**PROTEINS IN GROWTH REGULATION DURING EARLY
DEVELOPMENT OF THE CHICK EMBRYO**

Comprehensive Three Year Report

Norman W. Klein

**Department of Animal Genetics
University of Connecticut
Storrs, Connecticut 06268**

May, 1971 - May, 1974

**PREPARED FOR THE U. S. ATOMIC ENERGY COMMISSION
UNDER CONTRACT NO. AT(11-1)-3139**

NOTICE

This report was prepared as an account of work sponsored by the United States Government. Neither the United States nor the United States Atomic Energy Commission, nor any of their employees, nor any of their contractors, subcontractors, or their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness or usefulness of any information, apparatus, product or process disclosed, or represents that its use would not infringe privately owned rights.

MASTER

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

TABLE OF CONTENTS

	PAGE
Research Accomplishments.....	1
I Background.....	1
1. Introduction.....	1
2. Rationale.....	2
3. The requirement of embryos for nutrient protein.....	4
4. The requirement of embryos for specific nutrient proteins.....	7
5. Nutrient protein complementation.....	9
6. Quantitative analysis of protein utilization.....	13
7. Protein starvation during early development.....	17
8. Nutrition and the synthesis of specific proteins - Gel electrophoresis.....	25
9. Nutrition and the synthesis of specific proteins - Serum proteins.....	33
10. Miscellaneous studies.....	40
A. DNA and DNA dependent DNA polymerase activity.....	40
B. Electron microscopy and histology.....	42
C. Absolute protein synthetic rates.....	43
D. Affinity chromatography and nutrient protein uptake.....	44
E. Hydrolases and protein starvation.....	45
II Summary of research accomplishments of the last three years.....	47
1. Yolk proteins.....	47
2. Nutrient protein starvation.....	47
3. Nutrition and the synthesis of specific proteins - Gel electrophoresis.....	50
4. Nutrition and the synthesis of specific proteins - Serum proteins.....	52
5. Miscellaneous studies.....	54
Proposed Research.....	56
1. The stability of soluble proteins.....	57
2. Protein synthetic activity of ribosomes.....	59
3. Nutrition and the synthesis of specific proteins - Gel electrophoresis (completion of study).....	62
4. The synthesis and breakdown of microtubule protein.....	63
5. Nutrition and the synthesis of specific proteins - Serum proteins (completion of study).....	65
6. The selective uptake of serum proteins.....	66
7. The specificity of the nutrient protein requirement.....	68

State of Knowledge, significance, and needs for future studies on protein nutrition and growth regulation.....	69
Literature cited.....	75
Graduate students and post-doctorals.....	79
Graduate students.....	79
Post doctorals.....	80
Bibliography.....	81
Bibliography of principal investigator and publications associated with this Contract.....	81
Research completed - manuscripts in preparation.....	84
Federal support.....	86

RESEARCH ACCOMPLISHMENTS

I. BACKGROUND

1. Introduction

The present report is mainly concerned with our research activities of the last three years (1971-1974). During this period we directed our efforts toward those fundamental questions stated in our 1971 report and we have with considerable success followed the new approach proposed in that report. Thus, in 1971 (see Comprehensive Report of 1971, p. 10) I stated, "I feel, at this time, that our descriptive work is nearing completion. We have in some instances defined rather precisely both an experimental condition and a response. For the first time I feel that we can proceed to seek out the basic mechanisms relating an experimental condition to a response." During the last three years I feel that we have indeed proceeded to seek out basic mechanisms and have applied the most rigorous and precise methods of modern molecular biology to this end. Rather than attempting to work on new questions or slight modifications of the same questions at a constant and superficial level of analysis, we have, to a large extent, continued working on the same fundamental question but have greatly increased the specificity and depth of analysis. The success of this approach appears to this investigator to justify our continued research in this direction and provides the basis for this renewal request.

In an attempt to stress our research activities of the last three years, considerations of the rationale and background will be relatively brief. For a more comprehensive treatment of this material the reader is referred to the chapter prepared by invitation for Volume III of The Biochemistry of Animal Development, R. Weber, ed. This chapter entitled "Protein Nutrition In Growth Regulation" was prepared about a year ago and not only reviews our own work in this area but also shows the relationship of our work to the more general area of developmental nutrition.

With the hope of improving readability, the presentation of data and references to the work of other investigators have been minimized. For the sake of continuity our accomplishments of the last three years have been incorporated with our previous studies. A brief statement at the end of this section will summarize those specific research accomplishments of the last three years.

2. Rationale

Our research can be viewed as two distinct but interrelated interests. On the one hand, we are interested in the nutrition of embryos and particularly in the nutrition of proteins. We are interested in the specificity of the nutrient protein requirement of embryos and in each of the many steps required to convert a nutrient protein contained within the nutrient milieu into an embryo specific protein. On the other hand, we employ nutrition as an experimental tool to study the mechanisms regulating growth during development. For example, by changing the amount and type of nutrient protein, specific alterations

occur in the extent and pattern of embryo growth. Thus, in such the same manner as one employs a genetic mutant to expose a normal phenomenon, these growth-altered embryos can be used to examine mechanisms which may operate in the regulation of normal growth. As distinct as these two interests or approaches appear our overall working hypothesis stresses their interrelation in so far as we contend and have attempted to demonstrate that the nutrient proteins themselves may play an important role either directly or indirectly in the regulation of growth.

Excluding our own studies in support of the hypothesis that nutrient proteins play an important role in regulating embryonic growth, the following rationale was used in the initial formulation of the hypothesis. Many studies involving mature animals, mainly rats, have shown that dietary protein or amino acids can influence cellular activity. I refer here to such studies as those on the synthesis and breakdown of protein (1, 2), polysome stability (3) polysome functional activity (4) and amino acid transport (5). These same animals which respond so readily to dietary changes in protein and amino acids are also subject to signals derived from such regulators of cellular activity as the endocrine and nervous systems. It would therefore follow that in an organism, such as an embryo, which lacks functional endocrine and nervous systems, nutrition may play an even greater role in the regulation of cellular activity. Thus, an early embryo may be viewed as a bacterium which readily responds to nutrition. However, unlike a bacterium an embryo exhibits a precise pattern of organ and regional growth and undergoes progressive differentiation.

3. The Requirement of Embryos for Nutrient Protein.

As a starting point, we will assume that the present line of investigation originated with our attempts to culture early chick embryos (11-13 somites, approximately 40 hours of pre-incubation) on a chemically defined medium and to demonstrate a response to a deficiency of a single essential amino acid (6). There were several reasons for doing this original study. First, we were interested in developmental nutrition and believed that the ability to culture embryos on media which were defined and free of protein would make it possible to study a complete range of nutrients. Second, we were particularly interested in the affect of an amino acid deficiency on an embryo and how it might compare to the affects of an amino acid analogue. Finally, we felt that if it was possible to demonstrate some affect by omitting a single essential amino acid we would at the same time demonstrate that the chick embryo explant depended on the medium for these nutrients. This latter point was particularly crucial because the presence of residual yolk carried over from the egg could obscure attempts to study nutrition through alterations in the culture medium. In brief, we were able to demonstrate limited growth on a complete chemically defined medium and when either leucine or lysine were omitted from the medium, growth was reduced to the same extent as that achieved by omission of all amino acids.

