KEYSER, Encil Glen, 1929-
TERATOGENIC EFFECTS OF ANTIMETABOLITES IN THE CHICK EMBRYO AND THEIR RELATION TO PHYSICAL AND CHEMICAL PROPERTIES OF TISSUE PROTEINS.

The Ohio State University, Ph.D., 1971
Biochemistry

University Microfilms, A XEROX Company, Ann Arbor, Michigan
TERATOGENIC EFFECTS OF ANTIMETABOLITES IN THE CHICK EMBRYO
AND THEIR RELATION TO PHYSICAL AND CHEMICAL PROPERTIES
OF TISSUE PROTEINS

DISSERTATION

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

By
E. Glen Keyser, B.S., M.S.

The Ohio State University
1971

Approved by

[Signature]
Adviser
Department of Poultry Science
ACKNOWLEDGMENTS

It would be difficult to mention each person who has contributed in some way to the completion of this study. Therefore, I will mention the few without whose help the work would have been severely handicapped.

I wish to express my appreciation to my adviser, Dr. Edward C. Naber, for first stimulating my interest and then supplying me with the academic and personal guidance with which to carry this graduate study program to its completion.

I would like to extend thanks to the other members of my committee, Drs. Virginia Vivian, John Allred and David Cornwell for their counsel and advice during all phases of the graduate study program. I also thank the Ohio Agricultural Research and Development Center for providing monies and equipment for this project.

Finally, I wish to thank my wife, Nancy, and our children, all of whom have sacrificed so much that this program might be completed.
VITA

December 24, 1929 Born, Bellaire, Ohio

March 16, 1956... B. Sc. in Agr. The Ohio State University, Columbus, Ohio

1956-1960........ County Extension Agent, Agriculture Gratiot County, Ithaca, Michigan


1967-1967........ Graduate Student, Department of Animal Science, University of Arkansas, Fayetteville, Arkansas

January 1968..... M.S. University of Arkansas, Fayetteville, Arkansas

1967-Present..... Graduate Student, Department of Poultry Science, The Ohio State University, Columbus, Ohio

PUBLICATIONS


PUBLICATIONS (Cont'd.)


Dietary protein level and serum protein fractions in male chickens. Accepted for publication in Poultry Sci.

FIELDS OF STUDY

Major Field: Poultry Science


Studies in Physiological Chemistry. Professors D. G. Cornwell, H. W. Sprecher, R. H. Nuenke, and K. E. Richardson


Studies in Physiology. Professor H. S. Weiss
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>VITA</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>REVIEW</td>
<td>5</td>
</tr>
<tr>
<td>Antimetabolites in the Chick Embryo</td>
<td>8</td>
</tr>
<tr>
<td>Antibiotics Affecting Protein Biosynthesis</td>
<td>15</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>15</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>18</td>
</tr>
<tr>
<td>Puromycin</td>
<td>24</td>
</tr>
<tr>
<td>Other Antibiotics</td>
<td>30</td>
</tr>
<tr>
<td>Electrophoretic Studies in the Chick Embryo Proteins</td>
<td>31</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>33</td>
</tr>
<tr>
<td>Incubation</td>
<td>33</td>
</tr>
<tr>
<td>Embryo Harvest</td>
<td>35</td>
</tr>
<tr>
<td>Preparation of Protein Extracts</td>
<td>36</td>
</tr>
<tr>
<td>Polyacrylamide Gel Electrophoresis</td>
<td>40</td>
</tr>
<tr>
<td>Water soluble protein extracts</td>
<td>40</td>
</tr>
<tr>
<td>Saline-citrate soluble protein extracts</td>
<td>46</td>
</tr>
<tr>
<td>Acidic and/or histone protein extracts</td>
<td>49</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (Cont'd.)

Microdensitometric Evaluation of the Proteins Bands in the
  Gels. ................................................................. 49

RESULTS. ............................................................ 52
  Chloramphenicol ........................................ 53
  Actinomycin D .............................................. 68
  Puromycin ................................................... 69
  Time Study Effect .......................................... 76

DISCUSSION ..................................................... 126

SUMMARY .......................................................... 133

BIBLIOGRAPHY .................................................. 135
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Composition of Layer-Breeder Diet</td>
<td>34</td>
</tr>
<tr>
<td>2.</td>
<td>Composition of Solutions Used for Electrophoretic Separation of Water Soluble Protein Extracts</td>
<td>42</td>
</tr>
<tr>
<td>3.</td>
<td>Bounds of Disc Gel Electrophoresis Systems</td>
<td>43</td>
</tr>
<tr>
<td>4.</td>
<td>Composition of Solutions Used for Electrophoretic Separations of Saline Soluble Protein Extracts</td>
<td>47</td>
</tr>
<tr>
<td>5.</td>
<td>Composition of Solutions Used for Electrophoretic Separation of Acid Soluble Protein Extracts</td>
<td>50</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Place of protein biosynthesis inhibition by antibiotics</td>
<td>6</td>
</tr>
<tr>
<td>2.</td>
<td>Major steps of protein biosynthesis</td>
<td>7</td>
</tr>
<tr>
<td>3.</td>
<td>Structure of chloramphenicol</td>
<td>16</td>
</tr>
<tr>
<td>4.</td>
<td>Structure of antinomycin D</td>
<td>19</td>
</tr>
<tr>
<td>5.</td>
<td>Model of actinomycin binding to deoxyguanosine of DNA in β configuration</td>
<td>21</td>
</tr>
<tr>
<td>6.</td>
<td>Structure of puromycin</td>
<td>26</td>
</tr>
<tr>
<td>7.</td>
<td>Structure of the adenosine end of aminoacyl-sRNA</td>
<td>27</td>
</tr>
<tr>
<td>8.</td>
<td>Reactions showing amino acid activation and bonding with s-RNA in protein synthesis</td>
<td>28</td>
</tr>
<tr>
<td>9.</td>
<td>Diagram of embryonic chick tissue separation for experiment 6</td>
<td>37</td>
</tr>
<tr>
<td>10.</td>
<td>Diagram of embryonic chick tissue separation for experiment 7</td>
<td>38</td>
</tr>
<tr>
<td>11.</td>
<td>Diagram of embryonic chick tissue separation for experiment 8</td>
<td>39</td>
</tr>
<tr>
<td>12.</td>
<td>Arrangement of gel concentrations for a high resolution run of the β and higher polymer components of collagen</td>
<td>48</td>
</tr>
<tr>
<td>13.</td>
<td>Microdensitometric tracing of amido-Schwarz stained polyacrylamide gels of the head segment of untreated 120 hour old chick embryos</td>
<td>56</td>
</tr>
<tr>
<td>14.</td>
<td>Microdensitometric tracing of amido-Schwarz stained polyacrylamide gels of the body segment of untreated 120 hour old chick embryos</td>
<td>58</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>15.</td>
<td>Microdensitometric tracing of amido-Schwarz stained polyacrylamide gels of</td>
<td></td>
</tr>
<tr>
<td></td>
<td>the appendage buds of untreated 120 hour old chick embryos</td>
<td>60</td>
</tr>
<tr>
<td>16.</td>
<td>Microdensitometric tracing of amido-Schwarz stained polyacrylamide gels of</td>
<td></td>
</tr>
<tr>
<td></td>
<td>the head segment of chloramphenicol (1.0 mg) treated 120 hour old chick</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>embryos</td>
<td></td>
</tr>
<tr>
<td>17.</td>
<td>Microdensitometric tracing of amido-Schwarz stained polyacrylamide gels of</td>
<td></td>
</tr>
<tr>
<td></td>
<td>the body segment of chloramphenicol (1.0 mg) treated 120 hour old chick</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>embryos</td>
<td></td>
</tr>
<tr>
<td>18.</td>
<td>Microdensitometric tracing of amido-Schwarz stained polyacrylamide gels of</td>
<td></td>
</tr>
<tr>
<td></td>
<td>the appendage buds of chloramphenicol (1.0 mg) treated 120 hour old chick</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>embryos</td>
<td></td>
</tr>
<tr>
<td>19.</td>
<td>Amido-Schwarz stained electrophoretic separated gel of water soluble proteins</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>from the head segment of untreated 120 hour old chick embryos</td>
<td></td>
</tr>
<tr>
<td>20.</td>
<td>Microdensitometric tracing of amido-Schwarz stained polyacrylamide gels of</td>
<td></td>
</tr>
<tr>
<td></td>
<td>the head segment of actinomycin D (1.0 µg) treated 120 hour old chick</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>embryos</td>
<td></td>
</tr>
<tr>
<td>21.</td>
<td>Microdensitometric tracing of amido-Schwarz stained polyacrylamide gels of</td>
<td></td>
</tr>
<tr>
<td></td>
<td>the body segment of actinomycin D (1.0 µg) treated 120 hour old chick</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>embryos</td>
<td></td>
</tr>
<tr>
<td>22.</td>
<td>Microdensitometric tracing of amido-Schwarz stained polyacrylamide gels of</td>
<td></td>
</tr>
<tr>
<td></td>
<td>the appendage buds of actinomycin D (1.0 µg) treated 120 hour old chick</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>embryos</td>
<td></td>
</tr>
<tr>
<td>23.</td>
<td>Microdensitometric tracing of amido-Schwarz stained polyacrylamide gels of</td>
<td></td>
</tr>
<tr>
<td></td>
<td>the head segment of puromycin (0.5 µg) treated 120 hour old chick embryos</td>
<td>78</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>24.</td>
<td>Microdensitometric tracing of amido-Schwarz stained polyacrylamide gels of the body segment of puromycin (0.5 µg) treated 120 hour old chick embryos.</td>
<td>80</td>
</tr>
<tr>
<td>25.</td>
<td>Microdensitometric tracing of amido-Schwarz stained polyacrylamide gels of the appendage buds of puromycin (0.5 µg) treated 120 hour old chick embryos.</td>
<td>82</td>
</tr>
<tr>
<td>26.</td>
<td>Microdensitometric tracing of amido-Schwarz stained polyacrylamide gels from the head segment of water soluble protein extracts of untreated 120 hour old chick embryos.</td>
<td>85</td>
</tr>
<tr>
<td>27.</td>
<td>Ratio of the amounts of protein present in various protein components to component 1 from microdensitometric tracings of water soluble tissue proteins from 120 hour old chick embryos.</td>
<td>88</td>
</tr>
<tr>
<td>28.</td>
<td>Ratio of the amounts of protein present in various protein components to component 1 from microdensitometric tracings of water soluble tissue proteins from 120 hour old chloramphenicol (1.0 mg) treated embryos administered at 117 hours of development</td>
<td>90</td>
</tr>
<tr>
<td>29.</td>
<td>Ratio of the amounts of protein present in various protein components to component 1 from microdensitometric tracings of water soluble tissue proteins from 120 hour old chloramphenicol (1.0 mg) treated embryos administered at 114 hours of development</td>
<td>92</td>
</tr>
<tr>
<td>30.</td>
<td>Ratio of the amounts of protein present in various protein components to component 1 from microdensitometric tracings of water soluble tissue proteins from 120 hour old chloramphenicol (1.0 mg) treated embryos administered at 111 hours of development</td>
<td>94</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>31.</td>
<td>Ratio of the amounts of protein present in various protein components to component 1 from microdensitometric tracings of water soluble tissue proteins from 120 hour old chloramphenicol (1.0 mg) treated embryos administered at 108 hours of development.</td>
<td>96</td>
</tr>
<tr>
<td>32.</td>
<td>Ratio of the amounts of protein present in various protein components to component 1 from microdensitometric tracings of water soluble tissue proteins from 120 hour old chloramphenicol (1.0 mg) treated embryos administered at 96 hours of development.</td>
<td>98</td>
</tr>
<tr>
<td>33.</td>
<td>Ratio of the amounts of protein present in various protein components to component 1 from microdensitometric tracings of water soluble tissue proteins from 120 hour old chloramphenicol (1.0 mg) treated embryos administered at 90 hours of development.</td>
<td>100</td>
</tr>
<tr>
<td>34.</td>
<td>Ratio of the amounts of protein present in various protein components to component 1 from microdensitometric tracings of water soluble tissue proteins from 120 hour old chloramphenicol (1.0 mg) treated embryos administered at 84 hours of development.</td>
<td>102</td>
</tr>
<tr>
<td>35.</td>
<td>Ratio of the amounts of protein present in various protein components to component 1 from microdensitometric tracings of water soluble tissue proteins from 120 hour old chloramphenicol (1.0 mg) treated embryos administered at 72 hours of development.</td>
<td>104</td>
</tr>
<tr>
<td>36.</td>
<td>Ratio of the amounts of protein present in various protein components to component 1 from microdensitometric tracings of water soluble tissue proteins from 120 hour old chloramphenicol (1.0 mg) treated embryos administered at 48 hours of development.</td>
<td>106</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>37.</td>
<td>Ratio of the amounts of protein present in various components to component 1 from microdensitometric tracings of water soluble appendage bud protein extracts from 120 hour untreated and chloramphenicol treated (1.0 mg, 0.50 mg, and 0.25 mg) treated chick embryos treated at 114 hours, 108 hours, and 96 hours of development.</td>
<td>110</td>
</tr>
<tr>
<td>38.</td>
<td>Microdensitometric tracing of amido-Schwarz stained polyacrylamide gels of saline-citrate soluble protein extracts of untreated 120 hour old chick embryos.</td>
<td>112</td>
</tr>
<tr>
<td>39.</td>
<td>Microdensitometric tracing of amido-Schwarz stained polyacrylamide gels of 0.45 M sodium chloride soluble protein extracts of untreated 120 hour old chick embryos.</td>
<td>115</td>
</tr>
<tr>
<td>40.</td>
<td>Microdensitometric tracing of amido-Schwarz stained polyacrylamide gels of 0.03 M sodium citrate protein extracts of untreated 120 hour old chick embryos.</td>
<td>117</td>
</tr>
<tr>
<td>41.</td>
<td>Microdensitometric tracing of amido-Schwarz stained polyacrylamide gels of acid soluble protein extracts of untreated 120 hour old chick embryos.</td>
<td>120</td>
</tr>
<tr>
<td>42.</td>
<td>Microdensitometric tracing of amido-Schwarz stained polyacrylamide gels of acid soluble protein extracts of chloramphenicol (1.0 mg) treated 120 hour old chick embryos.</td>
<td>122</td>
</tr>
<tr>
<td>43.</td>
<td>Weights of 120 hour old untreated and chloramphenicol (0.25 mg) treated chick embryos treated at various stages of embryonic development.</td>
<td>124</td>
</tr>
<tr>
<td>44.</td>
<td>Day old chick weights of untreated and chloramphenicol (0.25 mg) treated chick embryo treated at various stages of embryonic development.</td>
<td>125</td>
</tr>
</tbody>
</table>
INTRODUCTION

Nutrition research during the past few decades has revealed much information concerning the gross metabolic and teratogenic defects produced by vitamin deficiency disease. Thus, the "end products" of avitaminosis are well known for most vitamins. The biochemist has been quite successful in establishing the roles of many vitamins as essential components of coenzymes that are necessary for specific metabolic reactions. At the chemical level therefore, the functions of many vitamins can be described in terms of enzymes that promote specific chemical transformations. However, little information is available that would permit an understanding of how specific biochemical defects are related to the gross physiological and anatomical defects resulting from vitamin deficiency during embryonic development. It seems possible that failures in morphogenesis which result in structural defects in tissues are preceded by formation of faulty physical and chemical structure in the macromolecules that make up the anomalous tissue or by synthesis of abnormal amounts of proteins that are normal components of tissue. Recent advances in molecular biology have demonstrated that defects in protein structure can account for the formation of biochemically and structurally defective tissues.

