medium in a 250 ml erlenmeyer flask, incubated until good growth was observed and then transferred to CM-211 medium containing 200 ug/ml streptomycin. Strains 63R3 and 63R4 were obtained by plating UV-irradiated cells directly onto charcoal agar containing 200 ug/ml streptomycin.

(b) Antibiotic resistance and growth requirements of streptomycin-resistant strains:

Resistance of the 15 original streptomycin-resistant strains to
the antibiotics used in this work is shown in Table III. Some strains developed by UV irradiation of parent strains 4 and 63 were resistant to streptomycin and chloramphenicol together and separately on charcoal agar and in CM-211 medium. Growth was pearly and homogeneous in CM-211 medium in the presence of chloramphenicol while flocculent growth was observed with and without streptomycin. The doubly resistant strains showed more rapid and abundant growth in the absence of chloramphenicol in both fluid and solid media. Growth of strain 4R3, which was also resistant to tetracycline and chlortetracycline, was inhibited considerably by these antibiotics but was normal in their absence. Growth was improved when the tetracycline and chlortetracycline concentration was lowered to 40 ug/ml in both types of media and had a pearly appearance at both the high and low concentrations in CM-211 medium.

Table III. Antibiotic resistance of strains originally selected for streptomycin resistance.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Streptomycin (5000 ug/ml)</th>
<th>Chloramphenicol (80 ug/ml)</th>
<th>Tetracycline (80 ug/ml)</th>
<th>Chlortetracycline (120 ug/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>23R</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>23R1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>23R2</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>23R3</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>23R4</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4R</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4R1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4R2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4R3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4R4</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>63R</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>63R1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>63R2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>63R3</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>63R4</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ Resistant
- Sensitive
Figures 6 and 7 show a comparison of growth with and without 80 ug/ml streptomycin in CM-211 medium. Strains 4R1, 63R2 and 63R3 gave slightly higher OD₅₇₅ readings in the presence of streptomycin than in its absence after 24 hours incubation in CM-211 medium. The presence or absence of streptomycin in the medium had no effect on 24 hour growth of strains 63R4, 23R2, 23R1 and 4R2, while the antibiotic inhibited growth of other strains to some extent.

Growth requirements of the original streptomycin-resistant strains (Table IV) were determined using starch and NAD dependence for the comparison of resistant and parent strains. All streptomycin-resistant strains were starch-independent and 8 of the 15 strains did not require NAD for growth in the chemically defined medium. Of the three spontaneous mutants, strain 23R was the only one having an altered NAD requirement.

Table IV. Growth requirements of strains originally selected for streptomycin resistance.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Starch Required</th>
<th>NAD Required</th>
</tr>
</thead>
<tbody>
<tr>
<td>23R</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>23R1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>23R2</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>23R3</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>23R4</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>4R</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>4R1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4R2</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>4R3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4R4</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>63R</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>63R1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>63R2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>63R3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>63R4</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ Resistant  - Sensitive
Figure 5. Growth of streptomycin-resistant strains $4R, 4R_1, 4R_2, 4R_3,$ and $4R_4$ in CM-211 medium with and without 80 μg/ml streptomycin.

- With streptomycin
- Without streptomycin
Figure 6. Growth of streptomycin-resistant strains 23R, 23R1, 23R2, 23R3, and 23R4 in CM-211 medium with and without 80 μg/ml streptomycin.

/ With streptomycin
- Without streptomycin
Figure 7. Growth of streptomycin-resistant strains 63R, 63R1, 63R2, 63R3, and 63R4 in CM-211 medium with and without 80 μg/ml streptomycin.

Optical Density (575 nm)
(5) Relationship between the period of UV irradiation exposure and the development of streptomycin resistance:

Cell suspensions of strains 63, 23 and 4 were irradiated 20, 15 and 10 minutes, respectively. Samples were plated at 5-minute intervals and the streptomycin resistance of survivors was determined at these times. All streptomycin-resistant colonies were also tested for resistance to tetracycline, chlortetracycline and chloramphenicol. The results shown in Table V represent a total of 60 survivor colonies of each strain from each exposure period.