These observations indicated that the chick embryo explant system provided a unique opportunity to study developmental nutrition. These cultures represented the only developmental system in which an early

intact embryo could be cultured for an extended period of time in the absence of nutrients carried over either from the egg cell or the maternal system. This encouraged us to attempt some improvements on the classical technique of chick embryo explantation (7). Eggs are incubated for approximately 40 hours. They are then broken into a bowl and the embryos with yolk-sac membrane intact are cut free of the yolk and vitelline membrane. By microscopic examination, embryos of precisely 11 to 13 somites are selected for experimentation (we average about three embryos per dozen eggs). The yolk sac peripheral to the sinus terminalis is cut away and the embryos with intact yolk-sac membranes are thoroughly rinsed to remove adhering yolk. Explants are then placed ventral surface down on the desired nutrient medium containing agar and cultured at 37.5°C . Embryo explants receive a mixture of 25% O_2 + 75% air for 0 to 24 hours and 100% O_2 thereafter. Employing a nutrient medium of fresh egg yolk and white (whole egg homogenate medium) such preparations will survive for 72 hours during which time morphological development and growth approximate that achieved in ovo. It should be noted that by selecting a very precise developmental stage for our studies we are able to work with a rather uniform group of embryos from one experiment to the next. Furthermore, we have perfected the procedure to the extent that in a single morning four or five experienced individuals can set up routinely 100 to 150 cultures. It should be noted that work is progressing on the culture of mammalian embryos. For example, Hsu (8) recently reported the successful culture of mouse embryos from the 2 cell to early somite stage. However, I

doubt if sufficient numbers can be cultured at this time to permit quantitative studies.

In the original studies on chemically defined media and amino acid deficiencies comparisons were made of the growth of embryos not only on a defined medium with and without certain amino acids but on whole egg homogenate medium as well. It was apparent in this early study that embryo growth was greater on whole egg than on defined nutrients (after 48 hours of culture embryo protein nitrogen values were for complete chemically defined 12.0 $\mu\text{g}/\text{embryo}$ and whole egg 16.3 $\mu\text{g}/\text{embryo}$). However with improvements in the technique of culturing embryos, embryo growth on whole egg increased greatly but growth on a chemically defined protein-free medium remained limited (after 48 hours of culture complete chemically defined 15.9 $\mu\text{g}/\text{embryo}$ and whole egg 53.3 $\mu\text{g}/\text{embryo}$). Attempts were made to improve embryo growth on defined nutrients (comparing various nutrient mixtures, different salts, etc.) but were unsuccessful. Although there were many differences between a defined medium and one containing a mixture of egg white and yolk, we became concerned with the difference in amino acid concentration. In the defined medium there were 0.2 gms of amino acids per 100 ml of medium while in the whole egg medium amino acids were mainly contained in protein and there were 6 - 7 gms of protein per 100 ml of medium. In an attempt to evaluate this large difference in amino acid-protein concentration we cultured embryos either on higher levels of free amino acids or alternatively diluted the protein of whole egg to a level comparable to the amino acid concentration of the defined medium.

Small increases in the concentration of amino acids were toxic. However, when egg protein was diluted, embryo growth on egg medium was comparable to that achieved with defined medium (9). Thus, we tentatively concluded that the value of egg medium was simply associated with its high level of protein. It also appeared possible that the embryo actually required nutrient protein because only in this form could amino acids be provided in sufficient concentration to support the needs of an embryo for rapid growth and still be compatible with survival.

It should be noted that in addition to our studies, reports from other laboratories also suggested that the chick embryo required nutrient protein (see 10 for review). The literature dealing with this question in mammalian embryos is contradictory. It is not clear at this time if the early mammalian embryos requires nutrient protein, can be cultured successfully on defined media containing free amino acids, or contains sufficient amounts of endogenous nutrients to grow and develop in the absence of an external source of nitrogen. The reader is referred to the chapter (10) enclosed with this report for a review of the literature on the mammalian embryo as well as the chick embryo. However, Heu (8) in his recent successful culture of mouse embryos does employ a serum containing medium as well as a collagen substrate.

4. The Requirement of Embryos for Specific Nutrient Proteins.

Our next series of studies followed directly from the tentative conclusion that egg proteins simply provided amino acids at a high concentration and in a form that could be tolerated. Thus, it was reasoned that the proteins of egg were not unique if this was their primary function

and it should be possible to culture embryos on a variety of proteins. The growth (protein and DNA accumulation) of chick embryo explants on a variety of proteins and sources of protein was compared and large differences were observed (9). After 48 hours of culture the greatest embryo growth was achieved with the whole egg homogenate medium (a mixture of egg yolk and white). This was closely followed in decreasing order by yolk alone, a mixture of horse serum and ovalbumin, and egg white. Ovalbumin alone was intermediate in that embryo growth on this protein was lower than the previously mentioned four sources of protein but considerably greater than the more limited growth achieved with sub-blastodermal fluid, bovine colostrum, horse serum, or bovine casein. Avian embryo extract, bovine milk, and bovine serum albumin failed to support embryo growth to any extent. Sera from the horse, hen, rooster, rabbit, and chicken (mixture of rooster and hen) gave comparable results. In this study considerable attention was given both to the concentration of protein in the medium as well as the non-protein constituents of the medium in an attempt to minimize these variables. Assuming this was achieved, these results would suggest that proteins derived from yolk or from the egg in general have unique properties to meet the needs of an embryo for rapid growth. Thus, the greatest growth was achieved on media containing yolk followed by media containing either egg white or the major protein of egg white, ovalbumin. These observations may indicate therefore that the proteins of yolk provide some factor or factors other than amino acids to the embryo, and that the proteins of egg white share a common factor (s). Alternatively, these results may indicate that,

rather than providing some unknown factor, the proteins of egg are uniquely suited to provide amino acids. For example, egg proteins may be more readily taken up by cells of the embryo than proteins from other sources or they may serve more readily as substrates for the proteolytic enzymes of the embryo.

Again the reader is referred to a review chapter (10) included with this report for studies that tend to support the above conclusion for both the chick and mammalian embryos. The recent studies by Hsu (8) are also relevant to this section and should be noted. He found a possible stage specific requirement for serum in that during the culture period at particular times he uses calf serum, followed by fetal calf serum, and finally human cord serum. Although this does not clearly implicate the proteins of serum, these sera would contain different proteins.

5. Nutrient Protein Complementation.

The first demonstration that a combination of proteins or sources of protein would support a higher level of embryo growth than could be achieved with either individually was made with ovalbumin and serum in the last study discussed on embryo growth with various proteins (9). The combination of serum and ovalbumin in a single medium was not fortuitous but was based on the observation that serum and ovalbumin supported different parts of the chick embryo explant system. Serum alone supported the development of a large and highly vascular extra-embryonic yolk-sac membrane with a relatively small embryo. Ovalbumin, on the other hand supported the growth of a relatively large embryo

but the membrane was small and poorly vascularized. In combination, serum and ovalbumin supported embryo growth comparable to that achieved on whole egg homogenate medium with a somewhat larger extra-embryonic membrane. In addition, gross observations of embryo cultured on either serum or ovalbumin suggested that nutritional factors could influence the relative growth of embryo regions. This was supported by quantitative determinations of the amounts of DNA, RNA and protein contained in isolated embryo regions following 48 hours of culture (10). The brain regions of explants cultured on ovalbumin contained a higher proportion of total embryo DNA, RNA and protein than did the brains of embryo cultured on serum. And, the regions composing the trunk of serum cultured embryos contained proportionately more of the total embryo DNA, RNA and protein than did the trunk regions of ovalbumin cultured embryos. It should be noted that comparisons with explants cultured on various dilutions of whole egg homogenate medium indicated that the results obtained with serum and ovalbumin were not simply due to differences in overall embryo growth.