The nutritive requirements of animals are generally thought to be elevated by pregnancy and reproduction. Thus it has generally been assumed that the nutritive requirements of the embryo are extremely
critical. During its development and growth, the chick embryo increases in size from 0.0002 grams at 1 day of incubation to about 40 grams on the 21st day (1). This means that the chick embryo increases its size 200,000 fold during a 20 day period. The tremendous proliferation of cells and organization of tissues that must accompany this rapid development serves to emphasize the needs of optimum nutrition.

The processes of induction, differentiation and organ or tissue development and growth are extremely complex and cannot be studied with unicellular organisms and conventional tissue cultures. While simple living systems are very useful for the study of biochemical events and structures within the cell, they provide very little basis for understanding the complex organization of cells into tissue and organs. There are many limitations on the ability to experimentally manipulate and observe the development of mammalian embryos. Birds, however, package their ova in sterile containers which undergo embryonic development outside of the maternal environment where it can be studied free from maternal influences other than those passed on through chromosomes and the nutrients contained in the egg. The developing chick embryo provides a biological system in which one can study tissue and organ development at various stages of complexity as these are related to biochemical function. Therefore, clues to the primary effects of antimetabolites on tissue growth and differentiation may be found in this system where one tissue is not highly dependent upon others for its welfare. While considerable research has been done on normal induction and differentiation of embryonic forms, few attempts have been made to relate tissue and organ development to specific metabolic defects produced by antimetabolites or vitamin deficiency.
Since the early developmental anatomy of the chick and the mammalian embryo is similar, studies of this type may yield information applicable to human nutrition research. If the chemical and physical processes that contribute to faulty embryogenesis are better understood these abnormal conditions may be counteracted or rectified. Nutrient deficiency states produced by many antivitamins and natural teratogens such as the lathrogens as well as antibiotics and other drugs are all capable of causing abnormal development. Because mammalian embryos are closely tied to maternal influences and are at the same time somewhat parasitic, it has been difficult to evaluate the effect of many drugs and dietary alterations on prepartum development. Nevertheless, the lessons learned from the prevention of antimetabolite induced teratogenesis should eventually result in dietary designs and patterns of drug administration that promote optimum human fetal development and minimize physical and mental defects.

The present study was designed to alter the synthesis of protein in the developing embryo with the use of antimetabolites. If the antimetabolite interferes with protein biosynthesis, then quantitative or qualitative changes in protein formation should take place and these changes could be determined by a study of the chemical and physical properties of proteins in embryonic tissues. This type of information would relate the knowledge between biochemical function and anatomical defect.

The specific aims of this study were: (1) to study the teratological properties of antimetabolites on the developing chick embryo, particularly those antimetabolites known to directly or indirectly interfere with protein formation, (2) to study the physical and chemical
properties of proteins derived from affected tissues and organs and compare them to those derived from normal tissue, and (3) to identify the macromolecules in abnormal tissue development induced by antimetabolites.
REVIEW

There are many places in the biosynthesis of proteins where antimetabolites might inhibit their formation or cause faulty synthesis of the proteins as illustrated in Miura's antibiotic and replication chart (Figure 1). Antibiotics could operate by eliminating the function of messenger ribonucleic acid (m-RNA) (sites A and D, figure 1) through competition for binding sites thus preventing the s-RNA-amino acid complex attachment to the ribosome surface. The antibiotics could also operate by inducing misreading (sites B and E, figure 1) of the 3 base "anticodon" on the transfer ribonucleic acid (t-RNA) pairs with the matching codon on the m-RNA strand. The antibiotic could also induce malfunction by forming a peptide bond with the free amino group of the antibiotic. Another way in which the antibiotic could limit protein synthesis would be to inhibit nucleic acid synthesis by inhibiting deoxyribonucleic acid (DNA) directed RNA synthesis through a binding of the antibiotic to the DNA (site F, figure 1). Thus RNA polymerase activity is suppressed.

A brief discussion of the major steps in protein biosynthesis will aid in understanding the places where selected antibiotics influence the biosynthesis of proteins. A simplified summary of protein biosynthesis is shown in Figure 2. The majority of free amino acids within the cell originate from one of two sources. They may be transported into the cell from extracellular fluid (step 1), or amino acids may be synthesized by the fixation of NH₃ with α-ketoglutarate to form glutamic acid which can
Figure 1. Places of protein biosynthesis inhibition by antibiotics.
Figure 2. Major steps in protein biosynthesis.
subsequently undergo transamination to form any of the non-essential amino acids (step 2). Intracellular amino acids arising from either of the two sources are activated in a reaction requiring adenosine triphosphate (ATP) to form amino-acid adenylates (step 3), which are then bound to soluble ribonucleic acid (s-RNA) forming the s-RNA-amino acid complexes (step 4). Separate and specific s-RNA exist for each of the amino acids. In a reaction requiring guanosine triphosphate (GTP), the s-RNA-amino acids are then transferred to ribosomes, where peptide bond formation occurs, and ribonucleoproteins are formed (step 5). The completed protein (step 6) is subsequently stripped off the ribosome particle and released into the cytoplasmic portion of the cell (2).

Three conditions to be fulfilled before protein formation can proceed would be (1) the presence of enough building blocks (amino acids), (2) the presence of enough energy (ATP) as well as (3) adequate machinery for the synthesis of the protein. This machinery involves a series of extremely complicated processes, such as (1) transfer of information from the nucleus to the cytoplasm, (2) activation of amino acids, and (3) assembly of amino acids into proteins with their special tri-dimensional arrangement.

Antimetabolites in the Chick Embryo

Ackerman and Taylor (3) were probably the first investigators to apply the antivitamin technique to the developing chick embryo. They observed that 3-acetyl pyridine, an analog of niacin, inhibited embryonic development. This inhibition was reversed by simultaneous administration of niacin. Nicotinic acid and tryptophan were far less effective than niacin in reversing the antimetabolite inhibition. This evidence
suggested that tryptophan and nicotinic acid could be converted to
niacin by the chick embryo but that the process was limiting.

Naber and Largent (4) investigated the teratogenic properties of
thalidomide in the developing chick embryo. When administered in a
glycerol suspension, thalidomide significantly increased the incidence
of embryonic teratogenesis, particularly phocomelia, rumplelessness,
hydrocephalous and ectopic viscera. One milligram of the drug produced
an incidence of 2 to 5 percent malformations which was not increased by
larger amounts of the drug. The low incidence of malformation made it
difficult to interpret data from reversal studies with B-vitamins. The
nicotinic acid antagonist, 3-acetylpyridine, was found to produce
thalidomide-like malformations. However, no potentiation of teratogenesis
from 3-acetylpyridine was obtained from simultaneous administration of
thalidomide. Nicotinic acid content of the developing chick embryos was
first depressed by thalidomide administration and then increased to
levels above the controls indicating a temporary depression in nicotinic
acid synthesis due to thalidomide. The teratogenic properties of thali­
domide may be due to a temporary disruption of nicotinic acid synthesis which
affect structures undergoing rapid morphogenesis during this period.

Cravens and Snell (5) showed that desoxypridoxine was toxic for
the young chick embryo and that pyridoxal hydrochloride could prevent
the toxic condition. Pyridoxal hydrochloride was more potent than
pyridoxamine or pyridoxine analogues in preventing the inhibition.
Pyridoxal hydrochloride is involved in the synthesis of nicotinic acid
from tryptophan. It was thought that nicotinic acid might modify the
inhibitory effects of the desoxypyrdoxine. Neither nicotinic acid nor
its amide affected the inhibitory properties of the antimetabolite.
Karnofsky, et al. (6) first demonstrated a relationship between several analogs of folacin. High levels of folacin did not, however, counteract the toxic effects of the inhibitors. Snell and Cravens (7) found the desoxyribosides were capable of reversing aminopterin inhibition, thus suggesting that folacin normally functions in nucleic acid biosynthesis.

Naber, Snell, and Cravens (8) observed that aminopterin was extremely toxic to the developing chick embryo. While the vitamin folacin was incapable of preventing aminopterin toxicity, nucleic acid derivatives were found to be effective in preventing toxicity of the antimetabolite. Specifically, thymidine plus certain purine derivatives were found to be most active. These data were interpreted to mean that aminopterin prevented embryonic development by preventing biosynthesis of nucleic acid derivatives. By supplying these purine derivatives preformed to the chick embryo aminopterin lost its toxic effect and normal embryonic development took place.

Naber, et al. (9) showed that two thiamine analogs (neopyrithiamine and oxythiamine) had differential effects on chick embryonic development and growth. They suggested that thiamine might have more than one function in normal development and growth. Boggs and Shorb (10) have shown that 2,5-dimethylbenzimidazole and 2-ethyl-5-methylbenzimidazole were toxic to the developing chick embryo and that cobalamin partially reversed this effect. Naber, et al. (11) observed that glycine toxicity was prevented by folacin. Dietary glycine failed to depress the folacin content of the tissues, nor did folacin depress the blood level of glycine. Liver glycogen was depressed by feeding glycine and elevated by folacin. Serine was innocuous at levels equivalent to those that produced glycine toxicity. Tyrosine and alanine depressed growth without correction.
by folacin. Naber, et al. (12) observed in growing chicks that the addition of desoxyribonucleic acid resulted in increased weight gains only when the chick was deficient in folacin further substantiating that folacin is involved in the biosynthesis of nucleic acid derivatives.

Beta-aminopropionitrile (BAPN) increases the fragility and solubility of collagen in the chick embryo (13). BAPN acts by preventing the formation of insoluble collagen from forms soluble in neutral salt solutions (14). Thus, BAPN directly or indirectly interferes with the ordered aggregation of soluble collagen protein into the higher molecular weight insoluble collagen found in collagen fibrils. Soluble collagen can be converted to insoluble collagen fibrils by heat precipitation. Martin, et al. (15) showed that the structure of heat precipitated collagen varies markedly depending on pH and ionic strength of the medium. Katchalsky, et al. (16) studied the binding of cations and alterations in solubility of alginic acid produced by cations. They found that the binding of divalent cations produced alterations in the solubility and other physical properties of the alginate fibril. Calcium and copper ions were bound strongly and greatly changed the solubility of the polyelectrolyte. However, magnesium was poorly bound and did not change solubility characteristics.

Gross (17) showed that collagen from lathyritic animals was easily resolubilized at 5°C. after thermal gelation at 37°C., whereas collagen from normal animals was only partially resoluble under these conditions. Other physical and chemical methods failed to reveal any other differences between lathyritic and normal collagen. Hausmann (18) found that the gelatinization rates of normal and lathyritic collagen do not differ but that lathyritic collagen shows marked resolubility at 4°C. when compared
to a normal gel. Veis and Drake (19) showed that formaldehyde may be used to introduce methylene bridge crosslinks into collagen thereby converting alpha particles to the gamma form (tropocollagen). Ehrhart (20) showed that BAPN reacts with formaldehyde, acetaldehyde and pyrvaldehyde at pH 7 and inhibits formation of products between these aldehydes and certain amino acids. BAPN was also found to inhibit the in vitro synthesis of the methylene bridged compound N-(1'-acetamido-methylene) alanine from alanine, formaldehyde and acetamide. Thus BAPN may interfere with aldehyde incorporation into collagen and thereby prevent covalent crosslink formation essential to convert alpha particles to gamma particles.

Naber and Blackwood (21) found that pantothenic acid and its derivatives did not alter BAPN toxicity or co-enzyme A content of the liver in chick embryos, but that calcium as pantothenate, gluconate or chloride reduced embryonic mortality and leg abnormalities in young chicks due to the antimetabolite. Blackwood (22) reported on the changing inhibition of early differentiation and general development in the chick embryo by 2-ethyl-5-methylbenzimidazole (EMB). This compound was found to inhibit early induction or differentiation of heart and trunk but did not affect induction. Therefore this antimetabolite (EMB) probably inhibits RNA synthesis.