Table V. Relationship between the period of UV irradiation exposure and development of streptomycin resistance.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Period of Irradiation (min.)</th>
<th>Sm-R Colonies*</th>
<th>Sm-R Colonies Resistant to</th>
<th>Cm</th>
<th>Tc</th>
<th>Clt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
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<td>0</td>
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<td>0</td>
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<tr>
<td></td>
<td>5</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>12</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>63</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>11</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>17</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Determined from a total of 60 survivor colonies from each irradiation period.

Sm Streptomycin
Cm Chloramphenicol
Tc Tetracycline
Clt Chlortetracycline

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A definite correlation exists between the UV irradiation sensitivity of strains 23, 4 and 63 and the development of streptomycin resistance. One-half the exposure time required for development of 17 resistant colonies of strain 63 resulted in development of 15 resistant colonies of strain 23. UV inactivation and development of streptomycin resistance in strain 4 was intermediate between these strains. A non-linear relationship appears to exist between the UV irradiation exposure period and induced mutation to streptomycin resistance although the small sample size was insufficient to give an accurate determination. This non-linear relationship between irradiation dose and mutation rate generally exists for UV irradiation while treatment with X-rays gives a linear relationship in bacteria.

Development of chloramphenicol resistance along with streptomycin resistance occurred in mutants of strains 4 and 63, while all mutants of strain 23 were resistant to streptomycin alone. The five original streptomycin-resistant strains of 23 were similarly sensitive to chloramphenicol. Of the 75 colonies tested, all were sensitive to tetracycline and chlortetracycline at the concentrations used in solid medium.

(6) Chloramphenicol resistance:

All attempts to obtain chloramphenicol-resistant strains of 23, 4 and 63 by inoculating charcoal agar, Bordet-Gengou agar or CM-211 medium containing chloramphenicol, with normal or UV-irradiated cells met with failure. Strains 63CmR, 63CmR1, 23CmR, 23CmR1, 4CmR and 4CmR1 which were resistant to at least 80 µg/ml of chloramphenicol in CM-211 medium or on charcoal agar were obtained by adding increasing concentrations of chloramphenicol to actively metabolizing cells. Growth of
these strains on charcoal agar and in CM-211 medium was considerably
better in the absence of the antibiotic in a 24-48 hour incubation per­
iod. Growth of all chloramphenicol-resistant strains was pearly and
homogeneous in CM-211 medium containing 80 ug/ml chloramphenicol; while
a flocculent growth, characteristic of the parent strain, occurred in
the absence of the antibiotic.

The antibiotic resistance of these strains is shown in Table VI.
All chloramphenicol-resistant strains were resistant to at least 80
ug/ml chloramphenicol in CM-211 medium or on charcoal agar. These
strains grew in media containing 100 ug/ml chloramphenicol but were not
tested at higher concentrations and no attempt was made to increase the
resistance level by subculturing cells in higher concentrations of the
antibiotic. None of these strains were resistant to 40 ug/ml strepto­
mycin and no cross resistance to tetracycline or chlortetracycline was
observed, indicating that the developed resistance was specific within
the range of antibiotics tested.

Table VI. Antibiotic resistance of strains originally selected for
chloramphenicol resistance.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Chloramphenicol (80 ug/ml)</th>
<th>Streptomycin (40 ug/ml)</th>
<th>Tetracycline (80 ug/ml)</th>
<th>Chlortetracycline (120 ug/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>63CmR</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>63CmR1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>23CmR</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>23CmR1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4CmR</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4CmR1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ Resistant
- Sensitive
The chloramphenicol-resistant strains did not require starch for growth and serial transfer in CM-211 medium and all required NAD for growth and serial transfer in the chemically defined medium. Stock cultures of these strains were maintained on charcoal agar without antibiotic and the chloramphenicol resistance characteristic was stable throughout the period required for completion of the experiments.

(7) Tetracycline resistance:

Tetracycline resistant strains were developed by adding increasing concentrations of the antibiotic to actively metabolizing cells, the procedure being similar to that used for the development of chloramphenicol-resistant strains. Antibiotic resistance of these strains is shown in Table VII.