A second example of nutrient protein complimentation was found in studies dealing with the two egg white proteins, ovalbumin and conalbumin. In previous studies we had made use of ovalbumin prepared by the classical salt precipitation procedure. In the section to be discussed on quantitative studies of protein utilization, a highly purified preparation of ovalbumin was desired and was prepared by column chromatography. Highly purified ovalbumin unlike salt precipitated ovalbumin would not support embryo growth. By systematically combining the various egg white protein with ovalbumin in a single nutrient

mixture, it was possible to show that conalbumin was the important protein (10). Conalbumin alone like ovalbumin would not support embryo growth but when these two proteins were combined (75% ovalbumin and 25% conalbumin) embryo growth was excellent. It was interesting to note that conalbumin like serum appeared to specifically support growth and development of the extraembryonic yolk-sac membrane. Attempts to determine the basis for the conalbumin requirement have been unsuccessful. For example, conalbumin binds iron but could not be replaced by adding iron to the medium. And, attempts to replace conalbumin with amino acid mixtures were also unsuccessful.

A third example of nutrient protein complementation was observed in the course of a comprehensive analysis of the ability of various yolk proteins and yolk protein fractions to support embryo growth and development (11). During the course of this study it was possible to make use of many of the published yolk protein isolation and some procedures_q were modified particularly for these studies (12). Although this study covered several aspects of yolk protein utilization the major finding with regard to nutrient protein complementation were as follows. Chick embryos could not be cultured on a medium containing only one yolk protein, however, embryos were successfully cultured on a medium containing both the protein lipovitellin and the proteins of the livetin fraction. When the livetin complex was resolved into six subfractions, the yolk transferrin fraction appeared particularly pure and supported high levels of embryo growth when combined with lipovitellin. This complementation appeared particularly significant because it was observed with the proteins of yolk. The proteins of yolk have been shown in

several studies to be used exclusively by the chick embryo in ovo at the time comparable to that studied in culture (see 10 page 17 for reference to this point). Thus, although the horse serum-ovalbumin complementation or the ovalbumin-conalbumin complementation represent unusual nutritional situation for the early chick embryo, the fact that such complementation can be demonstrated with the proteins of yolk suggested that this phenomenon may be of some developmental significance.

In a recent review (see 10 pages 30 to 33) I considered at some length various possible explanations for the observation that two particular proteins would support considerably higher levels of embryo growth than could be achieved with each individual protein. At this time I would like to suggest the following from a recent publication (11). Conalbumin (or ovotransferrin) of egg white and transferrin of the yolk livetin fraction are both iron binding glycoproteins and appear to differ only in their carbohydrate prosthetic group (13). Whole serum would also contain a transferrin fraction. Conalbumin, yolk transferrin and serum appear to have a similar effect on chick embryo explants. These three substances enhance the growth and development of the extra-embryonic yolk-sac membrane but do not support high levels of growth for the embryo proper. On the other hand, ovalbumin and lipovitellin do not support the growth and development of the membrane but rather appear essential for the growth of the embryo proper. From studies to be discussed in the next section as well as work with compounds such as puromycin, the high level of protein in the membranes of conalbumin, yolk transferrin, and serum cultured embryos, may actually represent

the presence of these nutrient proteins within the cells of the membrane. Thus, these proteins do not appear to be readily degraded in the membrane but do appear essential. Possibly, these proteins serve directly as essential serum proteins as has been previously suggested (14). Ovalbumin and lipovitellin, on the other hand, may be degraded to amino acids within the yolk-sac membrane and serve as a major source of these precursors for the embryo proper either directly or more likely after conversion into proteins (serum proteins) within the membrane. The breakdown of ovalbumin and its conversion to proteins (serum proteins) within the yolk-sac membrane has been demonstrated but this sequence of events can only be inferred for lipovitellin.

6. Quantitative Analysis of Protein Utilization.

In view of our interests in the protein nutrition of embryos it became naturally important to determine the manner by which an embryo utilizes a protein. Although quite a few studies had been reported dealing with this general subject, the literature was rather contradictory (10). On the one hand, there were many reports involving immunological detection of proteins and electron microscopic observations (particularly those of Bellairs) which suggested that intact nutrient proteins were taken into the cells of the embryo proper. On the other hand, there were several studies which indicated that high levels of proteolytic activity were associated with the yolk-sac which suggested that nutrient proteins could be extensively degraded following uptake by the membranes. In addition, Lambson (15) in an electron microscopic study did not find a structural basis for the passage of protein through the cells of the

yolk-sac and into the circulatory system. We believed that this apparent conflict in interpretation was due to the lack of quantitative information and proceeded therefore with the appropriate study (16).

The observation that chick embryos could be cultured on a medium containing a mixture of purified ovalbumin and conalbumin as the sole source of nitrogen provided the basis for these studies. Radioactive amino acids were injected into a laying hen and labeled ovalbumin and conalbumin were isolated. Rabbit antiserum was prepared against ovalbumin but not conalbumin. The study consisted of three basic experiments. Chick embryos of 11 to 13 somites were either (A) cultured for various periods up to 48 hours on a medium in which the protein consisted of 90% ^{14}C -ovalbumin and 10% unlabeled conalbumin, (B) cultured for 24 hours on this same medium and then transferred to a medium containing unlabeled ovalbumin and conalbumin, or (C) cultured for 48 hours on a medium containing 10% ^{14}C -conalbumin and 90% unlabeled ovalbumin. After the desired culture period the embryo explants were dissected into brain, trunk, and area pellucida, and the area opaca was divided into an inner and outer region. In the ovalbumin studies, the antibody-antigen precipitation reaction was used to quantitate intact ovalbumin in a particular region and radioactivity in the trichloroacetic acid soluble fraction provided the amount of ovalbumin as ovalbumin breakdown products (amino acids). The quantity of ovalbumin converted to embryonic protein was derived by subtracting the value obtained for intact ovalbumin from the total radioactivity in protein. It should be noted that the determination of ovalbumin specific activity made it possible to convert

radioactivity into μg of ovalbumin present in a particular region as either intact ovalbumin, ovalbumin as breakdown products, or ovalbumin converted to embryo protein. The data were expressed on the basis of the quantity of DNA in a particular region rather than protein content because regions such as the yolk-sac membrane contained considerable amounts of nutrient protein. In the absence of antisera, the conalbumin study was simply limited to an estimate of total uptake or utilization.

The first experiments in which embryos were simply cultured for various periods up to 48 hours on ^{14}C -ovalbumin showed that the area opaca of the yolk-sac was the primary site of protein uptake. This region contained eleven times as much intact ovalbumin on a per cell basis as the embryo proper after 48 hours of culture. In addition, the amount of ovalbumin as breakdown products (amino acids) in the area opaca far exceeded the amounts in the embryo proper suggesting that protein was not only taken up by the area opaca but also degraded in this region of the yolk-sac. In spite of these large differences between the yolk-sac and embryo proper, both converted comparable amounts of ovalbumin to newly synthesized protein.

The above study provided some insight into the overall pattern of nutrient protein utilization but did not permit an evaluation of the sequence of steps in this process. In the second series of experiments we concentrated on this point by transferring embryos after only 24 hours of culture on ^{14}C -ovalbumin to a medium containing unlabeled ovalbumin. During the second 24 hour period (after transfer) the amounts of intact ovalbumin and ovalbumin breakdown products in regions of the

embryo proper remained low and constant. Changes in the area opaca of the yolk-sac, however, were particularly interesting and informative. Thus, the amount of intact ovalbumin in the area opaca dropped precipitously after transfer but the amount of ovalbumin as breakdown products remained at a high level and showed a gradual decline. This suggested that the large breakdown pool was maintained through the proteolysis of ovalbumin in the area opaca. Furthermore, the difference in breakdown pools between yolk-sac and embryo proper would suggest that amino acids as such were not transferred in appreciable quantities to the embryo. However, it was not possible to exclude a gradual release of free amino acids from the membrane and rapid utilization by the embryo proper. Nevertheless, these studies along with the studies to be discussed on pulse labeling with amino acids prior to culture and studies of serum protein synthesis would suggest as a plausible alternative that most of the nutrient protein was converted to proteins (possibly serum proteins) in the membrane which were transferred to the embryo and served as a source of amino acids for the synthesis of embryo specific proteins.