Blackwood and Naber (23) compared several calcium salts for their effect on BAPN toxicity. Most calcium salts partially reverse the effects of the toxic agent when administered simultaneously with BAPN, but enhance its toxicity when given 6 days prior to the lathrogen. Copper and zinc were active in reversing BAPN toxicity under the same conditions that calcium was effective. Magnesium and manganese did not alter the lethal
effect of the lathrogen. Because calcium and copper bind strongly with negative polyelectrolytes to reduce their solubility and magnesium does not, the data suggest that certain divalent ions reduced BAPN toxicity by promoting collagen insolubility.

Naber, et al. (24) found that BAPN treatment reduced uptake of calcium\(^{45}\) (\(^{45}\)Ca) into embryonic bone during the first 23 hours following BAPN administration, but increased \(^{45}\)Ca uptake during the period from 48 to 72 hours following the BAPN treatment. Collagen extracts and the residue of bone from embryos treated with BAPN 72 hours previously showed increased uptake of the radioisotope. Salt soluble collagen extracts from lathyritic embryos contained less total calcium than similar extracts from normal embryos. When both salt soluble and acetic acid soluble collagen were purified, the collagen from BAPN treated embryos contained less calcium than preparations from normal embryos. The data suggest that the increased calcium uptake of tissues due to BAPN treatment may represent a compensatory action against the toxic effect of BAPN which initially depresses calcium uptake of these tissues.

Naber et al. (25) studied the effect of calcium on collagen formation in normal and BAPN treated embryos. Calcium gluconate and/or BAPN was injected into eggs after 7 days of incubation. Three days later proline \(^{14}\)C was given to all the surviving embryos. Six hours later tissue was harvested and collagen extracts were prepared in the cold, dialyzed and analyzed for proline and hydroxyproline radioactivity, calcium and nitrogen. Results showed that the increase in soluble collagen due to BAPN treatment is largely prevented by calcium administration. More hydroxyproline \(^{14}\)C was transferred to insoluble collagen when calcium was given to BAPN-treated embryos. It appears that the
ability of calcium in counteracting BAPN toxicity is mediated by its ability to promote the conversion of soluble to insoluble collagen in lathyritic embryos. Collagen extracts from 17 day old embryos showed that the lathyritic bones contain less non-dialyzable calcium than normal extracts. The amount of non-dialyzable calcium in collagen extracts decreases with increasing extractibility of collagen in both normal and lathyritic embryos.

Naber, et al. (26) conducted a series of experiments to study the relationship of functional aldehyde groups in normal and lathyritic collagen to their resolubility following thermal gelation. After dialysis of extracted collagen against 0.2% dimedon solution to react with available aldehyde groups in the collagen, thermal gelation of both normal and lathyritic collagen becomes easily reversible. Because greater changes in resolubility due to dimedon treatment take place in normal collagen than in lathyritic collagen, it was suggested that normal collagen contains more reactive aldehyde groups than its lathyritic counterpart. Determinations of the aldehyde content of collagen showed that both crude and purified collagen extracted by saline or acetic acid had a higher aldehyde content when prepared from normal embryos in contrast to that prepared from lathyritic embryos. Hence, BAPN apparently prevents the formation of aldehyde functions essential to molecular aggregation of the collagen macromolecule.

Naber, et al. (26) reacted purified collagen with formaldehyde in the cold to convert the alpha components of collagen to beta and gamma components. This process was studied with normal and lathyritic collagen by removing samples at various time intervals and determining the relative quantities of alpha, beta and gamma components by disc gel electrophoresis. While the conversion of alpha molecules to gamma molecules takes place in
both normal and lathyritic collagen, the reaction requires considerably more time in the case of the lathyritic collagen. The reduced \textit{in vitro} reactivity of the alpha components of lathyritic collagen may account for the failure of the BAPN treated embryo to convert alpha components to structurally sound collagen \textit{in vivo}. Hence, this lathrogen reduces the reactivity of \(\alpha\)-components to form dimers and trimers which are necessary to the structure of the tropcollagen molecule. This in turn increased the fragility and solubility of the connective tissues.

**Antibiotics Affecting Protein Biosynthesis**

1. Chloramphenicol

Chloramphenicol (Figure 3) has been studied extensively, particularly because of its selective ability to inhibit protein synthesis in bacteria. Chloramphenicol also commands interest because protein synthesis in mammalian cells and in mammalian cell-free systems is much more resistant than analogous microbial systems to inhibition by the drug. Chloramphenicol appears to compete with m-RNA for binding sites on the ribosome surface thus preventing RNA attachment to the ribosome. It has also been suggested (27) that protein biosynthesis in the intact mammalian cell is susceptible to inhibition by the drug only when new m-RNA is undergoing attachment to the ribosome and that the resistance of more mature cells could be due to the fact that the RNA is already attached.

Although chloramphenicol readily inhibits protein synthesis in microbial systems, protein synthesis in mammalian systems is markedly resistant to inhibition by chloramphenicol. The difference in sensitivity to chloramphenicol is most apparent in cell-free systems where cellular permeability is not a factor. For example, complete inhibition of protein
Figure 3. Structure of Chloramphenicol.
synthesis in E. coli cell-free systems can be obtained with 0.15 μmole of chloramphenicol/ml of reaction mixture (28). In contrast comparatively little inhibition of protein synthesis in mammalian cell-free systems has been obtained with 5-10 μmoles of chloramphenicol/ml of reaction mixture (29). Similarly, inhibition of amino acid incorporation in intact mammalian cells has been obtained only with amounts of chloramphenicol which greatly exceed concentrations employed to inhibit protein biosynthesis in bacteria and mammalian cell-free systems (30, 31, 32, 33). Certain clinical observations indicate that chloramphenicol may inhibit protein synthesis by mammalian cells to a greater extent than previously suspected. Although hematologic toxicity in patients receiving chloramphenicol is relatively uncommon, susceptibility to chloramphenicol toxicity is markedly increased in conditions characterized by proliferation or maturation of immature erythrocytes. The administration of chloramphenicol to patients with pernicious anemia in relapse prevents the reticulocyte response which is normally obtained with vitamin B₁₂ (34). Presumably the initial steps in the maturation of erythrocyte precursors require the formation and deposition of ribosomal template RNA capable of directing the synthesis of hemoglobin and other proteins necessary for cellular development.

Blackwood (22) reported that chloramphenicol administered at embryonic developmental stage 10 as proposed by Hamburger and Hamilton (35) at a dosage level of 1 mg. per fertile egg appeared to inhibit differentiation of the splanchnopleure resulting in a visculation of the heart, head and ventral trunk. Chloramphenicol markedly inhibited the rate of growth and development without grossly malforming throughout the treatment period and did not generally stop development if administered before developmental stage 10 (35). Billett et al. (36) also reported
abnormal development in chick embryos treated with 200-300 µg of chloramphenicol/ml of growth media when explanted at the fall streak to head fold stage. The most obvious features of the response to the antimetabolite are a failure of the neural tube to close and an inhibition of hemoglobin formation. In order to inhibit hemoglobin formation in the area vasculosa, it was necessary to treat the embryo several hours before the hemoglobin would appear. These abnormalities were also accompanied by a reduction in the area of the blastoderm and of the area vasculosa.

Vazquez (37) reported that there is a close relationship between the binding of $^{14}$C-chloramphenicol to ribosomes and the antibacterial activity of chloramphenicol and suggested that the primary site of action of chloramphenicol in bacteria is on the 50-S component of the ribosome. Vazquez (38) suggested that the binding of chloramphenicol to ribosomes so alters the ribosomal structure that the ribosomes are no longer functional and that chloramphenicol could stop protein synthesis without showing specific competition for the binding sites on the ribosome with either m-RNA or aminoacyl s-RNA, but nevertheless preventing growth of the peptide.

2. Actinomycin D

Actinomycins (Figure 4) are bright red peptide-containing antibiotics. Actinomycin inhibits nucleic acid synthesis. Reich et al. (39) showed that actinomycin inhibits DNA directed RNA synthesis by a bonding of the antibiotic to DNA. Actinomycin inhibits strand separation of helical DNA and suppresses DNA polymerase activity.

Due to the work of Brockmann (40) and Johnson (41), the actinomycins offer unique opportunities for correlating biological activity with
Figure 4. Structure of actinomycin D.
molecular structure. Many different naturally occurring actinomycins have been isolated and characterized (40). Complex formation between actinomycin and DNA shows an absolute specificity for guanine and for a single functional group of guanine, the amino group.

A model of the structure of actinomycin-DNA complexes, based on X-ray and model building studies, was proposed by Hamilton et al. (42). According to this model (Figure 5), actinomycin is visualized as bound in the minor groove of helical DNA with which it can form up to seven hydrogen bonds under these conditions. The properties of the complex deduced from the model fit most of the known facts concerning the reaction of actinomycin with DNA and its inhibition of DNA-dependent RNA synthesis.

Pierro (43) reported that the first visible effect of treatment with actinomycin D involved cell death and degeneration in the unsegmented somite mesoderm and undifferentiated mass of the primitive streak or trunk-tail node, depending on the developmental stage of the embryo. When the antimetabolite was administered at 48 or 72 hours of incubation rumplessness and missing trunk areas were observed (44). The extent to which cell death and degeneration occurs was variable and in some instances was so extensive that development of the posterior trunk region ceased entirely; thus trunkless embryos were formed and rumpless embryos in which degeneration was less extensive were also observed.

Brachet et al. (45) reported that levels of actinomycin D which did not stop cleavage in amphibian eggs, interfered with gastrulation and especially formation of the nervous system. Heilporn-Pohl (46) using explanted chick embryos treated with 0.115 μg/ml actinomycin D observed that when treatment was begun at the primitive streak stage, the nervous
Figure 5. Model of actinomycin binding to deoxyguanosine of DNA in β configuration. Hydrogen bonds between Actinomycin and DNA are indicated by ----, hydrogen bonds between guanine and cytosine in DNA are indicated by ....
system of the chick failed to develop while the embryo treated at the early medullary plate stage showed that the posterior half of the nervous system was poorly differentiated plus a considerable degree of lysis was observed in the caudal region of the embryo.

Klein and Pierro (47) demonstrated that protein N, DNA and RNA content of the posterior fraction of embryos explanted to media containing actinomycin D was reduced when compared to normal explants. Wilson (48) showed that actinomycin D administered to rats (200 μg/Kg. body weight) resulted in fetal malformations when given on the 8th, 9th, or 10th day of gestation. Denis (49, 50, 51) found that actinomycin suppressed the competence of the ectoderm and inductor power of the blastopore lip of Pleurodeles walthia cells. He also observed radiographically that nucleic acid and protein synthesis were altered by actinomycin D. Mazurov and Orekhovich (52) showed that collagen synthesis in the chick embryo cartilage was stimulated by actinomycin as determined by tritiated proline incorporation.

Gross and Cousineau (53) treated sea urchin eggs with actinomycin D and found that the antibiotic decreased DNA, slightly decreased DNA, and had no effect on protein biosynthesis judged by bulk labeling and radioautography. Kirk (54) observed an immediate inhibition of the synthesis of RNA, followed rapidly by inhibition of protein synthesis in Staphylococcus aureus cultures. Heilporn-Pohl (46) observed that the incorporation of 3H-uridine is inhibited between 50 and 80 percent after 5 to 8 hours of treatment with actinomycin D. This effect was observed mainly in the cytoplasm. Actinomycin D also seemed to inhibit the transfer of RNA from nucleus to cytoplasm. Reich et al. (55) reported
that actinomycin D, by inhibiting DNA-dependent RNA synthesis, blocks expression of the genetic potentiabilities of the host.

Spiegel, et al. (56) studied the proteins produced during early sea urchin development and compared eggs treated with actinomycin D to normal eggs. Labeling with valine -l-^14C and separating the soluble proteins by disc gel electrophoresis resulted in seven components and seven coincidental radioactive peaks from both normal and actinomycin D treated eggs. These authors suggested that the early synthesis of soluble proteins in sea urchin eggs is carried out by preformed m-RNA contained in the unfertilized egg. Billiar et al. (57) observed the activity and electrophoretic patterns of L-malate dehydrogenase in developing sea urchin embryos. Actinomycin D had no effect on the nature or activity of the enzyme although it inhibited protein synthesis as measured by incorporation of labeled amino acids. Since L-malate dehydrogenase is present in the unfertilized egg and its activity is not altered during embryonic development, the antibiotic would not seem to affect synthesis of proteins for which preformed RNA is present in the egg prior to fertilization and antibiotic treatment.

Pierro (44) reported that DNA, deoxyguanoxine and guanosine protect to varying degrees against the teratogenic action of actinomycin D, provided the compound was premixed with the teratogen 24 hours prior to injection. Hurwitz et al. (58) reported that the inhibition of the enzymatic synthesis of DNA and RNA by actinomycin D is competitive in nature and can be reversed by increasing concentration of DNA. These authors also observed that the synthesis of RNA is sensitive to the inhibition when primers such as human marrow DNA and heated thymus DNA
were employed and no inhibition was observed if synthetic oligonucleotides or polydeoxythymidylets were employed.

Gallera (59) treated chick embryos for a short period (30-150 minutes) with actinomycin D at concentrations ranging from 0.05 to 0.25 µg/ml of explanted media. Although the actinomycin caused cytolysis of the graft, neural structures were induced in all cases. In another experiment, actinomycin always elicited neural induction, except when the host blastoderm disintegrated precociously. However, these neural inductions were rapidly arrested in their differentiation if the development of the host was strongly inhibited. Even at the lowest dose, actinomycin exhibited antimitotic properties. The peripheral expansion of blastoderms was reduced; only at higher doses was the development of axial structures actually inhibited.