Strains 63TcR, 23TcR and 4TcR were resistant to 80 ug/ml tetracycline and 120 ug/ml chlortetracycline separately on charcoal agar and in CM-211 medium. Growth was inhibited considerably in the presence of these antibiotics but was normal in their absence. Presence of tetracycline or chlortetracycline in CM-211 medium cultures resulted in pearly,

Table VII. Antibiotic resistance of strains originally selected for tetracycline resistance.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Tetracycline (80 ug/ml)</th>
<th>Chlortetracycline (120 ug/ml)</th>
<th>Streptomycin (140 ug/ml)</th>
<th>Chloramphenicol (80 ug/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>63TcR</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>23TcR</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4TcR</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ Resistant
- Sensitive
homogeneous growth while growth without the antibiotics was flocculent, being similar to the parent strains.

Starch was not required for growth and serial transfer in CM-211 medium but NAD was required for growth in the chemically defined medium.

(8) Characteristics of starch-independent strains:

Strains not requiring starch for growth and serial transfer in CM-211 medium were developed from parent strains 63 and 4 by transferring a heavy inoculum of cells growing in the complete CM-211 medium to CM-211 medium without starch. Transfers were then made, gradually decreasing the inoculum size in starch-free medium until transfer of 1 ml from a starch-free culture would grow in 24 hours in fresh starch-free CM-211 medium. Attempts to recover a starch-independent strain of 23 by this method or variations of the method failed. Strains 63-ST and 4-ST were sensitive to 40 µg/ml streptomycin, 80 µg/ml chloramphenicol, 80 µg/ml tetracycline and 120 µg/ml chlortetracycline. These strains showed an overall reduced growth rate in CM-211 medium with and without starch and on charcoal agar. However, growth was increased in CM-211 medium containing starch as compared to the starch-free medium, indicating that these strains were not completely starch-independent.

(9) Transfer of resistance in strains 63R and 63CmR:

An attempt was made to detect a transfer of antibiotic resistance by incubating a mixed cell suspension of a streptomycin-resistant and a chloramphenicol-resistant strain for various periods and plating samples on media containing both antibiotics. Results from the 6-hour incubation period only, are given in Table VIII, since doubly resistant strains
were not recovered following 2 and 4 hours incubation. Control plates of streptomycin and chloramphenicol agar inoculated with each strain separately gave no visible colonies. No visible colonies were produced when a filtrate from an 18-hour CM-211 medium culture of each strain was mixed and incubated 6 hours with cells of the other strain.

Growth of the doubly resistant strains was similar to that of the chloramphenicol-resistant parent in the presence of 80 ug/ml chloramphenicol in CM-211 medium. In CM-211 medium with and without streptomycin, growth was flocculent and resembled that of the streptomycin-resistant parent. The seven doubly resistant strains did not require starch or NAD while the chloramphenicol-resistant parent had a NAD requirement.

Table VIII. Transfer of resistance in streptomycin-resistant strain 63R and chloramphenicol-resistant strain 63CmR.

<table>
<thead>
<tr>
<th>Experiment*</th>
<th>Doubly Resistant Colonies Isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>

* Approximately 1 x 10^8 cells were used as the inoculum in each experiment.
Previous studies on antibiotic resistance in *B. pertussis* have been carried out with the public health aspect of resistance in mind, with the exception of transformation studies done on the streptomycin resistance characteristic (Branefors, 1964). Antibiotic resistance has been used extensively as a genetic determinant in other organisms, and the subsequent alterations in nutritional requirements resulting from antibiotic resistance mutation have further increased the importance of the genetic aspect of antibiotic resistance.

Antibiotic resistance was chosen as the focal point in this preliminary genetic study, since selection of resistant cells appeared to be the only possible starting point. Lack of a chemically defined medium when this work was initiated ruled out selection of auxotrophic or prototrophic mutants. The idea of selecting for antibiotic resistance was also strengthened by the degradation of organisms from a virulent to an avirulent phase and subsequent alteration of morphology and nutritional requirements.

The chemically defined medium used for determining the NAD requirement of various antibiotic resistant strains developed in this study was a modification of a partially defined medium which would support the growth of Phase I strains of *B. pertussis* (Wilson, 1963). This defined medium was developed for use as a minimal medium supporting the growth of the typical strains of organisms employed in this work and...
did not support sufficient growth to be suitable for mass cultivation of the organism. Since this medium did not contain niacin, which is a growth factor for *B. pertussis* (Hornibrook, 1940; Jebb and Tomlinson, 1955; Turner, 1961) but did contain NAD, which can replace niacin (Fukimi et al., 1963; Jebb and Tomlinson, 1955; Proom, 1955), it is not known whether niacin could have replaced NAD in this medium. Possibly the deletion of constituents from this medium such as nucleic acid derivatives, ATP and certain amino acids would result in a less complex minimal medium and would not affect growth appreciably.