In this study of ^{14}C -ovalbumin utilization we determined the contribution of ovalbumin to the newly formed protein (quantitative difference in protein content between zero-time and 48 hours of culture). In the total embryo explant (yolk-sac membrane plus embryo) only 57% was derived from ovalbumin. For this reason we cultured embryo explants on ^{14}C -conalbumin to determine the contribution from this protein. It was possible to demonstrate that the balance or 43% was derived from conalbumin.

It should be clearly noted that the medium in this study contained 90% ovalbumin and only 10% ^{14}C -conalbumin of the total protein of the medium. Thus, in spite of a nine fold difference in the medium the difference in utilization came to only 14%. This clearly indicated that conalbumin relative to ovalbumin was preferentially taken up. However, in the absence of conalbumin antiserum it was impossible to determine the amount of conalbumin present as the intact protein from that amount converted to embryo protein.

The above studies provided the first quantitative evaluation of nutrient protein utilization by an embryo. The major limitation of this study, I believe, was the protein employed in the study. As previously noted, the chick embryo utilizes the proteins of yolk during the phase of development studied while ovalbumin and conalbumin are egg white proteins. Comparable studies with yolk proteins are feasible and should be conducted. Although studies on protein utilization by mammalian embryos have not been reported, one worker (17) has indicated that such studies may be forthcoming.

7. Protein Starvation During Early Development.

Several years ago, we developed procedures for the study of embryos cultured on a protein-free medium. We reasoned that the functional significance of many nutrients (vitamins, minerals, amino acids, etc.) had been demonstrated through the study of specific deficiencies and possibly the same approach could be used to study protein nutrition. More precisely, it was hoped that embryos starved of protein could be used to study the basis by which one organ or region of an embryo accumulates

protein, RNA and DNA faster than another organ or region. For example, the brain of an early embryo accumulates protein at a more rapid rate than the heart, somites, or neural tube. If an embryo is starved of nutrient protein or provided with a limited supply would the brain respond like a tumor and continue to grow at a more rapid rate and at the expense of other organs and regions (actually the precise opposite was observed). However, of possibly greater significance, we were particularly interested in examining the interrelationship of macromolecular synthesis to breakdown in the regulation of growth of particular organs and regions of an early embryo. At this time the use of protein starved embryos has exceeded our original plans in so far as the starved embryo has become one of our basic experimental systems by which to study growth (and protein nutrition) related phenomenon. Furthermore, the cultured chick embryo has become the only system in which to study the affects of protein starvation on an early embryo. When pregnant rats were given a protein free diet during the first ten days of pregnancy, a large percentage of the positively mated females failed to litter (18). This difficulty has been traced to deficiencies of various maternal hormones rather than to a direct affect of protein starvation on the embryo. Thus, it would be quite difficult to study protein starvation during early development in such animals.

We have developed two techniques for the culture of early chick embryos under the condition of protein starvation. In one procedure we make use of the non-nutritive polysaccharide, Dextran, as a substitute for the osmotic properties of protein. An aqueous sterile solution of Dextran is placed within the dialysis bag and allowed to

equilibrate against a mixture of fresh egg yolk and white in the cold for five days. The equilibrated Dextran in combination with agar is then used directly as the nutrient substrate. The second procedure simply involves the culture of embryo explants on squares of dialysis tubing which are in turn placed directly on the standard whole egg homogenate medium. To date, where comparisons have been made, comparable results have been obtained with both techniques. Dextran has been used more extensively because the yolk-sac membrane tends to adhere strongly to the dialysis tubing which makes the transfer of explants to a fresh medium difficult.

Our preliminary studies suggested that all regions of the embryo were not affected to the same extent by starvation. In order to quantitate this response procedures were developed which would allow the dissection of embryos in a reproducible manner so that the quantities of DNA, RNA, and protein in these regions could be determined. Embryos can now be dissected routinely into brain, neural tube, somite, heart, and extraembryonic membrane. Relative to embryo explants cultured on whole egg "growth" medium, in those cultured on protein starvation medium the accumulation of macromolecules in the brain was most restricted and the heart least.

As an initial approach to an analysis of the differences between embryo regions in sensitivity to starvation as well as the relationship of synthesis to degradation, chick embryo explants were exposed to ^{14}C -amino acids for three hours in liquid medium, cultured on unlabeled media for six hours to reduce free ^{14}C -amino acids and

then cultured for various periods up to 48 hours on either growth or protein starvation media (19). During culture on starvation medium the greatest loss of radioactivity from protein occurred in the brain which also showed the smallest increase in protein content. The brain was followed in order of decreasing loss of radioactivity and increasing accumulation of protein by neural tube, somites, and heart. This sensitivity of the brain and specifically the developing nervous system to protein starvation has been confirmed by histological as well as electron microscopic analysis. When embryo explants were cultured on growth medium, radioactivity was not lost from regions of the embryo proper but the yolk-sac membrane lost a considerable amount of protein radioactivity. Referring back to the previous discussion on the transfer of proteins (possibly serum proteins) from yolk-sac to embryo proper, the observed loss of protein radioactivity from membrane protein supports this interpretation.

More recently, we have shown that the loss of radioactivity from RNA (uridine labeling) followed the same pattern as that observed for protein (20). In addition, of the total radioactivity lost from sensitive regions (brain and neural tube) 40 to 45% of the protein label and 60 to 70% of the RNA label were derived from the ribosome fraction (21).

In our most recent series of experiments (see manuscript included with this report, "Polysome Activity in Relation to Growth and Protein Starvation in Brains and Hearts of Cultured Early Chick Embryos" (22a) we considered two questions related to our studies with the protein

starved embryo. First, we have been concerned with the possible affects of protein starvation on protein synthesis. For example, it appeared possible that all regions of an embryo may degrade comparable amounts of protein during starvation and that our results simply reflected the ability of a particular region or organ to reutilize the breakdown products for synthesis of new protein. Our first attempts to evaluate this possibility involved the determination of ¹⁴C-amino acid incorporation into proteins of the various regions of the embryo after periods of culture on starvation or growth media. The results suggested that regional differences in the synthesis of protein was not a factor and that our results indicated regional difference in protein degradation. However, in this study protein incorporation was only corrected for differences between regions in the uptake of labeled amino acids rather than by the actual specific activity of the precursor pool. Therefore we attempted to determine the absolute rate of protein synthesis in embryo regions by correcting protein incorporation values for precursor specific activity. Considering the amino acid attached to transfer RNA as the most immediate precursor to protein, we attempted to determine the specific activity of that amino acid employing the elegant technique of Regier and Kafatos (22b). While working on this procedure, it became apparent that a more direct and reasonable approach would be to work directly with polysomes and to examine their activity in a cell-free protein synthesizing system. Thus, although we have not completely discontinued our attempts to examine absolute rates of protein synthesis in the intact embryo, we have been working primarily with isolated polysomes. The second question considered in this recent study

followed from the observation that the ribosome fraction contributed large amounts of the total radioactivity lost from protein and RNA of the sensitive regions during starvation. Thus, we attempted to determine if ribosomes isolated from a sensitive region (brain) were more sensitive to degradation in vitro than ribosomes isolated from a region resistant to protein starvation (heart).