3. Puromycin

Puromycin was discovered in culture filtrates of Streptomyces albo-niger on the basis of its antimicrobial activity (60). Puromycin has a broad range of biological activity, inhibiting growth of bacteria, protozoa, and mammalian cells. Although the hoped-for clinical usefulness of puromycin was never realized owing primarily to its nephrotoxicity, it has proved of great value in investigative work as a specific, reversible inhibitor of protein synthesis.

The first indication that puromycin affects protein synthesis was the observation of Creaser (61) that the antibiotic inhibits induced enzyme formation in bacteria. In growing bacteria the over-all rate of protein synthesis is also markedly reduced by puromycin, but RNA and
DNA synthesis continue at an approximately normal rate; subsequent removal of the antimetabolite results in restoration of protein synthesis and growth (62).

Studies on the mechanism of inhibition of protein synthesis by puromycin began with the observation of Yarmolinsky and de la Haba (63) that the structure of the antibiotic is related to that of an intermediate in protein synthesis, aminoacyl-sRNA. The degradative and synthetic studies of Baker et al. (64) had established the structure of puromycin as a 3'-aminonucleoside of dimethyl-adenosine with L-o-methyl tyrosine linked via an amide bond to the 3' amino group (Figure 6). The identification of aminoacyl-s-RNA, the activated intermediate of protein synthesis, as a 2' or 3' aminoacyl ester of the adenosine end of s-RNA (Figure 7) led Yarmolinsky and de la Haba (63) to propose that puromycin inhibits protein synthesis by acting as a structural analogue of esterified s-RNA.

The biosynthesis of proteins proceeds via an initial activation of each amino acid with the formation of enzyme-bound aminoacyl-adenylate residue of s-RNA with the activated aminoacyl group (reaction 1, Figure 8). The aminoacyl-s-RNA, aligned on the ribosome-template RNA complex, appears to serve as the acceptor of the peptide chain, leading to stepwise elongation of the polypeptide (reaction 2, Figure 8). Puromycin does not affect amino acid activation or the esterification of s-RNA (reaction 1, Figure 8), but does markedly inhibit the transfer of aminoacyl residues from s-RNA into long chain polypeptides, both in animals and microbial preparations (63).
Figure 6. Structure of Puromycin.
Figure 7. Structure of the adenosine end of aminoacyl-sRNA.
Figure 8. Reactions showing amino acid activation and bonding with s-RNA in protein biosynthesis.
Brachet (65) demonstrated unfavorable effects of puromycin (5 to 30 µg/ml) on development of amphibian eggs and inhibition of protein synthesis which strongly affected the tip of the alga where particular proteins are accumulated and where the incorporation of labeled methionine was considerable. Malpoix and Limbosch (66) working with chick embryos reported that puromycin (8 µg/ml of explanted medium) not only inhibited the synthesis of protein, notable hemoglobin, but also that of nucleic acid. A secondary inhibitory effect on heme synthesis was also observed. At a low level of puromycin treatment (1 µg/ml of explanted medium), the antimetabolite inhibited ribosomal RNA synthesis selectively. An inhibition of the order of 50 percent was observed for 28S RNA and of 30 percent for 16S RNA; low molecular weight RNA's (4S) were unaffected by the antimetabolite. When the level was doubled the synthesis of all types of RNA was inhibited. Brachet et al. (67) reported that puromycin (30 µg/ml of reaction mixture) inhibited nervous system differentiation in amphibian eggs. Puromycin also inhibited growth in both nucleate and anucleate halves of Acetabularia. Hultin (68) studied the inhibitory effect of puromycin on amino acid incorporation in Paracention eggs and observed that only 50 percent of the eggs ceased development in a 10^{-4} M puromycin solution after the clear streak stage of the first division while the remaining 50 percent reached a 2 cell stage.

Juva and Prockop (69) demonstrated, using gel filtration, that puromycin inhibited synthesis of hydroxyproline-containing peptides of molecular weight of 10^4 or more in collagen extracts of chick embryonic cartilage.
Billiar et al. (57) observed the activity and electrophoretic patterns of $\Delta$-malate dehydrogenase in developing sea urchin embryos and reported that puromycin had no effect on the nature or activity of the enzyme, although the antimetabolite inhibited protein synthesis as measured by incorporation of labeled amino acids. Puromycin did not alter the activity of $\Delta$-malate dehydrogenase in the developing embryo.

Duprat (70) added puromycin to a culture medium of embryonic cells of Pleurodeles walthia and Ambystoma mexicanum. He observed cytological damage of the nucleolus, chromatin and mitochondria and cellular morphological differentiation was inhibited although other cellular functions remain active. An autoradiographic study demonstrated that puromycin inhibited $^{3}\text{H}$-uridine incorporation into the nucleolus but had no effect on that into the nucleoplasm. Similarly, the RNA migration from nucleus to cytoplasm does not appear to be affected by the antimetabolite.

4. Other antibiotics

In addition to the work with the previous antimetabolites, other investigators have studied other compounds for their effects on morphogenesis and metabolism.

Siegel and Sisler (71) showed that cycloheximide inhibited the incorporation of $^{14}\text{C}$-leucine into ribosomal protein in a cell free system. These authors suggested the site of action of cycloheximide apparently involves the transfer of aminoacyl t-RNA to the ribosomes and subsequent protein formation.
Rosenkranz et al. (72), using hydroxyurea (0.1 M and 0.2 M solutions) induced a reversible inhibition of DNA synthesis. Scholtissek (73) showed that proflavine strongly inhibited the incorporation of radioactive precursors ($^{32}$P and $^{14}$C-uridine) into RNA of chick fibroblasts. The low molecular weight RNA contained high amounts of uridine monophosphate and cytosine monophosphate (84% pyrimidine) while the high-molecular weight RNA is low in guanadine monophosphate and cytosine monophosphate.

Verrett and Mutchler (74) reported a 30 percent incidence of malformations of the skeleton in chick embryos involving the spinal column in studies with streptomycin (10 mg/egg). They also reported that penicillin (2 mg/egg) gave a low incidence of a non-specific malformation. In combination with streptomycin, no increased incidence was observed; however, the combination seemed to enhance the severity of the malformation produced by the streptomycin alone. These authors also observed a 10 percent incidence of leg malformations due to oxytetracycline (10 mg/egg) treatment.

Kury and Graig (75) working with chick embryos observed many tissue and morphological abnormalities when mitomycin C (2-28 µg/egg at 72 or 96 hours of incubation) was administered. These authors observed a retardation of growth, cerebral hemorrhage, bilateral microphthalmia and malformations of the lower extremities. Histologic examination revealed necrotic changes involving the developing retina, lens, cerebral vesicles, mesenchyme and heart.

Electrophoretic Studies in the Chick Embryo Proteins

Amin (76) reported on the separation of chick embryo proteins by starch gel electrophoresis and observed 9 components or peaks in embryonic
tissue at day 10 of incubation. He reported a similarity in patterns on
day 13, 16, and 19 with 12 components present. The protein components
observed at day 10 of the developing chick embryo were also observed in
the day-old chick. Geelhoed and Conklin (77) worked with the 7 day old
chick embryos in which they separated the proteins by acrylamide gel
electrophoresis found in the serum, allantoic fluid and amnionic fluid.
They observed 9 fractions in the serum, 8 fractions in the allantoic fluid
of which 6 had identical Rf values with the serum fractions, and 7 fractions
in the amnionic fluid of which 4 had identical Rf values with the serum
fractions.

Kischer et al. (78) separated nuclear histones isolated from chick
embryos from gastrulation to 7 days of age by disc gel electrophoresis
and found no reflection of ontogenic changes in continuing embryonic
development. A gradual increase in the basic/acidic amino-acid ratio
of the histone preparations as gastrulation was completed suggested an
association of histones with other nuclear proteins during this early
stage.

Results of these studies are evidence that certain antimetabolites
do, indeed, interfere with protein biosynthesis. This interference may be
related to failures in embryogenesis which may have as their basis the
formation of inadequate amounts of protein precursors or the formation of
abnormal protein precursors which affect tissue formation.
MATERIALS AND METHODS

A series of ten experiments was conducted in this investigation. The first three experiments were designed to determine the effect of the amount of antimetabolite and the stage of embryonic development in the chick at the time the antimetabolite was administered on embryogenesis and protein formation. In experiments 4 through 8 the detailed effects of chloramphenicol on the formation of water soluble, saline-citrate soluble, and acid soluble tissue proteins were determined. In experiments 9 and 10 the effects on teratogenesis and subsequent survival and growth of the treated embryo were determined.

Untreated or control embryos were observed in each experiment. A sample of embryonic tissue consisted of the tissue from 24 embryos. Four replicates of each experimental treatment were observed. Antimetabolites used in this investigation were chloramphenicol, actinomycin D, and puromycin. Chloramphenicol and puromycin were secured from Sigma Chemical Company. The actinomycin D was secured from Mann Research Laboratories. The chloramphenicol was solubilized in a 20% ethanol solution and the actinomycin D and puromycin were solubilized in water.

1. Incubation

In all experiments chick embryos cultivated in ovo were used. Fertile eggs from White Leghorns fed complete diets (Table 1) were used as a source of embryos for this investigation. The fertile eggs were
**TABLE 1**

**COMPOSITION OF LAYER-BREEDER DIET**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amount (gm/kg of Feed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ground Yellow Corn</td>
<td>672.9</td>
</tr>
<tr>
<td>44% Soybean Meal</td>
<td>155.5</td>
</tr>
<tr>
<td>50% Meat and Bone Scrap</td>
<td>25.0</td>
</tr>
<tr>
<td>60% Dried Fish Solubles</td>
<td>15.0</td>
</tr>
<tr>
<td>17% Alfalfa Meal</td>
<td>25.0</td>
</tr>
<tr>
<td>16% Dried Whey Product</td>
<td>25.0</td>
</tr>
<tr>
<td>Dicalcium Phosphate (16% Ca; 21% P)</td>
<td>12.0</td>
</tr>
<tr>
<td>Feeding Limestone (35% Ca)</td>
<td>65.0</td>
</tr>
<tr>
<td>Iodized Salt</td>
<td>4.8</td>
</tr>
</tbody>
</table>

**Micronutrients Added**

<table>
<thead>
<tr>
<th>Micronutrient</th>
<th>Amount (mg/kg of Feed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Riboflavin</td>
<td>1.77</td>
</tr>
<tr>
<td>Pantothenic Acid</td>
<td>3.85</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>0.06</td>
</tr>
<tr>
<td>Methionine hydroxy analog</td>
<td>489.00</td>
</tr>
<tr>
<td>Butylated hydroxy toluene</td>
<td>124.00</td>
</tr>
<tr>
<td>Zinc (Zinc oxide)</td>
<td>137.50</td>
</tr>
<tr>
<td>Manganese (Manganous oxide)</td>
<td>116.50</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>I.U./kg of Feed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A</td>
<td>2,200</td>
</tr>
<tr>
<td>Vitamin D$_3$</td>
<td>1,056</td>
</tr>
</tbody>
</table>
cleansed with 70% ethanol solution and manipulated under aseptic conditions. Prior to putting the fertile eggs in a Jamesway Model 252 incubator, the incubator and trays were cleansed and then rinsed in a 800 ppm quaternary ammonia compound (Roccal). All solutions, equipment, and glassware were sterilized in a model DS-2052 steam heated Barnstead sterilizer at a chamber pressure of 15 pound per sq inch of pressure and a temperature of 121°C for 30 minutes. The incubator temperature had a range of 37.5°C - 38°C and a wet bulb range of 29.4 - 30.5°C. At the specified time for treatment of the fertile egg, the antimetabolite was injected into the egg using a 1 ml tuberculin syringe with a 27 gauge, 1/2 inch needle. The egg shell was pierced by drilling a small hole (1 mm in diameter) using a Dremel drill, Model No. 1, in the shell of the ethanol-cleansed fertile egg directly over the air cell. The antimetabolite was deposited on the inner membrane of the air cell. Following deposition of the antimetabolite on the inner shell membrane of the air cell, the egg shell was sealed with flexible collodion. The diluent that was used to solubilize the antimetabolite was deposited on the inner membrane of the air cell of the control group of chick embryos.

2. Embryo harvest.

After a total of 120 hours of incubation, the developing eggs were removed from the incubator. The embryo was removed from the fertile egg by breaking the shell with a Tri-R Egg Punch, peeling back the inner shell membrane and removing the embryo with a pair of tweezers. The allantoic and amnionic sac were separated from the embryo and the external surface of the embryo rinsed five times with sterile distilled water to remove any foreign protein which might conceivably be adhering
to the embryo. The embryo was then sectioned into (1) head segment, (2) body cavity, and (3) the limb buds and tail node (appendage buds). These segments were then centrifuged (100 x G) for 5 minutes at 2°C. to remove excess liquids from the external surface of the segmented tissue.

3. Preparation of Protein Extracts

Different procedures were employed in the extraction of the tissue proteins but in general the methods of Shepherd and Gurley (79) and Kischer et al. (78) were used with slight modifications. The tissue proteins extracted in experiments 1, 2, and 3 were homogenized only with water. In experiments 4, 5, and 6 the extractions were made as shown in Figure 9. The extractions in experiment 7 were made as shown in Figure 10. Figure 11 demonstrates the protein extraction procedure for experiment 8. In general, the segments were homogenized in a Tenbroeck tissue grinder with a pestle clearance of 0.004 inches and the proteins homogenized with water (2.5 ml of H2O/gm of tissue). This homogenate was centrifuged in a Sorvall RC2-B automatic refrigerated centrifuge (30,000 x G) for 20 minutes at 2°C. The precipitate was re-extracted three times by homogenization in the water solution with subsequent centrifugation. The supernatant was collected and stored under refrigeration (-20°C.) for not longer than 48 hours before analysis.