Preliminary UV irradiation inactivation studies were carried out to determine the sensitivity of various strains to UV irradiation in the system designed for inducing mutation to streptomycin resistance. The rate of inactivation was decreased by irradiating the cell suspension in a 100 ml beaker giving a depth of 10 mm and by placing the UV source 1 cm from the surface of the cell suspension. This system, similar to that used by Wachsman and Mangalo (1962) to enrich for auxotrophic mutants of *B. megaterium*, probably allows a maximum number of cells to receive some irradiation damage without being damaged to the extent that they will no longer divide.

The inactivation curves of *B. pertussis* show a trend toward leveling off between 2.5 and 5 minutes exposure time. This change of slope of the inactivation curve has been observed in various organisms including *Shigella sonnei* (Ramage, 1961) and *Neisseria meningitidis* (Lie, 1965). However, in most inactivation studies UV irradiation treatment is not extended beyond 1 percent survival and cells representing $10^{-3}$ percent survivors or less are overlooked. This bipartite
survival curve could result from a portion of the cell population being of a different radiation resistant genotype. Another factor to consider is the stage of development of the cell population since cells in the log phase are most susceptible to the inactivating effects of UV irradiation. The most resistant population may have been composed of cells which were not actively dividing at the time that cells were harvested for UV treatment. Another possibility is that the high percentage of inactivated cells may protect the viable cells by absorbing the UV irradiation. This absorption factor would be especially important in the irradiation procedure used in this work. The increased resistance to UV irradiation observed in the atypical strains 16, 11 and 2 may have been due to the increased ability of survivor cells to form visible colonies, since these strains showed better overall growth than the typical strains. Since all cultures were incubated 18 hours prior to irradiation, the increased growth rate of the atypical strains may have resulted in their being beyond the log growth phase at the time of irradiation treatment.

The spontaneous mutation rate of B. pertussis to streptomycin resistance has been reported to be approximately $10^{-10}$ (Alexander and Redman, 1949). Although this rate is quite low, more spontaneously resistant cells should have been recovered in the screening of strains 23, 63 and 4. Cells transferred to plates of charcoal or Bordet-Gengou agar containing 80-200 ug/ml streptomycin may have failed to produce visible colonies due to drying of the plates over an incubation period of 72-96 hours, since the controlled incubation system was not in use at this time. Failure to observe growth in CM-211 medium containing
80-200 ug/ml streptomycin may have resulted from the inability of a small number of resistant organisms to give detectable growth in this medium. Throughout these experiments an inoculum of approximately $10^4$ cells per 50 ml of CM-211 medium was required to give consistently good growth. Perhaps the most important factor governing the growth of resistant organisms in media containing streptomycin is the phenotypic lag in cells which have just received the streptomycin resistance characteristic. In organisms having extended generation times, full phenotypic expression of streptomycin resistance may take up to 5-9 hours (Bovre, 1964). During this period the bactericidal action of streptomycin could result in death or at least inactivation of the potentially resistant cells to a point where they would fail to divide.

The mutation rate for streptomycin resistance in *B. pertussis* was markedly increased by UV irradiation and was related to the period of exposure in the procedure used for this study. However, attempts to obtain streptomycin-resistant organisms from a UV-irradiated population by transferring treated cells directly to media containing streptomycin met with limited success as did attempts to obtain spontaneously resistant cells by direct plating. In the UV-treated cells a lag induced by irradiation may have influenced the expression of resistance in conjunction with the phenotypic lag. Investigators have found this lag in the onset of division after UV irradiation, the duration being related to irradiation intensity (Hollaender and Duggar, 1938, Demeric, 1946; Lie, 1965). The duration of the UV-induced lag in strains 23, 63 and H was not determined but could be expected to be reasonably long due to the extended exposure times used throughout this work.
Possibly the UV-induced lag alone may have resulted in the failure to observe streptomycin resistance expression or may have worked in combination with the phenotypic lag to give this effect in the presence of streptomycin. Decreased mutant frequency has been observed following UV irradiation exposure when irradiated cells were exposed to protein synthesis inhibitors, such as chloramphenicol and base analogs, or the RNA synthesis inhibitor 6-azauracil during the critical post-irradiation period (Witkin and Theil, 1960). Transferring UV-irradiated cells of B. pertussis directly to media containing chloramphenicol or streptomycin may inhibit synthesis of enzymes necessary for DNA replication and preclude the development of cells having a potentially resistant genotype.