Polysomes isolated from brains were more active (on a per polysome basis) in cell-free protein synthesis than those from the heart (the same enzyme fraction, isolated from whole four-day chick embryos, was used for both groups of polysomes). This was true for polysomes isolated from brains and hearts of growing as well as starved embryos. Protein starvation reduced polysome activity but this reduction occurred for both brain and heart polysomes. Thus, these results confirmed our previous studies which suggested that protein starvation did not specifically inhibit the synthesis of protein in those regions of the embryo which were sensitive to starvation. In this study we also examined polysome-ribosome profiles because differences in the proportion of active polysomes to inactive ribosomes could contribute to the differences observed in synthetic activity. The profiles were quite similar except both the brain and heart of starved embryos had 6% fewer ribosomes as polysomes (greater than dimers) than the comparable regions from growing embryos. The possibility that the differences between regions and culture conditions for polysome activity could be due to differences in polysome stability was also considered. Others have demonstrated that these ribosome-

messenger RNA complexes differ in stability in relation to particular organs, age of animal, and nutritional state. In general, it was not possible to relate polysome stability (postmitochondrial supernatants were incubated for 60 minutes at 37.5° prior to placement on sucrose gradient) to polysome protein synthetic activity. In addition, we examined ribosome-associated ribonuclease activity (¹⁴C-polyuridylic acid was used as a substrate) which again could influence polysome protein synthetic activity. Ribosome-associated ribonuclease activity could not be related to polysome synthetic activity but ribonuclease activity was directly related to polysome stability.

In the second series of studies a direct attempt was made to determine a possible basis for the sensitivity of brain and insensitivity of heart to starvation. Ribosomes prelabeled with either ¹⁴C-amino acids or ¹⁴C-uridine were treated with either trypsin, ribonuclease, or no enzyme (autodegradation) and the release of radioactivity into a trichloroacetic acid soluble fraction was determined. With both enzymes the differences between brain and heart, and growing and starved were small but the heart rather than the brain ribosomes (as would be predicted) appeared more readily degraded. However, measurements of autodegradation were generally in agreement with the differences in sensitivity to starvation in that brain ribosomes appeared to be more readily degraded than those of the heart. The significance of this observation was difficult to interpret in view of the conflicting observations with trypsin and ribonuclease degradation.

It was of particular interest to compare our results with

those of Henshaw et al.(4). In the fasting rat a reduction in liver protein synthetic activity could be attributed by these workers to reductions in polysome activity, the proportion of ribosomes as polysomes and the total amount of ribosomes in the liver. Similarly, we have shown that starvation leads to a reduction in polysome activity and the proportion of ribosomes as polysomes. Furthermore, in a previous study we reported (21) that starvation leads to a reduction in ribosomal RNA per cell (DNA). However, of possibly greater significance, these same workers (4) observed that variations in the protein synthetic activity of livers of fed animals was due to polysome activity alone and specifically to a soluble factor (s) associated with polysomes. In our study, the difference between brains and hearts of growing embryos also appeared limited to polysome activity. During the phase of development studied the accumulation of protein by the brain of the early chick embryo has just started to exceed that of other regions. Thus, the difference between brain and heart polysomes provides a basis for the regulating of regional growth differences. And, to this writer's knowledge this observation provides the first possible mechanism by which growth can be regulated during early development. It should be noted that differences in polysome activity as a regulatory mechanism is not unique to the early embryo. For example, in addition to the studies of Henshaw et al.(4) with the rat liver, the changes in protein synthetic activity with maturation in the mouse brain also appears regulated by polysome activity and possibly a soluble factor (s) associated with the polysomes (23, 24). Additional studies

will be required to determine if a specific soluble factor (s) regulates protein synthetic activity in the early chick embryo.

8. Nutrition and the Synthesis of Specific Proteins - Gel Electrophoresis

One of our primary objectives has been to evaluate the effects of nutrition on the synthesis of specific proteins during early embryonic development. This objective follows from our working hypothesis that an early embryo is, unlike the adult organism, particularly susceptible to nutrition in the regulation of cellular activity. Several approaches are presently being used to evaluate the synthesis of specific proteins by embryo explant cultured under various conditions of nutrition. One approach, that of gel electrophoresis of total supernatant proteins, provides what might be considered a general analysis of a large number of different specific proteins. By combining gel electrophoresis with the technique of double label and coelectrophoretic analysis of two different mixtures of proteins, rather precise quantitative estimates can be made of the degree of similarity or dissimilarity of the two mixtures. We have used this procedure to evaluate the effects of nutrition on the synthesis of proteins in regions of embryo explants.

In brief, high speed supernatant fractions (100,000 x g) from regions of embryos labeled with either ^3H or ^{14}C -valine are combined and placed on a polyacrylamide disc gel (usually 7.5% acrylamide). The gels are stained to visualize the protein bands and they are then cut into 0.7 mm. slices. Individual slices are placed in counting solution for analysis of radioactivity by liquid scintillation procedures. This procedure generates a great deal of data and we have developed,

like many other investigators, a computer program to meet our needs. The computer makes various corrections (background, channel spillover, quenching, and counting efficiency) and provides various expressions and plots of the data such as percentage distribution per slice, overall percent difference, ratios of ^3H to ^{14}C , ratios normalized to an overall ratio, and statistical analysis so that a particular slice is brought to statistical significance through combination with the next slice. Many of these procedures have been reported in the literature (25, 26) and although each appears to have certain advantages in expression they seem to provide the same general results.

In our experiments we first evaluated controls in which embryos cultured under one condition were exposed for three hours at the end of the culture period to either ^3H or ^{14}C -valine and supernatants of regions were combined. This provided an estimate of the sensitivity of the technique. The effects of nutrition involved comparisons between embryo explants cultured on protein starvation medium and whole egg homogenate growth medium, and in a second series embryos cultured on ovalbumin-conalbumin medium ^{were compared} with whole egg (terminal labeling). In the course of these studies we also did some comparisons between starved and zero-time embryos as well as ovalbumin-conalbumin and zero time. Finally, we have attempted to determine changes with starvation or growth by labeling embryos at zero-time and then culturing for 45 hours prior to dissection and analysis (post labeling culture). Part of this work was presented at a meeting (27) and we are presently preparing a publication based on these experiments.

Controls were run with embryo explants cultured for 45 hours on whole egg homogenate growth medium. After the culture period explants were divided into two groups and placed into a flask containing either ^{14}C or ^3H valine in chick Ringer's bicarbonate solution. The flasks were attached to a wrist action shaker for three hours and dissected into the following regions; brain, neural tube, somites, heart and extraembryonic yolk-sac membrane. High speed supernatant fractions were then combined for analysis by gel electrophoresis. The results were calculated as total absolute percentage difference which involves the calculation of percent of total counts in each slice for ^3H and ^{14}C and the summation of the differences in percentages for ^3H and ^{14}C . Thus, theoretically the values could range from zero for identical protein mixtures to 200 for maximum difference. The control values (brain in combination with brain, somites with somites, etc.) ranged from 5.6 for brain to 8.7 for heart. In contrast, when brain supernatants were combined with those of a different region (brain combined with heart, brain with somites, etc) the values ranged from 9.4 to 14.5. Visual inspection of the gels or the plots (graphs) of the count data supported these quantitative values and showed that different regions did synthesize (or contained) distinct groups of proteins. Thus, although the various regions analyzed contained mixtures of cell types and certainly many proteins in common, they synthesized protein mixtures which could be distinguished both quantitatively and qualitatively.