The precipitate remaining from the water extraction was homogenized in a solution containing 0.14 M NaCl and 0.01 M sodium citrate at pH 5.8 (2.5 ml/gm of tissue). The homogenate was centrifuged (30,000 x G) for 20 minutes at 2°C. The precipitate was re-extracted three times by homogenization in the saline-citrate solution with subsequent centrifugation. The supernatant was collected and stored under refrigeration (-20°C.) for no longer than 48 hours before analysis.
Figure 9. Diagram of embryonic chick tissue extractions for experiment 6.
Homogenized Embryonic Tissue
Extraction with water (3x)
Centrifuged (30,000 x G) 20 minutes, 2°C (3x)

Supernatant
(water soluble proteins)

Precipitate

Extraction with 0.14 M NaCl - 0.01 M Na Citrate (3x)
Centrifuged (30,000 xG) 20 minutes, 2°C (3x)

Supernatant
(acid soluble proteins)

Precipitate
discarded

Extraction with pH 1.0, H₂SO₄
Centrifuged (30,000 xG) 20 minutes, 2°C (3x)

Supernatant
discarded

Precipitate

Add pH 1.0, H₂SO₄ to solubilize

Acid saline-citrate soluble proteins

Figure 10. Diagram of embryonic chick tissue extractions for experiment 7.
Figure 11. Diagram of embryonic chick tissue extractions for experiment.
Homogenized Embryonic Tissue

1. Extraction with saline-citrate (3x) G) 20 minutes, 2°C (3x) centrifuged (30,000 x G) 20 minutes, 2°C (3x)
   - Supernatant discarded
   - Supernatant (Water soluble Proteins)

2. Extraction with water (3x)
   - Supernatant discarded

3. Extraction with 0.14 M NaCl - 0.01 M Na Citrate (3x) centrifuged (30,000 x G) 20 minutes, 2°C (3x)
   - Supernatant
   - Supernatant (Saline citrate discarded solute proteins)
   - Precipitate discarded

4. Extraction with 0.03 M Na Citrate (3x) centrifuged (30,000 x G) 20 minutes, 2°C (3x)
   - Supernatant
   - Supernatant (Citrate soluble proteins)
   - Precipitate discarded

5. Add 2.8 volumes of cold 95% ethanol
   - Store at 5°C, 18 hours
   - Centrifuged (16,000 x G), 15 minutes 2°C

6. Washed with cold absolute ethanol
   - Resuspend in water, 2°C
   - Store at 5°C, 30 minutes
   - Centrifuged (38,000 x G) 1 hour, 2°C
   - Supernatant discarded
   - Precipitate discarded

Tissue extractions for experiment R.
The precipitate was washed three times with sterile distilled water and then resuspended and extracted with pH 1.8, H$_2$SO$_4$ (2.5 ml per gram of tissue). This homogenate was centrifuged (30,000 x G) for 20 minutes at 2°C. In experiment 8 the precipitate was subsequently re-extracted by homogenization in a pH 1.0, H$_2$SO$_4$ solution and re-extracted a third time in a pH 0.6, H$_2$SO$_4$ solution with subsequent centrifugation. The precipitate remaining after the final extraction with acid was discarded. The three acid supernatants were pooled, 2.8 volumes of cold 95% ethanol were added, and the mixture allowed to stand for 18 hours at 4°C. The white precipitate which formed was collected by centrifugation (16,000 x G) for 15 minutes at 2°C, washed with cold absolute ethanol (2.5 ml per gram of tissue), and resuspended in distilled water (1.0 ml per gram of tissue). After 0.5 hour, the suspension was centrifuged (38,000 x G) for 1 hour, the clear supernatant was collected and stored under refrigeration (~20°C.) for not longer than 24 hours before analysis.

In experiment 8 the extraction of the proteins was accomplished without first extracting the water soluble proteins as shown in Figure 11. In addition the extraction of saline-citrate soluble proteins was accomplished by homogenization using a 0.45 M NaCl solution followed by a 0.03 M Na citrate solution.

4. Polyacrylamide gel electrophoresis

A. Water soluble protein extracts

The method of Ornstein (80) and Davis (81) with slight modifications was used to electrophoretically separate the water soluble proteins. The technique involves the use of 7% acrylamide gels at a pH 8.9 with a pH 8.3,
0.15 M glycine cathode buffer with 0.00012% bromophenol blue dye and a pH 4.0, 0.15 M glycine anode buffer. Electrophoresis was carried out in a Canalco Model 24 disc electrophoresis apparatus from cathode to anode for approximately 60 minutes at 3.3 mA per gel. This apparatus is designed to hold 24 columns (inside diameter 5 mm). In these experiments a column length of 6.5 cm was used with a separating gel length of 5.0 cm. Duplicate samples were subjected to electrophoretic separation of the proteins. Gel stock solutions and working solutions were prepared as indicated in Table 2, and attempts were made to stay within the bounds on gel solutions (Table 3) set up by Williams and Reisfield (82).

Sample gel was prepared by mixing 150 μl of sample with 0.50 ml of stacking gel solution. The sample gel (0.20 ml) was placed directly in a sample column base cap. A glass sample column (5 mm x 6.5 cm) was inserted into this cap as far as it would go and adjusted so that the column was exactly vertical. Distilled water (0.10 ml) was added to the column to form a layer over the sample gel mixture eliminating the meniscus which would otherwise cause curved bands of separated components. The sample gels were polymerized by positioning the photopolymerizing light source as close as possible to the gel. Polymerization (a slight opalescence) was completed in approximately 30 minutes.

The water layer was removed and the polymerized gel was rinsed 3 times with the rinse solution. The column was rocked gently so that the rinse solution contacted the entire inner surface of the column. The rinse solution was removed by careful aspiration using a hypodermic needle connected to a water pump to insure complete removal of the rinse solution. Stacking gel solution (0.15 ml) was added to the column.
### TABLE 2
COMPOSITION OF SOLUTIONS USED FOR ELECTROPHORETIC SEPARATION
OF WATER SOLUBLE PROTEIN EXTRACTS

<table>
<thead>
<tr>
<th>Stock Solutions</th>
<th>Solution A</th>
<th>Solution B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 N HCl</td>
<td>1 N HCl</td>
</tr>
<tr>
<td></td>
<td>36.3 gm</td>
<td>5.98 gm</td>
</tr>
<tr>
<td></td>
<td>H₂O to make</td>
<td>H₂O to make</td>
</tr>
<tr>
<td></td>
<td>100 ml</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

|                 | Solution C | Solution D |
|                 | Acrylamide | Acrylamide |
|                 | 28.0 gm    | 10 gm      |
|                 | 0.735 gm   | 2.5 gm     |
|                 | H₂O to make| H₂O to make|
|                 | 100 ml     | 100 ml     |

|                 | Solution E | Solution F |
|                 | Riboflavin | Sucrose    |
|                 | 4.0 mg     | 40 gm      |
|                 | H₂O to make| H₂O to make|
|                 | 100 ml     | 100 ml     |

|                 | Solution G | Solution H (Buffer) |
|                 | Ammonium persulfate | Tris |
|                 | 0.14 gm | 3.0 gm |
|                 | H₂O to make | Glycine |
|                 | 100 ml | 14.4 gm |

|                 | H₂O to make | 1000 ml |
|                 |             |         |

**Specimen Stain**
Anallne Black 0.5 gm
7% Acetic Acid to make 100 ml

<table>
<thead>
<tr>
<th>Working Solutions</th>
<th>Separating gel solution</th>
<th>Stacking gel solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 part (A)</td>
<td>1 part (B)</td>
</tr>
<tr>
<td></td>
<td>2 parts (C)</td>
<td>2 parts (D)</td>
</tr>
<tr>
<td></td>
<td>1 part H₂O</td>
<td>1 part (E)</td>
</tr>
<tr>
<td></td>
<td>To form gel, combine</td>
<td>4 parts (F)</td>
</tr>
<tr>
<td></td>
<td>1:1 with (G)</td>
<td></td>
</tr>
</tbody>
</table>

1. **Tris** = 2-Amino-2-Hydroxymethyl 1-3 propanediol
2. **Temed** = N, N, N', N' - Tetramethylethylenediamine
3. **Bis** = N, N¹-methylenebisacyrlamide
### TABLE 3
**BOUNDS OF DISC ELECTROPHORESIS SYSTEM**

<table>
<thead>
<tr>
<th>Anionic system with running gel pH of 8.9</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$pK_a$ of trailing ion</td>
<td>8.9 - 9.9</td>
</tr>
<tr>
<td>pH of sample gel</td>
<td>6.8 - 7.8</td>
</tr>
<tr>
<td>$pK_a$ of buffer</td>
<td>7.9 - 8.9</td>
</tr>
<tr>
<td>pH of electrode buffer</td>
<td>8.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cationic system with running gel pH of 4.3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$pK_a$ of trailing ion</td>
<td>3.3 - 4.3</td>
</tr>
<tr>
<td>pH of sample gel</td>
<td>5.6 - 6.6</td>
</tr>
<tr>
<td>$pK_a$ of buffer</td>
<td>4.3 - 5.3</td>
</tr>
<tr>
<td>pH of electrode buffer</td>
<td>5.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cationic system with running gel pH of 2.3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$pK_a$ of trailing ion</td>
<td>1.6 - 2.3</td>
</tr>
<tr>
<td>pH of sample gel</td>
<td>5.0 - 6.0</td>
</tr>
<tr>
<td>$pK_a$ of buffer</td>
<td>2.3 - 3.3</td>
</tr>
<tr>
<td>pH of electrode buffer</td>
<td>4.0</td>
</tr>
</tbody>
</table>
and layered with 0.10 ml of distilled water. The column was placed approximately 30 cm from the photopolymerizing light source and as soon as photopolymerization began (as evidenced by the appearance of opalescence about 15 minutes after exposure was started), the column was moved close to the light source for an additional 15 minutes to insure total polymerization. Rapid polymerization results in a gel of uneven pore size and in poor sample separation. The water layer was removed and rinsed as previously described.

Equal parts of separating gel solution and solution G (catalyst) were mixed by gently swirling and the column was filled with this mixture, adding enough excess gel solution to form an inverted meniscus at the top. The column was sealed at the top by gently positioning a patch of aluminum foil approximately 10 mm x 10 mm to form a flat surface. The column was protected from light and permitted to stand undisturbed in darkness for 30 minutes while chemical polymerization occurred. The aluminum foil was removed. The sample column base was removed carefully from the column to avoid tearing or deforming the gel.

The column was inserted in the upper bath column adapter with the sample end toward the cathode. The upper end of the column was flush with the top surface of the adapter. A drop of anode buffer was placed on the bottom of the surface of each column to prevent entrapment of air bubbles with the anode buffer. A drop of buffer was placed on top of this gel to insure that no air bubbles are entrapped with the cathode buffer. The cathode buffer assembly was put into position so the lower ends of the column extended approximately 1 cm below the surface of the anode buffer. The lid was inserted over the cathode buffer assembly
and the electrodes attached and the current supply adjusted till a reading of 3.3 mA per tube was reached. Excessive current causes ohmic heating within the gel, resulting in band distortion.

Within a few minutes after starting the flow of current, a thin disc of tracking dye passes through the interface between the stacking and separating gels this thin disc divides into two distinct discs. The leading disc is the free-bromophenol blue dye; the trailing disc is a dye-albumin complex. Electrophoresis was continued until the tracking dye band was approximately 0.5 cm from the bottom of the column. During electrophoresis, separation of many protein components can be observed by the formation of refractile lines in the separating gel.

At the conclusion of the run, the lid and cathode bath assembly were removed. The buffer was drained from this assembly. The column was removed from the upper bath column adapter and the gel was removed with the aid of a syringe and needle on a tygon tube attached to a water line under very low pressure. The needle was inserted between the glass and the gel and as the column was rotated the gel came free and slipped out of the column. During this procedure the needle must be kept flat against the glass surface to avoid scratching the gel. This gel was placed in a 10 x 75 mm test tube containing approximately 2 ml of specimen stain containing anlaine black (Amido-Schwarz) for a period of 1 hour. The protein fractions in the gel will diffuse rapidly if they are not properly fixed.

After staining was complete, the excess stain was decanted from the test tube and the gel was rinsed twice with 7% acetic acid solution. The stained gel was transferred to a previously prepared destaining tube,
plugged with 0.25 ml of stacking gel with the sample end toward the cathode. The destaining tubes were inserted in the bath assembly, (care being taken to insure that no air bubbles exist in the destaining tubes) 7% acetic acid (1400 ml) is then put in each assembly and the current supply adjusted until a reading of 8.3 mA per tube is reached. When the sections of the gel containing no separated fractions are entirely clear of stain, the power supply is disconnected, gels removed from the destaining tube and transferred to test tubes (10 x 75 mm), filled with 7% acetic acid and stoppered for future viewing and storage.

B. Saline-citrate soluble protein extracts

The methods of Nagai et al. (83) and Clark and Veis (84) were used with slight modifications. The technique involves the use of 7.5% acrylamide gel at pH of 4.3 with a pH 5.0, 0.3 M glycine cathode buffer and a pH 5.0, 0.3 M beta alanine anode buffer. Electrophoresis is carried out from anode to cathode for approximately 60 minutes in a cold room (4°C.) at 3.3 mA per gel.

Gel stacking solution and working solutions were prepared as indicated in Table 4, staying within the bounds on gel solutions (Table 3) as set up by Williams and Reisfield (82).