The procedure used for obtaining UV-induced streptomycin-resistant strains in this study permitted the cells to overcome the UV-induced lag and show full phenotypic expression of the streptomycin resistance characteristic in the absence of the antibiotic. Possibly the spontaneous mutation rate of B. pertussis to streptomycin resistance would be higher than $10^{-10}$ if full phenotypic expression occurred before cells were transferred to media containing streptomycin. Although investigations of spontaneous mutation to chloramphenicol resistance in B. pertussis have been unsuccessful, the occurrence of chloramphenicol and streptomycin resistance together in UV-induced mutants indicates that this mutation could occur spontaneously. Testing a population of B. pertussis for chloramphenicol resistance might prove successful if full phenotypic expression of this trait occurred prior to introduction of the antibiotic. Knowing the amount of time required for full phenotypic expression of streptomycin and chloramphenicol resistance in this organism would be of value in future experiments.
A procedure for allowing full phenotypic expression of streptomycin resistance in *Moraxella* involves incubation of cells on normal agar for a period sufficient to give full expression. The inoculated agar is then placed on agar containing streptomycin which diffuses to the cells (Bovre, 1964). The usefulness of this method for obtaining streptomycin-resistant cells of *B. pertussis* would be limited since a thin fluid layer must cover the agar medium to give consistently good colony production under the special conditions used in this study. From these results it appears that the only reliable method of testing a population of *B. pertussis* for induced mutation to streptomycin resistance is to grow selected colonies on normal agar and test the resulting cells for resistance. However, this procedure has its limitations for determining the spontaneous mutation rate since mutations can be expected to occur during the period required for full phenotypic expression in previously existing mutants.

Plans to compare the effectiveness of UV irradiation for inducing streptomycin resistance mutations in the typical strains 23, 63 and 4 and the atypical strains 11, 16 and 2 were discontinued when the latter strains were found to be resistant to 200 ug/ml streptomycin and 80 ug/ml chloramphenicol. Whether the antibiotic resistance in these degraded strains is a coincidence or whether degradation to phase 4 is accompanied by development of resistance to streptomycin and chloramphenicol is not known. Of the 15 original strains selected for streptomycin resistance, those also resistant to chloramphenicol were most similar to the atypical strains. Five out of seven of these strains did not require NAD for growth in the chemically defined medium and
were starch-independent, as were all streptomycin-resistant strains isolated. The mutation for streptomycin resistance is believed to modify the ribosome in order to increase its affinity for typical messengers to compensate for abnormalities resulting from the presence of streptomycin (Lederberg et al., 1964). If degradation and streptomycin resistance development are related in *B. pertussis*, a ribosome modification analogous to that occurring in resistance mutation may accompany or cause degradation to the atypical phase 4 of the organism.

Although the streptomycin-resistant strains originally isolated were resistant to high streptomycin concentrations, these strains did not show streptomycin dependence. Gabor (1965) suggested that if both streptomycin-resistant and dependent markers are multisite in nature, the possibility exists that they possess shared mutations in their region of overlap. If this is true, perhaps in the *B. pertussis* strains mutations did not occur at sufficient sites to impart the dependence characteristic. Possibly the procedure used for obtaining most mutants in this work did not permit the outgrowth of streptomycin-dependent strains due to the absence of streptomycin in post-irradiation media. Investigations with *Diplococcus* have shown that streptomycin-dependent strains cannot be obtained from sensitive strains but that dependent mutants can be isolated from resistant strains (Mishra and Ravin, 1966). If this were true in *B. pertussis*, the potent mutagenic effect of UV irradiation could be used to obtain dependent mutations in a streptomycin-resistant population. Isolation of dependent strains may provide some interesting nutritional studies on the organism since this mutation may involve a streptomycin-suppressible defect in a structural gene with the resulting
strain requiring either a specific metabolite or streptomycin (Gorini and Kataja, 1964).