During the course of these studies, we initiated studies on the synthesis of serum proteins. These studies will be discussed in the next section. In brief, we have been able to show that serum proteins were synthesized exclusively by the extraembryonic yolk-sac membrane and we were able to obtain radioactively labeled serum proteins. By comparing these radioactive serum proteins with the supernatant proteins derived from the various regions it was possible to show that at least two of the serum proteins co-migrated with radioactive supernatant proteins. One appeared to be transferrin and the other the so called "embryo specific serum protein." The regions contained different amounts of the embryo specific protein relative to other proteins and of particular interest, the largest amount was present in the brain. Insofar as the growth of the brain exceeds that of other regions at the time of development studied, we were (are) intrigued by a possible relationship between growth and the presence of this protein. Another aspect of serum proteins in relation to the synthesis of proteins by a particular region should be noted. The embryos in these experiments were exposed to label for only three hours but this short time period was sufficient for the synthesis of serum proteins in the yolk-sac and their transport to the embryo proper. If embryo explants were radioactively labeled without their yolk-sacs, these radioactive serum proteins were not detected. Another interesting aspect of serum protein synthesis was observed in supernatant fractions of the yolk-sac itself. Serum proteins were essentially absent from the region (yolk-sac) which was exclusively involved in their synthesis.

This suggested that serum proteins were rapidly transported out of the cells of the yolk-sac following synthesis.

The effects of nutrition on the synthesis of specific proteins was studied first with the condition of protein starvation. It should be noted that this condition involved not only an alteration in nutrition but a severe reduction in growth (or the total accumulation of protein). Thus, these studies provided the opportunity to relate growth as well as nutrition to the synthesis of specific proteins. Standard 7.5% gels as well as sodium dodecyl sulfate gels (separation on basis of molecular weight) were used in this study and provided comparable results. Supernatant fractions were combined from regions of explants cultured for 45 hours on either starvation or growth medium and exposed to ^3H or ^{14}C -valine between 45-48 hours. The largest difference for regions of the embryo proper between starved and growing explants was observed in the brain region which was followed by the neural tube. The somites and heart regions did not differ from control values. These differences were in agreement with our previous studies which indicated that the brain was particularly sensitive to starvation. Indeed, the large difference between starved and growing supernatant proteins of brain appeared in part due to a less distinct protein pattern for the starved brain. That is to say, in the starved brain supernatants, a greater proportion of radioactivity was noted between protein peaks than was observed for the growing brain. This appeared to indicate that proteins were being broken down (or not completed, mistranslation followed by rapid breakdown, etc.) in the starved brain during the

relatively short (three hours) labeling period. This was confirmed by the presence of a relatively large band (less than 13,000 molecular weight) in sodium dodecyl sulfate gels of supernatants from starved brains. In addition to this general pattern of breakdown, the starved brains lacked a major protein band present in the growing brain. We next compared zero-time supernatants with those of starved and growing embryos in an attempt to determine if during 45 hours of growth the synthesis of "new" proteins could be detected and if starvation inhibited the temporal synthesis of new proteins. Or, to restate, we attempted to determine if the onset of synthesis of "new" proteins were growth or time dependent. In comparing zero-time and 45 hours on growth medium the greatest change in proteins was observed in the neural tube followed by heart, brain and somites. In ranking these regions for difference between zero-time and 45 hours of starvation, however, the heart provided the greatest difference and was followed in sequence by somites, brain and neural tube. Thus, it would appear that regions resistant to starvation (somites and heart) could continue to initiate the synthesis of new proteins while regions sensitive to starvation were inhibited. Thus, growth appeared essential for the onset of synthesis for some proteins. This was particularly well illustrated by a specific protein present in brain supernatant extracts. This protein appeared as a major component of the brain of embryos cultured for 45 hours on growth medium. However, this protein was absent at zero-time as well as after 45 hours of culture on starvation medium.

Next, embryo explants were cultured on a medium in which the protein consisted of 75% ovalbumin and 25% conalbumin and compared with embryo explants cultured on whole egg (standard growth medium). Ovalbumin-conalbumin culture resulted in embryos containing slightly less total protein than whole egg cultured embryos but considerably more than protein starved embryos. Thus, ovalbumin-conalbumin provided a medium quite distinct from whole egg in protein composition but allowed considerable growth. This permitted a consideration of a nutritional change without a growth restriction. Similar to previous experiments embryo explants were cultured on either ovalbumin-conalbumin or whole egg growth media for 45 hours and then labeled with either ^3H or ^{14}C -valine for 45-48 hours. The quantitative differences (total percentage difference) was greatest for brain and somewhat lower for neural tube. Heart and somites did not differ appreciably from control values (controls were whole egg cultured run against whole egg cultured). Thus, similar to starvation cultured embryos, a difference was only noted for the brain and neural tube indicating the responsiveness of these two regions to nutrition. However, the response to ovalbumin-conalbumin was quite distinct from that of starvation. During culture on whole egg, four proteins appear in the brain which were not present at zero-time. Three appear in the starved brain at concentrations quite low relative to brains of whole egg cultured embryos while the fourth was absent from the starved brain. In the brains of ovalbumin-conalbumin embryos all four protein peaks were present and at concentrations exceeding that of the brains of whole egg embryos. Furthermore, several minor peaks present in whole

egg brains were greatly increased with ovalbumin-conalbumin. This suggested that the ovalbumin-conalbumin brains might be precocious relative to those of whole egg cultured embryos. Comparisons between zero-time brains and those of ovalbumin-conalbumin cultured embryo (as well as other zero-time comparisons) supported this interpretation. Thus, for brains, zero-time against ovalbumin-conalbumin gave a total percentage difference of 16.0, zero-time against whole egg gave a value of 12.5, and for zero-time against starved this value was 9.0. This suggested that relative to zero-time, the brains of ovalbumin-conalbumin embryos had "differentiated" to a greater extent than those of embryos cultured on either whole egg growth medium or protein starvation medium.

In the most recent series of experiments we attempted to determine if protein turnover occurred during rapid growth and if it involved specific proteins. In addition, we were interested in determining if during protein starvation specific proteins were degraded in those embryo regions sensitive to starvation. The studies on growing embryos has been tentatively completed but additional work must be done with the starved embryos and will not be considered at this time. In these studies, embryo explants were exposed to labeled valine at zero-time (either ^3H or ^{14}C) cultured for six hours to reduce the free pool of amino acids and then one group was dissected and the other group was cultured for approximately 35 hours on either whole egg growth medium or protein starvation medium (but starvation will not be considered). The total percentage difference for all regions

between zero-time versus 35 hours of post-label culture on whole egg growth medium was considerably greater than that of the zero-time versus terminal three hour labeling after 45 hours of culture on growth medium. That is to say, relative to zero-time, labeling followed by culture led to greater differences for all regions than terminal labeling. This suggested that during culture on growth medium breakdown of some specific proteins occurred. Thus, on the one hand, if all proteins were involved in turnover the values for zero-time versus post label culture should approximate those of zero-time versus terminal labeling. On the other hand, if turnover did not occur during culture on growth medium the zero-time and post label culture difference should have been minimal and certainly smaller than the zero-time versus terminal label difference. This interpretation could be supported by two specific proteins which decreases in radioactivity to a greater extent than the other proteins in a gel of brain supernatant prepared from embryos cultured for 35 hours after labeling. One protein had a molecular weight of approximately 15,000 and the other 25,000. The 25,000 molecular weight protein appeared to correspond to the embryo specific serum protein. This suggested that this serum protein may be utilized by the embryo as a source of amino acids or serve a more specific function.