The gel column is constructed in the same manner as previously described except that photopolymerization time is increased to approximately 1 hour and is carried out in a cold room (4°C.). The tracking dye will not migrate to the cathode, so it was necessary to follow the process of the separation of the protein components by closely observing the movement of the leading refractile line in the separating gel. In experiment 8, the separating gel was stacked as shown in Figure 12.
TABLE 4

COMPOSITION OF SOLUTIONS USED FOR ELECTROPHORETIC SEPARATION OF SALINE SOLUBLE PROTEIN EXTRACTS

Stock solutions: Same as for the Standard 7% gel, pH 8.9 except:

<table>
<thead>
<tr>
<th>Solution A</th>
<th>Solution B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 N KOH 48 ml</td>
<td>1 N KOH 48 ml</td>
</tr>
<tr>
<td>Glacial acetic acid 17.2 ml</td>
<td>Glacial acetic acid 2.87 ml</td>
</tr>
<tr>
<td>Temed 4.0 ml</td>
<td>Temed 0.46 ml</td>
</tr>
<tr>
<td>H₂O to make 100 ml</td>
<td>H₂O to make 100 ml</td>
</tr>
<tr>
<td>pH:4.3</td>
<td>pH:6.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solution C</th>
<th>Solution G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide 30 gm</td>
<td>Ammonium Persulfate 0.28 gm</td>
</tr>
<tr>
<td>Bis 0.8 gm</td>
<td>H₂O to make 100 ml</td>
</tr>
<tr>
<td>H₂O to make 100 ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solution H (Anode)</th>
<th>Solution H (Cathode)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta Alanine 31.2 gm</td>
<td>Glycine 28.1 gm</td>
</tr>
<tr>
<td>Glacial Acetic Acid 8.0 ml</td>
<td>Glacial Acetic Acid 2.06 ml</td>
</tr>
<tr>
<td>H₂O to make 1000 ml</td>
<td>H₂O to make 1000 ml</td>
</tr>
<tr>
<td>pH:5.0</td>
<td>pH:5.0</td>
</tr>
</tbody>
</table>

Working Solutions

Same as described in Table 2.
Figure 12. Arrangement of gel concentrations for a high resolution run of the \( \beta \) and higher polymer components of collagen.
Extreme care had to be used in the removal of the gel from the column following electrophoresis to avoid gel separation at the interface between the different gels.

C. Acidic and/or histone protein extracts

The methods of Shepherd and Gurley (85), Gurley and Shepherd (86), Reisfield et al. (87), and Bustin and Cole (88) were all employed with slight modifications. This technique involves the use of a 15% acrylamide gel at a pH of 4.3 with a pH 4.0, 0.3 M glycine cathode buffer and a pH 4.0, 0.3 M beta alanine anode buffer. Electrophoresis is carried out from anode to cathode for approximately 60 minutes at 3.3 mA per gel.

Gel stock solution and working solutions were prepared as indicated in Table 5. The gel column is constructed in the same manner as previously described in the saline-citrate protein separation except for the separating gel.

5. Microdensitometric evaluation of the protein bands in the gels.

The stained gels were inserted in a carrier and placed in a Joyce-Lobel chromoscan microdensitometer. The instrument was used with a 2.0 optical density wedge, an Amido-Schwarz dye deflection cam, an aperture slit openings of 0.1 mm in width by 0.5 mm height, a gain control setting of 5, an Amido-Schwarz dye-matched color filter, and a 100 watt tungsten lamp. All gels were scanned by transmittance.

The gel was scanned at a speed of 0.18 mm/sec. Any imbalance between the reference light beam and the sample beam will activate a signal in the photomultiplier, which is amplified to drive the optical wedge and a response from the recorder pen will give a microdensitometric tracing of
TABLE 5

COMPOSITION OF SOLUTIONS USED FOR ELECTROPHORETIC SEPARATION OF ACID SOLUBLE PROTEIN EXTRACTS

Stock solutions: Same as for the 7.5% gel, pH 4.3 except:

<table>
<thead>
<tr>
<th>Solution C</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>60 gm</td>
<td></td>
</tr>
<tr>
<td>Bis</td>
<td>0.4 gm</td>
<td></td>
</tr>
<tr>
<td>H₂O to make</td>
<td>100 ml</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solution H (anode)</th>
<th></th>
<th>Solution H (cathode)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta alanine</td>
<td>31.2 gm</td>
<td>Glycine</td>
<td>28.1 gm</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>12.0 ml</td>
<td>Glacial acetic acid</td>
<td>3.05 ml</td>
</tr>
<tr>
<td>H₂O to make</td>
<td>1000 ml</td>
<td>H₂O to make</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH:4.0</td>
<td></td>
<td>pH:4.0</td>
<td></td>
</tr>
</tbody>
</table>

Working solutions: Same as described in Table 2.
the protein components separated in the gel. The instrument is equipped with an integrator that counts at a rate proportional to the pen deflections and has a range of 250:1 over the 150 mm length of the pen excursions. Duplicate tracings were made of each of the stained gels.

Following scanning, the area under each of the peaks was determined by measuring the height of the component tracing and the width at a point which was 1/2 the height (Area = height x width at 1/2 height). The integrator counter on the microdensitometer was not employed in these experiments as the components of the gel tracing with a Rf value of less than 0.50 had a great deal of overlapping. Due to this difficulty it was impossible to determine the amount of protein attributable to the overlap and the amount which was for that particular component.

The Canalco electrophoresis apparatus, the gel composition, and the Joyce-Lobel chromoscan microdensitometer used in these experiments were selected because of the high resolution attainable by this equipment.
RESULTS

The first three experiments were designed to explore the effect of chloramphenicol, actinomycin D, and puromycin on embryonic development and protein formation. Factors which were studied included: (1) Dosage level of the antimetabolites to produce teratogenesis. (2) Stage in embryogenesis at the time of administration of the antimetabolite. (3) Stage in embryogenesis at which teratogenesis can be observed, both grossly and with the aid of a low power microscope. (4) Nature of malformations and their relationship to protein biosynthesis and growth inhibition of existing structures. (5) Effect of the antimetabolites on the physical and chemical nature of proteins produced by the developing chick embryo by polyacrylamide gel electrophoresis.

The dosage of the antimetabolites was varied in the first three experiments. Chloramphenicol was administered as previously described at levels of 0.5 mg, 1.0 mg, and 2.0 mg per developing embryo. Actinomycin D and puromycin were administered at levels of 0.25 µg, 0.5 µg, 1.0 µg, and 2.0 µg per developing embryo. The treatments were administered at 72 hours of incubation. Blackwood (21) used 0.5 mg and 1.0 mg of chloramphenicol in her investigation of the antimetabolites and used sterile distilled water as a diluent while injection 0.1 ml to obtain this amount of chloramphenicol; however, the solubility of this antibiotic is 2.5 mg/ml of water (89). This
indicates that Blackwood used a suspension while the present study employed the same amount of 20% ethanol solution to solubilize the anti-metabolite.

The chick embryos were harvested after 120 hours of incubation except in experiment 3 where the embryos were harvested at 96 hours. Harvesting chick embryos at 96 hours of development was a more difficult task. The embryo at this stage of development weighed only 50 mgs (1), and sectioning of the embryo was rather difficult. The allantoic and amnionic sacs were more difficult to remove without disturbing tissue within the developing embryo and increased the time required to harvest the embryos. This created a variable which affected the results. During the 96 to 120 hour period of development, the size of the embryo increases nearly 3 fold (1).

Chloramphenicol

Gross and microscopic examination of the embryos that were treated with chloramphenicol (1.0 mg) revealed the following defects: (a) omphalocephaly, (b) hydrocephaly (c) unclosed neural canal of the trunk region, (d) retarded or missing appendage buds, (3) amnion malformation, and (f) embryonic size reduced to approximately 1/3 the size of the control. Chloramphenicol appeared to retard development without disturbing differentiation as much as the other two antimetabolites studied. The interruption of differentiation was expressed in a limited number of types of malformations most of which involved vesiculation resulting in vesicles at the sides of the trunk neural canal, vesicular ventral trunk, and possibly a vesicular heart.

Microdensitometric tracings of the stained polyacrylamide gels (Figure 19) resulting from the electrophoretic separation of the protein
extracts of chloramphenicol (1.0 mg at 72 hrs.) treated embryos from experiment 1 (Figures 16-18) reveal a reduction in the number of high mobility proteins when compared with the control group (Figure 13-15). Components 2-4, 6 and 7 are missing from the head segment tracings of the chloramphenicol (1.0 mg) treated embryos which were present in the control embryos (Figure 13). The microdensitometric tracings of the stained polyacrylamide gels derived from the body cavity-section of the treated embryos (Figure 17) showed additional missing components. Nine of the higher mobility components present in the controls (Figure 14) are absent in the treated group (Figure 17). The microdensitometric tracings of the polyacrylamide gels derived from the appendage buds of the treated embryos (Figure 18) presented a tracing closely resembling what was observed in the head segment. Component 5 is the only additional component which is absent in the chloramphenicol (1.0 mg) treated embryo that is present in the control group (Figure 15) of the high mobility proteins.

When embryos were treated with 2.0 mg of chloramphenicol per embryo, 96% of the embryos treated at 72 hours of incubation did not survive beyond 84-96 hours of development. Those that did survive were examined but there was not enough tissue for study of protein patterns. The embryos which were treated with 0.5 mg of chloramphenicol developed without teratological effects.

Embryos which were not treated and those which were treated only with the diluent used in solubilizing the antimetabolite were the same size, showed no morphological structure impairment, and microdensitometric tracings of the proteins were identical. Thus the control groups were identical.
Figure 13. Microdensitometric tracings of amido-Schwarz stained polyacrylamide gels of the head segment of untreated 120 hour old chick embryos.
Figure 14. Microdensitometric tracings of amido-Schwarz stained polyacrylamide gels of the body segment of untreated 120 hour old chick embryos.
Figure 15. Microdensitometric tracings of amido-Schwarz stained polyacrylamide gels of the appendage buds of untreated 120 hour old chick embryos.
Figure 16. Microdensitometric tracings of amido-Schwarz stained polyacrylamide gels of the head segment of chloramphenicol (1.0 mg) treated 120 hour old chick embryos.
Figure 17. Microdensitometric tracings of amido-Schwarz stained polyacrylamide gels of the body segment of chloramphenicol (1.0 mg) treated 120 hour old chick embryos.
Figure 18. Microdensitometric tracings of amido-Schwarz stained polyacrylamide gels of the appendage buds of chloramphenicol (1.0 mg) treated 120 hour old chick embryos.
Figure 19. Amido-Schwarz stained electrophoretic separated gel of water soluble proteins from the head segment of untreated 120 hour old chick embryos.
Gross and microscopic examination of embryos that were treated with 1 \( \mu g \) actinomycin D revealed the following abnormalities: (a) retardation of growth in excess of what was observed in the chloramphenicol embryos, (b) unclosed neural canal of the trunk region, (c) cerebral hemorrhage, (d) hemorrhage within the body cavity, especially in area of kidney and liver development, (e) retarded or missing appendage buds, (f) inhibition of notochord formation and (g) apparent cell death.

The extent to which cell death or degeneration occurred was quite variable. Some embryos appeared as if the development of the posterior trunk region ceased entirely, others showed no cell death while yet other embryos showed some growth and nerve formation but exhibited absence of structures dorsal to the tail node. Actinomycin D treated embryos exhibited more undifferentiated mass of the primitive streak or trunk-tail node areas than the chloramphenicol treated embryos.

The embryos that were treated with 0.5 \( \mu g \) actinomycin D exhibited little effect from the antimetabolite. Apparently the passage of this small dose across the inner membrane was impaired or its dilution in the albumen after transport across the inner membrane was so great that less than 5\% of the embryos were affected. In those embryos treated with 0.25 \( \mu g \) of actinomycin D, no teratogenic effects could be observed. Those embryos treated with 2.0 \( \mu g \) of actinomycin D died within 2 hours of the treatment with the antimetabolite as evidenced by lysis of the tissue.
Microdensitometric tracings of the stained polyacrylamide gels resulting from electrophoretic separation of the protein extracts of actinomycin D (1.0 µg) treated embryos (Figures 20-22) reveals a different type of pattern than that which was observed in the chloramphenicol (1.0 mg) treated embryos. Component 12 from the head segment (Figure 20), component 21 from the body cavity (Figure 21) and component 13 from the appendage bud segments (Figure 22) show a predominate change in the amount of protein present in these components when they are compared with the segment control groups (Figures 13-15).

These three components of the polyacrylamide gels designated above were removed, resolubilized and diluted. This diluted protein extract was again subjected to electrophoretic separation. Only one component was present in this microdensitometric tracing. Thus, only one component was present in the original tracing of the original protein separations of these segment tissues.

The only effect of the antimetabolite actinomycin D, on protein synthesis in the high mobility portion appears to be in the appendage bud segment (Figure 22) where components 2 and 6 are missing when compared to the control embryos (Figure 15).

Puromycin

Gross and microscopic examination of embryos which were treated with 0.5 µg of puromycin revealed the following defects: (a) retardation of growth and (b) inhibition of further nervous system development following treatment. The embryo appeared as if cell division or growth ceased shortly after the administration of the antimetabolite. No effect could
Figure 20. Microdensitometric tracings of amido-Schwarz stained polyacrylamide gels of the head segment of actinomycin D (1.0 µg) treated 120 hour old chick embryos.
Figure 21. Microdensitometric tracings of amido-Schwartz stained polyacrylamide gels of the body segment of actinomycin D (1.0 μg) treated 120 hour old chick embryos.
Figure 22. Microdensitometric tracings of amino-Schwarz stained polyacrylamide gels of the appendage buds of actinomycin D (1.0 μg) treated 120 hour old chick embryos.
be observed from treatment with 0.25 μg of puromycin and the embryos treated with 1.0 μg and 2.0 μg of puromycin survived only 1 to 2 hours post treatment. Microdensitometric tracings of the stained polyacrylamide gels from embryos treated with 0.5 μg at 72 hours (Figures 23, 24 and 25) exhibited patterns similar to those for the chloramphenicol treated embryos for the head and appendage buds but not for the body segment.