The isolation of strains of *B. pertussis* showing resistance to more than one antibiotic indicates that multiple resistance is fairly common in UV-induced antibiotic resistant mutants of this organism. Since strains were selected for streptomycin resistance before being tested for resistance to the other antibiotics, combinations such as chloramphenicol and tetracycline or chlortetracycline resistance would not have been detected. This procedure may have resulted in the frequent occurrence of streptomycin and chloramphenicol resistance together. Since cross resistance to chlortetracycline was observed in tetracycline-resistant strains the possibility exists that strain 4R3, which was resistant to the four antibiotics used, possessed the tetracycline resistance determinant but showed cross resistance to chlortetracycline. Similarly, resistance to tetracycline may have been due to the presence of a chlortetracycline determinant. Due to similarities in structure of these tetracycline antibiotics, resistance could be controlled by the same genetic determinant in *B. pertussis*.

Failure to observe chloramphenicol resistance in any of the streptomycin-resistant strains of 23 may indicate that this strain differs from strains 63 and 4 in its genetic determinants for antibiotic resistance. The possibility exists that the responsible factors for multiple drug resistance in *B. pertussis* are episome based and that single resistance factors are of the integrated type. If this were the case, strain 23 may not possess episome-based resistance or sensitivity factors. The subject of multiple drug resistance, its episomal nature
and its occurrence in various organisms has been reviewed thoroughly (Watanabe, 1963). The limited amount of information gathered in this study does not permit any definite conclusions to be drawn concerning multiple drug resistance in *B. pertussis*.

Recently, streptomycin resistance and dependence in bacteria have come to be viewed as accommodations permitting or requiring certain alterations by streptomycin of the expression of other genetic traits carried by the organism. The relationship between development of streptomycin resistance and starch independence observed in this work is in agreement with transformation experiments (Branefors, 1963). Although controversy exists as to the exact function of starch, the mutation to streptomycin resistance may result in a subsequent alteration in metabolism. If starch functions to absorb a toxic product produced by the cell (Pollock, 1949; Dawson *et al.*, 1950), development of starch independence indicates that the toxic product is no longer produced or that this product is no longer toxic to the cell.

This problem is further complicated by the development of starch independence in chloramphenicol and tetracycline-resistant strains. It appears unlikely that development of resistance to the three antibiotics separately would result in the same metabolic alteration. Therefore, the possibility of altered permeability being responsible for development of starch independence in these strains must be considered. The relationship between altered permeability and tetracycline and chloramphenicol resistance development has been shown (Okamoto and Mizuno, 1962, 1964; Franklin and Godfrey, 1965; Izaki *et al.*, 1965). Although the mechanism of streptomycin resistance development does not involve a
permeability alteration, such an alteration could be associated with the streptomycin resistance mutation. Development of drug resistance to *B. pertussis* may result in a decreased uptake of the toxic product. Further studies directed toward identifying the toxic product and determining whether or not it is produced and taken up by antibiotic resistant and atypical strains should clear up this problem.

Since the antibiotic-resistant strains developed in this study were starch-independent, the possibility that strains selected for starch independence could be antibiotic resistant was investigated. Characteristics of the starch-independent strains 63-ST and 4-ST indicate that they are not necessarily antibiotic-resistant and are not NAD-independent. Colonial and cellular morphology were similar to the parent strains, ruling out the possibility that these strains resulted from selection of degraded cells in the culture. The decreased growth rate with and without starch indicates that they are not completely starch-independent and may have metabolic differences. Failure to isolate a starch-independent strain of 23 is difficult to explain, since all antibiotic-resistant strains developed from the 23 parent were starch-independent.