9. Nutrition and the Synthesis of Specific Proteins - Serum Proteins.

The possible role of serum protein in the regulation of early embryonic growth has been a basic component of our working hypothesis. The possible importance of serum proteins was derived from the

observation that protein (ovalbumin) was taken up by the yolk-sac membrane and degraded to amino acids within the membrane (16). Amino acids did not appear to be transported as free molecules to the embryo but were probably utilized by the yolk-sac for the synthesis of protein which were in turn transported to the embryo. We proposed that the transport protein (s) was a serum protein(s). We have for some time attempted to deal with experimental approaches to the study of serum proteins but were generally frustrated in our attempts. The quantity of serum was minute and the only approach appeared to involve withdrawal from the equally minute blood vessels or heart.

During the last year we have been fortunate to find an approach which permits not only the study of serum protein synthesis but also the collection of reasonable quantities of serum proteins. This procedure, we believe, will provide the basis for many interesting and pertinent future studies. To date, we have worked to define conditions and have initiated some experimentation.

The procedure is quite simple. Extraembryonic yolk-sac membranes from which the embryos are removed (under low power-dissecting microscope) are placed in a flask and shaken (wrist action) according to our routine procedure for labeling embryo explants with radioactive precursors. The medium consists only of our standard chick Ringer's-bicarbonate solution and radioactive precursors or other materials we may wish to study. After the desired period the chick Ringer's solution is centrifuged first at low speed (10,000 rpm - 30 minutes)

and then at a higher speed (42,000 rpm - 2 hours). This procedure removes blood cells, yolk particles, etc. The supernatant is dialyzed against water for two days and then placed on a standard 7.5% polyacrylamide gel for electrophoretic analysis. If radioactive amino acids are used, the gels are sliced and the radioactivity is analysed by either low background planchette counting or liquid scintillation counting. Such problems as the number of membranes per ml of chick Ringer's and length of shaking period have now been established.

The chick Ringer's was found to contain five major proteins when yolk-sacs from explants cultured for 48 hours on whole egg growth medium were used. We established that these five proteins were indeed serum proteins by showing that they co-migrated with serum proteins derived from the circulatory system of embryo explants cultured on the same whole egg medium for 48 hours. When membranes were shaken in the presence of ^{14}C -valine, three of the major bands contained radioactivity. The other two proteins did not contain radioactivity and appeared to be derived from proteins of the culture medium. We have identified one protein as serum transferrin and a second as the embryonic specific serum protein (this protein could not be detected in the serum of adult chickens). At this time we are not certain of the identity of the third protein.

In one of our first experiments, we attempted to define the site of serum protein synthesis. Embryos without extraembryonic yolk-sac membranes were shaken in chick Ringer's containing ^{14}C -valine and the Ringer's was analysed. The five serum proteins could be identified

in a stained gel but they did not contain radioactivity. Thus, although the embryo proper contained serum proteins, they clearly were not synthesized by the embryo proper. The synthesis of serum proteins was found to be restricted to the extraembryonic yolk-sac membrane.

In another series of experiments we attempted to determine the developmental stage at which serum protein synthesis was initiated. Previous studies have for the most part been limited to embryos of four or five days of incubation and older (after the circulatory system became functional). Some studies with adult serum antibodies, however, were performed on earlier embryos. These studies suggested that serum proteins were simply derived from intact yolk proteins or possibly from degenerative cells of a primitive line of red blood cells (28,29). Although our studies have not been completed (we will attempt to work with still younger embryos), yolk-sac membranes from embryos of only 24 hours of in ovo incubation (2-5 somites) synthesized serum proteins (yolk-sac membranes were shaken in the presence of ^{14}C -valine and the chick Ringer's solution was analysed by gel electrophoresis). Thus, serum proteins were synthesized by the yolk-sac prior to the establishment of a circulatory system (similar to globin) and clearly they were not simply derived from intact yolk proteins. The time required for synthesis (we shake for only three hours) of serum proteins as well as the fact that the primitive red blood cells do not decline until the fourth day of incubation, suggested that it was unlikely that serum proteins could be derived from

degenerative red blood cells.

We have also analyzed serum from embryos of 4, 8, 9, and 11 days of incubation employing fast green to stain gels and spectrophotometric scans at 650 nm. This procedure permits rather precise quantitation within a limited range of protein concentrations (30). These studies have verified previous studies (31) which showed that serum albumin first appeared at approximately day four of incubation and then increased rapidly relative to serum transferrin and the embryo specific serum protein.

We next turned to the question more directly related to our primary interest. Does nutrition effect the synthesis of serum proteins? Before considering our preliminary studies related to this question, a limitation of the technique we have developed for the study of serum protein synthesis should be noted. Although we could use staining of gels coupled with spectrophotometric scanning to quantitate protein in serum of embryos, the presence of proteins from the nutrient medium used to culture embryo explants in our chick Ringer's shaking solutions has made the use of this procedure difficult in these studies. We have attempted to correct for this "contamination" by using one protein band derived from the nutrient medium that does not co-migrate with serum proteins and another protein that was found exclusively in the serum. However, we have not been terribly pleased with this form of correction. We are still working on this problem but at this point we can only determine from radioactivity, the presence of a particular protein and the amount synthesized relative to the other serum proteins.

It should also be noted that two approaches can be used in the study of nutrition and serum protein synthesis. First, yolk-sac membranes can be placed in chick Ringer's shaking solution from embryo explants cultured for various periods on a particular nutrient medium of interest. Alternatively, a particular nutrient (e.g. a nutrient protein) can be added directly to the chick Ringer's shaking solution. In our preliminary studies we have been mainly concerned with the first approach but we have also considered briefly the second approach.

Yolk-sac membranes from embryo explants cultured for 48 hours on protein starvation medium were shaken in chick Ringer's containing ¹⁴C-valine. The Ringer's was analyzed by gel electrophoresis and the radioactivity was measured. In comparison to yolk-sacs from embryo explants cultured on whole egg growth medium, the "starved" yolk-sacs contained the same three major proteins and the proportion of radioactivity in each of the proteins was comparable to that of "whole egg" membranes. This experiment has been repeated several times with various modifications and generally the synthesis of serum proteins appears similar for starved and growing embryo explants. Again, these results do not represent quantitative estimates of synthesis but rather relative synthesis of the three major serum proteins. In one preliminary experiment we examined serum protein synthesis by yolk-sacs from embryo explants cultured 48 hours on ovalbumin-conalbumin medium. The results indicated that relative to yolk-sacs of whole egg cultured explants, there was a change in the relative synthesis of the serum proteins. Thus, serum transferrin contained 35% of the radioactivity

with ovalbumin-conalbumin and 23% with whole egg. This increased synthesis of transferrin could be attributed to a decreased synthesis of a second serum protein (unidentified) while the relative synthesis of the embryo specific serum protein was the same with both culture media. Although this study may indicate that nutrition can influence the synthesis of serum proteins, it should be clearly noted that these last data were taken from a single preliminary study.

As previously mentioned, we have just started to consider the effects of adding various nutrients, such as proteins, directly to the chick Ringer's used as the shaking (or yolk-sac suspension) solution. The serum proteins themselves were the first to be studied. Serum proteins at a concentration equivalent to that achieved after 3 hours of shaking, were added at zero-time. The yolk-sacs were derived from embryo explants previously cultured for 48 hours on growth medium. After these yolk-sacs were shaken for two hours in the presence of serum proteins, there were reductions relative to controls (no protein added) by 27% in protein released and 70% in protein radioactivity released. The reduction in the quantity of protein released, suggested that the added serum affected the amount of newly formed serum protein released. However, the reduction in radioactivity released could simply reflect a dilution of the added radioactive amino acid caused by the uptake and subsequent breakdown of serum proteins to amino acids by the yolk-sacs. Indeed, this was strongly suggested by the discrepancy between the release of protein and protein radioactivity. We are presently studying the effects of adding other proteins such as

ovalbumin, conalbumin, bovine serum albumin, etc. to the shaking solution. And, we plan to evaluate the response both quantitatively and qualitatively.