After evaluation of the first three experiments, it was decided that more consistent results were achieved by treating embryos with chloramphenicol. Not only was the consistency of the results obtained with chloramphenicol more reproducible, but there appeared to be a greater dose range over which teratogenesis began and embryonic death occurred than was the case with either actinomycin D or puromycin.

Therefore, studies with chloramphenicol should yield more useful information about protein formation and its relationship to teratogenesis than either actinomycin D or puromycin without inducing death.

Time-Study Effect

The next two experiments were designed to study the changes in protein patterns following administration of chloramphenicol. In experiment 4, embryos were treated with 0.5 mg and 1.0 mg of chloramphenicol at 24, 36, 48, 72, 84, and 96 hours of development and the embryos from all previous treated groups were harvested at 72, 84, 96, 108 and 120 hours of development. The embryos treated at 24 and 36 hours did not survive more than 12 hours following treatment of the antimetabolite. The 48 and 72 hour treatment groups were very similar to those observed in the first three experiments. The retardation of growth and a hemorrhagic condition in the liver and kidney areas were the only observed malformations in the 84, 96, and 108 hour treatment
Figure 23. Microdensitometric tracings of amido-Schwarz stained polyacrylamide gels of the head segment of puromycin (0.5 μg) treated 120 hour old chick embryos.
Figure 24. Microdensitometric tracings of amido-Schwarz stained polyacrylamide gels of the body segment of puromycin (0.5 μg) treated 120 hour old chick embryos.
Figure 25. Microdensitometric tracings of amido-Schwarz stained polyacrylamide gels of the appendage buds of puromycin (0.5 μg) treated 120 hour old chick embryos.
groups; however, embryonic growth of the 84 hour treatment group was about 1/2 of the control group and the 96 hour treatment group was approximately 2/3 the size of the control group while embryos in the 108 hour treatment group were approximately the same size as those in the control group. A problem was experienced in harvesting embryos having five different treatments. Sometimes within a treatment group embryonic size would increase as much as one fold during the period of time it took to harvest a treated group and its replicates. This increase in size is accompanied by a change in the separated protein pattern even within a treatment group. This changing protein pattern made it impossible to interpret any results between treatment groups.

The results of experiment 4 showed that new protein components were being formed from 72 to 120 hours of development. Control embryos which were harvested at 72 hours, showed only 24 of the components that were present in 120 hour embryo. The tracing of the control embryos harvested at 84 hours showed 14 new components that were not present on the 72 hour tracing. Components 2-8, 10-12, 17, and 20-22 (Figure 26) were the new components not observed at 72 hours. The tracing of the 96 hour embryo revealed 3 new protein components that were not present at 84 hours. The new components were 14, 15, and 16. No new components were observed in the extract during the period between 96 hours and 120 hours of embryonic development.

In experiment 5, the embryos were treated with 1.0 mg of chloramphenicol at 48, 72, 84, 90, 96, 99, 102, 105, 108, 111, 114 and 117 hours of incubation. The embryos were all harvested at 120 hours of
Figure 26. Microdensitometric tracings of amino-Schwarz stained polyacrylamide gels from the head segment of water soluble protein extracts of untreated 120 hour old chick embryos.
development. Hence the changes in the pattern of proteins due to age of the embryo at the time of harvest were eliminated. The tracings of the electrophoretic separated proteins were more reproducible due to both a staggering of groups and also to the greater the amount of embryonic tissue at 120 hours of development. The gross and microscopic examination of treated embryos revealed (a) omphalocephaly, (b) hydrocephaly, (c) unclosed neural canal of the trunk region, (d) retardation of growth in embryos treated prior to 108 hours, (e) retarded or missing appendage buds in groups treated before 72 hours of development, (f) amnion malformation and (g) hemorrhagic areas in the liver and kidney areas in groups treated after 102 hours of development.

A typical microdensitometric tracing (Figure 26) of stained gels from the electrophoretic separation of the water soluble proteins was obtained from head segments of untreated 120 hour embryos. More components are distinguishable in this tracing than were observed in the previous series of experiments, because adjustments were made in sensitivity of the Joyce Lobel chromoscan microdensitometer. It was quite obvious that component 1 was present in every tracing and that it was present in an amount related to total protein present. It became evident that this component could be used as an internal control and that it could be used as a standard with which to compare the other protein components in the system. Hence it was found convenient to compare the amounts of proteins in each peak to the amount present in peak 1 in the form of a ratio. Figures 27-36 show these ratios for the control and the chloramphenicol (1.0 mg) treated embryos which were treated at 48, 72, 84, 90, 96, 108, 111, 114 and 117 hours of development which were all harvested
Figure 27. Ratios of the amounts of protein present in various protein components to component 1 from microdensitometric tracings of water soluble tissue proteins from untreated 120 hour old chick embryos.
Figure 28. Ratios of the amounts of protein present in various protein components to component 1 from microdensitometric tracings of water soluble tissue protein from 120 hour old chloramphenicol (1.0 mg) treated chick embryos administered at 117 hours of development.
Figure 29. Ratios of the amounts of protein present in various protein components to component 1 from microdensitometric tracings of water soluble tissue proteins from 120 hour old chloramphenicol (1.0 mg) treated chick embryos administered at 114 hours of development.
The graph illustrates the ratio distribution across different components.

- **Head Segment**
- **Body Segment**
- **Appendage Buds**

The components are labeled from 3 to 39, with peaks indicating higher ratios. Component 19 and Component 26 show notable spikes, suggesting significant ratios in these categories.
Figure 30. Ratios of the amounts of protein present in various protein components to component 1 from microdensitometric tracings of water soluble tissue proteins from 120 hour old chloramphenicol (1.0 mg) treated chick embryos administered at 111 hours of development.
Graph showing the ratio of different components:

- Head Segment
- Body Segment
- Appendage Buds

The graph has a vertical axis labeled 'Ratio' and a horizontal axis labeled 'Component'. The x-axis ranges from 3 to 39, with specific labeled components at 19, 25, 27, and 28.
Figure 31. Ratios of the amounts of protein present in various protein components to component 1 from microdensitometric tracings of water soluble tissue proteins from 120 hour old chloramphenicol (1.0 mg) treated chick embryos administered at 108 hours of development.
Figure 32. Ratios of the amounts of protein present in various protein components to component 1 from microdensitometric tracings of water soluble tissue proteins from 120 hour old chloramphenicol (1.0 mg) treated chick embryos administered at 96 hours of development.
Figure 33. Ratios of the amounts of protein present in various protein components to component 1 from microdensitometric tracings of water soluble tissue proteins from 120 hour old chloramphenicol (1.0 mg) treated chick embryos administered at 90 hours of development.
Figure 34. Ratios of the amounts of protein present in various protein components to component 1 from microdensitometric tracings of water soluble tissue proteins from 120 hour old chloramphenicol (1.0 mg) treated chick embryos administered at 84 hours of development.
Figure 35. Ratios of the amounts of protein present in various protein components to component 1 from microdensitometric tracings of water soluble tissue proteins from 120 hour old chloramphenicol (1.0 mg) treated chick embryos administered at 72 hours of development.
Figure 36. Ratios of the amounts of protein present in various protein components to component 1 from microdensitometric tracings of water soluble tissue proteins from 120 hour old chloramphenicol (1.0 mg) treated chick embryos administered at 48 hours of development.
at 120 hours of development. Treatment at 117 hours of development (Figure 28) resulted in a large decrease in the water soluble protein components which have a low mobility (components 18-43) in the gel separation procedure. The high mobility components (components 1-17) have shown a decrease at this 117 hours of development treatment. For those embryos treated at 114 hours of development both high and low mobility water soluble protein fractions from head and body segment had almost entirely disappeared. The amounts of water soluble components from the appendage buds have decreased, but there is less effect of this antimetabolite on the appendage buds than from the head and body segments. However, for the embryos treated at 111 hours of development (Figure 30), the amounts of protein components have decreased in all segments to the point where they are no longer distinguishable by polyacrylamide gel electrophoresis. The group treated 108 hours (Figure 31) of development showed almost complete absence of these water soluble protein components except for components 26, 27 and 28 which were present in lesser amounts.

Beginning with the embryos which were treated at 96 hours of development (Figure 32) there is evidence for the beginning of a recovery process due to a reappearance of the water soluble protein components (components 26, 27, 28, 29, 30, 35, 36 and 39) in the lower mobility area of the tracings from protein separated on the polyacrylamide gels. This recovery phenonomen is further depicted in Figures 33-36 with the pattern of the tracings at 72 hours (Figure 36) prior to harvest representing complete recovery and these tracings are similar to those observed for the controls (Figure 27). These results are different from those obtained in the first three experiments where recovery was not demonstrated.
Therefore, the disruption in protein synthesis by chloramphenicol (1.0 mg) is not of a permanent nature in surviving embryos. This suggests that chloramphenicol is no longer functional and that competition no longer exists with m-RNA for binding sites on the ribosome surface.

The effects of chloramphenicol on the salt soluble and acid soluble proteins of the embryo as well as the water soluble proteins were examined in experiments 6, 7, and 8. The tissue extraction procedures used in experiments 6 and 7 are as indicated in figures 9 and 10 respectively. The microdensitometric tracings of the electrophoretic separated proteins of water soluble extracts (Figure 37) revealed that the 1.0 mg treatment reduced the amount of protein in components 18 and 19 as soon as 114 hours of development. Treatment at 96 and 108 hours of development revealed little or no effect on protein synthesis at the 1.0 mg level of chloramphenicol. When the 0.5 mg level of chloramphenicol was administered reduction in the amount of protein in components 18 and 19 was not noted before the 96 hour treatment. The 0.25 mg treatment apparently had no effect on protein synthesis. Hence, both the amount of chloramphenicol and the time relationships are consistent with a competitive relationship with m-RNA.

The collagen fibrils are composed of long rigid rod shaped molecules. These molecules are composed of three polypeptide chains, one of which differs to some extent in amino acid composition from the other two. Pies et al. (90) designated the two chains with identical amino acid composition α1 and the other α2. In an acid extract, these investigators also observed two different dimers in addition to the α subunits, one composed of two α1 chains bonded together and a second hybrid consisting of one α1 and one α2. The two α1 was designated β2 and the one α1 and one α2 was designated β1. Later a small fraction of collagen, which
Figure 37. Ratios of the amounts of protein present in various protein components to component 1 from microdensitometric tracings of water soluble appendage bud protein extracts from 120 hour untreated and chloramphenicol (1.0 mg, 0.5 mg and 0.25 mg) treated chick embryos treated at 114 hours, 108 hours and 96 hours of development.
Figure 38. Microdensitometric tracing of amido-Schwarz stained polyacrylamide gels of saline citrate soluble protein extracts of untreated 120 hour old chick embryos.
appeared to be a trimer in which all three chains were cross-linked was observed and this designated $\gamma$.

In experiment 6 the salt soluble protein extracts subjected to polyacrylamide gel electrophoresis showed a rather consistant pattern (Figure 38) in the lowest mobility area of the gel. This pattern was similar to that of Clark and Veis (84) in their investigations of collagen polymer components extracted from calf thymus by extraction with various saline and citrate solutions. There were no differences observed between the tracings of the saline-citrate extracted controls and saline-citrate extracted chloramphenicol treated embryos.

The separation of the acid (pH 2.3) soluble protein extracts revealed no differences between control embryos and chloramphenicol (1.0 mg) treated embryos. Since the histones are soluble in water these protein components would have been absent in the acid extract used since the tissue was previously extracted with water.

In experiment 7 electrophoretic examination of the proteins extracted in the 0.45 M saline solution using a variable acrylamide separating gel (Figure 12), revealed a pattern of separation similar to that obtained by Clark and Veis (84). The $\alpha$ components were resolved in the 7.5% gel (Figure 39). The $\alpha_1$ appears to be very close to 5.5-7.5% gel boundary. The $\beta$ components are resolved in the 5.5% gel while the $\gamma$ components appear to be near the 4.0-5.5% interface. The $\alpha$ components are predominant in the 0.45 M saline extract while the $\beta$ components are present in smaller quantities. The $\beta$ components are predominant in the 0.03 M citrate extract and they are well resolved in 5.5% gel (Figure 40). The $\gamma$ component is resolved at the rear of the 5.5% gel. The separation of these saline and
Figure 39. Microdensitometric tracing of amido-Schwarz stained polyacrylamide gels of 0.45 M sodium chloride soluble protein extracts of untreated 120 hour old chick embryos.
Figure 40. Microdensitometric tracing of amido-Schwarz stained polyacrylamide gels of 0.03 M citrate soluble protein extracts of untreated 120 hour old chick embryos.
citrate extracted proteins are quite similar to the separations obtained by Clark and Veis (84) and Nagai et al. (83). The separation of the saline-citrate extract was identical to that obtained in the previous experiment. It would appear that chloramphenicol does not exhibit any inhibiting effect on the synthesis of collagen components. Collagen synthesis could be more resistant to chloramphenicol inhibition than that of the water soluble proteins. The s-RNA available for collagen synthesis could be present in larger amounts than s-RNA is for synthesis of water soluble proteins. The s-RNA complexes which are most susceptible to inhibition by chloramphenicol could be those which affect the synthesis of proteins with basic amino acids.