The reduced growth rate of chloramphenicol and tetracycline-resistant strains in the presence of their respective antibiotic may have resulted from incomplete resistance development. Tetracycline-resistant cells of *E. coli* accumulate much less tetracycline than do sensitive cells and this decreased accumulation is directly related to development of increased resistance (Franklin and Godfrey, 1965; Izaki et al., 1965). The inhibition by chloramphenicol of amino acid
incorporation in cell-free systems of chloramphenicol-resistant *E. coli* (Okamoto and Mizuni, 1962, 1964) and the stepwise development of increased resistance also suggest a relationship between accumulation and resistance. Strains of *B. pertussis* showing incomplete resistance development may have accumulated the antibiotics to a smaller extent due to decreased permeability. However, the amount of antibiotic entering the cell or accumulated by the cell was sufficient to result in considerable growth inhibition. Subculture of these strains in the presence of increased concentrations of their respective antibiotic may have decreased this inhibition. The partial growth inhibition resulting from the presence of chloramphenicol and tetracyclines could explain the pearly, homogeneous appearance of CM-211 medium cultures containing these antibiotics.

Attempts to show a transfer of resistance between a streptomycin-resistant and chloramphenicol-resistant strain of *B. pertussis* were complicated by lack of a method for allowing full phenotypic expression to occur and the limited number of genetic markers available. If the doubly resistant strains actually resulted from the transfer of a resistance determinant, the question of which determinant was transferred remains unanswered. Since the chloramphenicol-resistant parent was NAD-dependent and all doubly resistant strains were NAD-independent, transfer of streptomycin resistance and NAD-independence must have occurred together. Transfer of chloramphenicol resistance to the streptomycin-resistant, NAD-independent parent would have resulted in the doubly resistant, NAD-independent genotype. Similarly, spontaneous mutation to chloramphenicol resistance could have occurred alone,
whereas streptomycin resistance and NAD independence mutations would have had to occur together if doubly resistant strains resulted from spontaneous mutation.

Spontaneous mutations from a specific auxotrophic type to the prototrophic type occur at an average rate of $1 \times 10^{-7}$ (Tatum and Ledeburg, 1947). Since the spontaneous mutation rate for streptomycin resistance in *B. pertussis* is $1 \times 10^{-10}$ (Alexander and Redman, 1949), simultaneous mutations for NAD independence and streptomycin resistance would occur at rates as low as $1 \times 10^{-17}$. Therefore, the frequency at which doubly resistant, NAD-independent cells occurred in this work indicates that they did not arise from spontaneous mutations in the chloramphenicol-resistant strain. The possibility exists that spontaneous mutations for chloramphenicol resistance occurred in the streptomycin-resistant parent although this mutation has not been reported previously in *B. pertussis*.

Future experiments involving transfer of genetic determinants in this organism may be more successful if strains are developed which have markers permitting selection of recombinants without the use of antibiotics.

From the data obtained in this preliminary genetic study, it appears that *B. pertussis* possesses a number of stable, genetically determined characteristics which can serve as markers. The potent mutagenic effect of UV irradiation on inducing mutations for antibiotic resistance and growth requirement alterations, along with refinements in culturing systems, should facilitate future genetic investigations on this organism. Further characterization of mutants regarding altered immunological, virulence and metabolic properties may eventually provide information on the mechanism of the degradation phenomenon.
CHAPTER VI

SUMMARY

1. A preliminary genetic study centered around streptomycin, chloramphenicol, tetracycline and chlortetracycline resistance development and accompanying growth requirement alterations was made with *Bordetella pertussis*.

2. Ultraviolet irradiation was found to be a very effective mutagenic agent for inducing single and multiple antibiotic resistant mutations along with NAD-independent mutations.

3. Chloramphenicol and tetracycline-resistant strains were developed by subculturing cells in the presence of increasing concentrations of the antibiotics. Chloramphenicol-resistant strains showed no cross resistance while tetracycline-resistant strains were cross resistant to chlortetracycline.

4. All antibiotic resistant strains were starch-independent while strains selected for starch independence showed no antibiotic resistance.

5. Three atypical strains examined were more resistant to UV inactivation than the typical strains. These strains were also NAD and starch-independent and streptomycin and chloramphenicol-resistant.

6. An attempt to demonstrate antibiotic resistance transfer between a streptomycin-resistant, NAD-independent strain and a chloramphenicol-resistant strain resulted in the development of seven doubly resistant NAD-independent strains.
7. Antibiotic resistance and nutritional characteristics of all strains were stable during the period required for completion of this work.

8. The possibility of elucidating the mechanism of degradation from the virulent to the avirulent phase through further characterization of nutritional and antibiotic resistant mutants is discussed.
BIBLIOGRAPHY