The electrophoretic pattern of the proteins in the acid (pH 2.3) soluble protein extracts separated on polyacrylamide gels (Figures 41 and 42), revealed missing components in the high mobility area from embryos treated with 1.0 mg of chloramphenicol at 72 hours of development. The missing components, 1, 2, 4, and 6-11, appear to be the same as those in the pattern that was exhibited by the water soluble protein extracts studied previously (Figures 16 and 26). The acid soluble protein extracts were then diluted with water and the pH adjusted to 8.3. The acid soluble proteins precipitated out and this solution was then centrifuged (30,000 x g) for 20 minutes at 2°C. The precipitate was discarded and a sample of the supernatent subjected to polyacrylamide disc gel electrophoresis. Protein components from this basic (pH 8.3) extract appeared at the following Rf's: 0.91, 0.85, 0.83, 0.80, 0.78, 0.74, 0.57, 0.55, and 0.52 in the controls; however no components appeared in the extracts from chloramphenicol treated embryos. Hence, it would appear that chloramphenicol has an inhibiting effect on histone formation.
Figure 41. Microdensitometric tracing of amido-Schwarz stained polyacrylamide gels of acid soluble protein extracts of untreated 120 hour old chick embryos.
Figure 42. Microdensitometric tracing of amido-Schwarz stained polyacrylamide gels of acid soluble protein extracts of chloramphenicol (1.0 mg) treated 120 hour old chick embryos.
Further investigations (Experiment 9 and 10) were conducted to evaluate the effect of the antimetabolite on retardation of growth. Chloramphenicol, was administered in a quantity that would produce teratogenesis in the chick embryo, but which would permit survival to hatching. The eggs were treated 36, 48, 60, 72, 84, 96 and 108 and 114 hours of incubation with 0.5 mg of chloramphenicol. Figure 43 shows the embryonic weights at 120 hours of incubation while Figure 44 shows the weights of the day old hatched chick. No embryo with a missing appendage bud survived the period of embryonic development. However, omphalocaphaly was observed in every embryo treated at 36 and 48 hours of development. A fold in the skin covering the top of the head did not close in over 60% of the day old hatched chicks treated before 60 hours of development. The embryos treated after 48 hours of development did not exhibit any malformations except for retardation of growth.

Chloramphenicol appears to have an effect on embryonic growth in all the treatments administered. The retardation of growth is evidenced in the day old chick, thus the effect of chloramphenicol appears to be permanent. Chloramphenicol retarded embryonic growth in those embryos treated at 36 hours and they were only 1/4 the size of the controls at 120 hours of development. However, this treatment group appears to regain some of the relative difference between 120 hours of development and hatching. The embryos treated prior to 96 hours of development are approximately the same size at 120 hours of development as the results obtained by Romanoff (1) at the times when treatments were administered in this experiment.

Therefore, chloramphenicol appears to inhibit growth of the chick embryo throughout embryonic development when administered at a 0.25 mg per embryo level during early embryogenesis.
Figure 43. Weights of 120 hour old untreated and chloramphenicol (0.25 mg.) treated chick embryos treated at various stages in embryonic development.
Figure 44. Day old chick weights of untreated and chloramphenicol (0.25 mg.) treated at various stages of embryonic development.
DISCUSSION

The results of this study suggest that chloramphenicol did not disturb induction but did selectively inhibit differentiation of the ventral trunk and appendage buds and retarded growth of the chick embryo. Actinomycin D and puromycin indiscriminately inhibited or altered either induction and/or early phases of differentiation. The biochemical processes interrupted by these compounds are necessary for the corresponding normal embryonic processes which the antimetabolites altered. It is well established that chloramphenicol has little inhibiting effect on RNA synthesis but selectively inhibits protein synthesis both in microorganisms (91) and in mammalian tissue (92). Chloramphenicol appears to act by inhibiting the transfer of amino acids from aminoacyl-RNA to protein by competition with m-RNA for binding sites on the ribosomes.

Actinomycin D inhibits DNA-directed RNA synthesis by a binding of the antimetabolite to DNA thus inhibiting strand separation of helical DNA and suppresses DNA polymerase activity at corresponding concentrations (93). Puromycin inhibits protein synthesis by acting as a structural analogue of esterified s-RNA (62). In metabolic inhibitor studies it has been found that although there are differences in effectiveness and specificity among analogues and even homologues of a chemical series, the biochemical process inhibited or enhanced by one compound is also affected by other members of the series (94). The principle is, in fact, the basis of all antimetabolite work.

126
Differences in the action of chloramphenicol, actinomycin D, and puromycin could conceivably be attributed to differences in the degree of cellular penetration by the three compounds. Higher rates of diffusion by actinomycin D and puromycin than of chloramphenicol could account for the ability of actinomycin D and puromycin to affect the embryo earlier in development than chloramphenicol and thereby produce larger undifferentiated masses in the developing embryo. Embryonic regional differences in penetration by the antimetabolite could also explain the teratogenic differences. Failure of the antimetabolite to malform structures after a critical period of development had passed could be due to a development of a resistance in the primordial structure to the penetration of the antimetabolite. However the most likely explanation for differences between these antimetabolites lies in their differing sites of action in the mechanism for protein synthesis.

Actinomycin D and puromycin were not explored after the dosage level, embryonic stage of development at time of treatment and harvest, the teratogenic effect, and the electrophoretic pattern of the proteins produced under the influence of these drugs were established. Chloramphenicol was studied further because it produced consistent changes in the electrophoretic patterns of the proteins obtained from treated embryos.

The effectiveness of chloramphenicol in reducing the amount of proteins found on electrophoretic examination decreased as the age of the embryo at treatment increased. This could indicate that the rate of protein synthesis becomes progressively less critical. Alternatively, as the embryo develops it may increase its capacity to detoxify
chloramphenicol and thereby decrease chloramphenicol-inhibition. The evidence of Waddington et al. (95) supports the view that protein synthesis has a declining importance because chloramphenicol inhibits general development without necessarily producing malformations and permits development for a longer period after treatment with increased age.

The retardation of growth in the chloramphenicol treated embryos after 72 hours of incubation suggests an inhibition in cell growth in the embryo since no malformations were observed in the treated embryos.

More components were distinguishable in this study than have been reported by previous investigators. It is quite possible that the electrophoretic solutions which were used in the formation of the polyacrylamide gels permitted higher resolution and a more uniform sieving effect. Another factor which would give rise to a greater number of components was the small area scanned by the microdensitometer. This contributed to a higher resolution of the protein discs as individual units.

The use of variable gel concentration gradients in polyacrylamide electrophoresis permits one to analyze mixtures of components with wide variation in molecular weight, charge and mobility in a single run. As was demonstrated in the separation of collagen, reproducible results can be obtained by casting the separating gel in sections, each of which has a uniform gel concentration. The analysis can be carried out without spreading the moving boundaries, and in fact the boundaries appear to be sharpened. This higher resolution can be used to show some differences in net migration rates that can be detected between similar components of different collagen preparations. It also is apparent at this higher resolution that the subunit composition of the soluble collagen may be...
somewhat more complex than anticipated. The differences may reside in variations in degree of hydroxylation as proposed by Bornstein (96).

It appears that chloramphenicol first inhibits the synthesis of high mobility-low molecular weight proteins (probably histones) in both the water soluble and acid soluble extracts. Elimination of the differences between control and treated embryos by basic precipitation of the proteins in these extracts suggests that the proteins affected are histones. Histones may be regulators of genetic activity. It is known that histones display molecular heterogeneity (95), a possible provision for selective regulation of genetic activity widely thought to occur in tissue differentiation. Tissues also differ in the way that their histones exhibit heterogeneity.

The biological processes of differentiation and development must involve a differential expression of the genome. The mechanisms for control of such expression are as yet unknown. A genetic regulatory function has been postulated, however, for certain basic cellular proteins, the histones (95). The presence of histones within the cell nucleus, their association with DNA and RNA, and their molecular heterogeneity give support to this concept (97). Furthermore, interactions between histones and in vitro DNA-dependent RNA synthesis systems have been reported (98). In the developing embryo, differentiation might be reflected by changes in the nuclear histone complement as new and differentiated tissues are formed, especially during the early stages of embryogenesis such as gastrulation when the primary organ rudiments are first formed.

The inhibitory role of histones was originally surmised by Stedman and Stedman (99). There is also biochemical evidence that histones do block DNA function. It seems possible that the suppression of RNA synthesis by histones is due to a complex formation between the added
histones and the DNA needed is a primer in the RNA polymerase reaction. The correlation between DNA binding and the inhibition of RNA synthesis is evident in the experiments of Huang and Bonner (100), who showed that histones which protected DNA from thermal denaturation also blocked its primer action.

The turnover rate of protein within the embryonic tissue is another variable which is a factor for consideration. The turnover rate for histones is relatively fast and may be one reason for the greater effect of chloramphenicol on histone synthesis in these experiments. While the turnover rate of collagen is very slow, previous workers (25) have demonstrated that the amount of proline incorporated into hydroxyproline of collagen fractions increased 12 fold from 72 hours of development to 120 hours of development in the embryo. Collagen formation from 72 hours to 120 hours of development does not represent collagen turnover; only the biosynthesis of collagen for growth and development of the chick embryo. If chloramphenicol reduced the synthesis of all proteins to the same degree, then these experiments should have revealed little increase in the amount of collagen from treated embryonic tissue. Actually, the amount of collagen extracted at 72 hours of development increased from 21 mg to 129 mg of extracted collagen at 120 hours. The growth data presented in Figure 43 shows that embryos treated at 72 hours of development are 51% of the weight of control embryos when observed at 120 hours. No differences were observed between treated and untreated embryos in the amount of collagen extracted when the difference in size is considered. The electrophoretic patterns of collagen extracts appear to be identical at 72, 84, 96 and 120 hours of
development. Hence, chloramphenicol apparently had little effect in inhibiting the collagen synthesis.

One might hypothesize that chloramphenicol has selectively altered the synthesis of proteins. Chloramphenicol may have a secondary mode of action in addition to its competing with m-RNA for binding sites on the ribosome. Chloramphenicol may selectively affect certain m-RNAs predestined for the synthesis of the low molecular weight-high mobility proteins in the embryonic tissue extract.

The experiments in this study showed that the administration of chloramphenicol (1.0 mg) to chick embryos was followed within a period of three hours by a decrease in the amount of histones being formed. If there are ribosomes with specificity for histone production and chloramphenicol acts by competition with m-RNA for binding sites on ribosomes then the selective effect of chloramphenicol on histone formation might be due to an increased specificity of chloramphenicol for binding sites on those ribosomes that are predestined for histone synthesis. If this hypothesis is correct, there should be a decrease in histone synthesis which was evidenced in this study by a reduction in the high mobility-low molecular weight components of both the water and acid soluble extracts. With reduced histone synthesis there may also be less complex formation between histones and nuclear DNA, and thus an increase in the synthesis of m-RNA and as a consequence increased competition for binding sites on the ribosome with chloramphenicol which could result in increased histone synthesis during recovery from chloramphenicol inhibition. This is suggested by the data in Figures 33 through 36 where the recovery from the suppression by chloramphenicol is shown. The specificity of the
process is evidenced in that some of the water soluble protein extracts were not affected by chloramphenicol and that the antimetabolite had little effect on collagen synthesis.

Since ribosomes are constructed mainly of RNA and basic proteins (histones), and chloramphenicol appears to interfere with histone synthesis, normal ribosome formation may be retarded and hence in time the synthesis of other proteins. The evidence in this study demonstrated that chloramphenicol reduced histone synthesis and the synthesis of other proteins was not greatly affected. If the decreased histone synthesis involved faulty ribosomes, then one might expect to observe many faulty proteins being synthesized. Also, with decreased histone synthesis, ribosomal synthesis would not only become faulty, but progressively cease to function and the recovery observed in these experiments would not have occurred.

Another hypothesis is that different m-RNA's compete to differing extents for binding sites on the ribosome with chloramphenicol. It would seem possible that competition between chloramphenicol and specific m-RNA's might take place with competition being most effective for m-RNA's which are concerned with histone production.

Chloramphenicol interferes with protein synthesis by competition with m-RNA for binding sites on the ribosome and apparently selectively alters protein synthesis. Histones were observed to be more affected. The effect may be on the feedback mechanism involving the histone - DNA complex which affects the total amount of m-RNA which could detoxify the chloramphenicol action. Histones also have a relatively fast turnover rate in relation to the other proteins.
A series of ten experiments were conducted in this investigation (1) to study the teratogenic properties of antimetabolites on the developing chick embryo, (2) to study the physical and chemical properties of proteins derived from affected tissues and organs and compare them to those derived from normal tissue and (3) to identify the macromolecules in abnormal tissue development induced by the antimetabolites.

The results obtained in this investigation suggest that chloramphenicol selectively inhibited differentiation of the ventral trunk and appendage buds and retarded growth of the chick embryo. Actinomycin D and puromycin indiscriminately inhibited or altered either induction and/or early phases of differentiation. Differences between these antimetabolites probably arise from their differing sites of action in the mechanism for protein synthesis.

The effect of chloramphenicol decreased as the ages of the embryos at the time of antimetabolite administration increased. This suggests that as the embryo develops it may increase its capacity to detoxify chloramphenicol and thus decrease chloramphenicol inhibition.

The retardation of growth in chloramphenicol (0.50-1.0 mg) treated embryos appears to be present throughout embryonic development when the antimetabolite was administered in early embryogenesis. The day-old chick of the chloramphenicol-treated embryos exhibited no teratogenic effects if the antimetabolite was administered past 60 hours of development.
Apparently cell growth was affected by the chloramphenicol and this effect seemed to be of a permanent nature.

The synthesis of histones seem to be most affected by the administration of chloramphenicol (1.0 mg) at 72 to 117 hours of development while formation of collagen is not greatly affected. Many histone proteins are being synthesized in early embryogenesis. Chloramphenicol, by competing with m-RNA for binding sites on the ribosomes, inhibits histone formation. This reduction in histone synthesis may result in less complex formation between DNA and histones. As a consequence increased synthesis of m-RNA and increased competition for binding sites on the ribosome with chloramphenicol may take place and increased histone formation may result.