10. Miscellaneous Studies

The following studies are presented to provide the overall scope of our activities particularly of the last three years. For the most part they represent lines of investigation that have either been quite recently initiated, continuing for some time but at what might be considered a "snail's pace", or temporarily discontinued in order to follow a more productive direction.

A. DNA and DNA Dependent DNA Polymerase Activity. One of the most striking regional differences that has been observed was that of DNA accumulation. Thus, when embryo explants were cultured on growth medium the accumulation of DNA by the brain exceeded that of other regions but was most severely restricted when explants were cultured on protein starvation medium.

In previous studies, we had investigated protein and RNA synthesis and breakdown, and have attempted similar studies with DNA. Embryo explants of 11 to 13 somites were labeled with ^{14}C -thymidine and then cultured for periods up to 48 hours on either growth (whole egg homogenate) or protein starvation media. Unlabeled (^{12}C) thymidine was added to the media in an attempt to block reutilization. Embryos were dissected into our standard regions, and the DNA was isolated (32) for quantitation (33) and the determination of radioactivity. This experiment has been completed only once and we are in the process of repeating the experiment. Nevertheless, the results were of interest

and although we don't expect precise quantitative reproducibility the general pattern should be reproduced. The results of the first experiment were quite unexpected as they were distinct from our previous protein and RNA studies. With both protein and RNA, radioactivity was not lost during culture on growth mediums. However, DNA radioactivity was lost. The neural tube lost 47% after 48 hours of culture and the brain lost 27% while radioactivity was not lost from the DNA of somites and heart regions. These losses were not expected in view of our past studies with protein and RNA and the fact that these regions rapidly accumulated DNA. One simple interpretation of this observation would be that unlike protein and RNA, the products of DNA breakdown were not reutilized (free thymidine was rapidly degraded). This interpretation, however, would suggest a rather extensive turnover of DNA as well as protein and RNA during rapid embryonic growth. Alternatively we could be dealing with "normal" cell death in the nervous system as has been reported (34) or some form of extensive DNA repair which may occur with rapid cell division. During culture on protein starvation medium, as might be expected, radioactivity was lost from the various regions with the heart maintaining the highest level relative to zero-time.

DNA dependent DNA polymerase activity was determined for the various regions from embryo explants cultured for 48 hours on either growth or protein starvation media. High speed supernatants were prepared after removal of nuclei and served as a source of enzyme following the procedure of Margolis (35). Heat denatured calf thymus

DNA was used as a substrate and the product was analyzed according to published procedures (36). The various controls required in such a study have been studied (absence of enzyme, precursors, etc.). DNA polymerase activity (expressed per μg DNA or μg protein) was found to be directly proportional to our estimates of "net" DNA synthesis for the various regions as well as for growth and starvation culture conditions. The estimate of "net" DNA synthesis was derived by combining the determination of DNA accumulation with the value for DNA lost derived from the loss of DNA radioactivity. This correlation between "net" DNA synthesis and DNA polymerase activity, suggested that this enzyme may be central in regulating the cellularity of a particular region as well as this important aspect of the growth process.

B. Electron Microscopy and Histology. At various times we have examined histological as well as electron microscopic preparations of embryo explants cultured on various media. For the most part, however, we have examined explants cultured on growth and protein starvation media. These studies have generally confirmed our biochemical work by indicating that the developing nervous system was particularly sensitive to protein starvation. Thus, histological preparation have shown in protein starved embryos that there was a delay in the formation of neural crest cells, neural ectoderm lost their columnar arrangement, and toward the end of 48 hours of incubation degenerative changes appeared in the neural ectoderm. Electron micrographs of neural ectoderm from starved embryos exhibited a reduction in microtubules as might

be expected from the loss of columnar arrangement. We are presently attempting to quantitate tubulin, the major protein associated with microtubules. The recent work of Messier and Auclair (37) should be noted. These investigators observed in chick embryos that inhibiting microtubule formation blocked nuclear migration in the neural ectoderm. Nuclear migration may be required for these cells to divide. Thus, a blockage of microtubule formation or a breakdown of existing microtubules could block cell division and DNA synthesis and ultimately be responsible for the sensitivity to protein starvation of nervous tissue in an early embryo. Although microtubules appear to be generally present in all types of cells, the phenomenon of nuclear migration coupled to cell division may be more restricted.

C. Absolute Protein Synthetic Rates. In our original studies (19) on the effects of protein starvation, during early embryonic development, it became essential to determine if starvation had an effect on protein synthesis. Thus, although we observed regional differences in protein breakdown, we were concerned with the possibility that breakdown could be occurring equally in all regions and the regional differences in breakdown that were observed were actually the result of regional differences in reutilization or synthesis. Our initial studies of the effects of starvation on protein synthesis did not take into account precursor pool specific activity but simply precursor uptake. In order to determine more precisely protein synthetic rates, we turned to the elegant method developed by Regier and Kafatos (38). This procedure was based on the formation of

a ^{14}C -2,4,dinitrofluorobenzene derivative of the total pool of a particular amino acid. Thus, by employing a ^3H labeled amino acid to follow incorporation into protein, the ratio of ^{14}C to ^3H would provide the mathematical factor for "pool dilution". We also became concerned with the problem of amino acid compartmentation within cells and decided that we must deal with the extent of precursor dilution in the most immediate precursor. Thus, we attempted to determine the precursor (amino acid) specific activity attached to transfer RNA. We have worked on most of the many steps involved in these procedures. However, we ultimately turned to studies with isolated polysomes and "cell-free protein synthesis". In brief, the cell-free system appeared to be a more direct approach and one that could be analyzed in depth. Although we have not completely given up on "absolute synthetic rates" in the intact embryos, at present the cell free system (this was previously discussed) appears to be a more productive approach.

D. Affinity Chromatography and Nutrient Protein Uptake.

Several examples of nutrient protein complementation and specificity in the nutrient protein requirement of early chick embryos were previously discussed in this report. Specificity of receptors for nutrient protein uptake on the cellular membranes of cells of the yolk-sac could provide a possible basis for some of these observations. A particularly favorable example where such a possibility appeared feasible was the difference between embryo explants cultured on conalbumin and bovine serum albumin. With conalbumin in the nutrient medium, the yolk-sacs accumulated large amounts of protein. On the other hand, the yolk-sacs were poorly developed and contained little

protein when embryo explants were cultured on bovine serum albumin. Thus, we attempted to consider the possibility that this difference between conalbumin and bovine serum albumin could be the result of a lack of receptors for bovine serum albumin or the presence of numerous receptors for conalbumin.

The methods developed by Cuatrecasas (39) for affinity chromatography and the "isolation" of cell membrane receptors was used. Working with cyanogen bromide activated-Sepharose 4B, derivatives containing conalbumin and bovine serum albumin were prepared and poured into a column. Radioactive membrane preparations were passed through the column and after sufficient buffer was used to rinse the non-adhering radioactivity the ionic strength of the buffer was increased. With the increased ionic strength, two distinct peaks of radioactivity appeared. This was very encouraging but we observed the same general pattern for both conalbumin and bovine serum albumin. There were many possible variations with the technique that were not studied. We hope to return to the use of affinity chromatography with this problem in the near future.

E. Hydrolases and Protein Starvation. As previously discussed, regional differences have been observed in the response of the early chick embryo to protein starvation. This regional difference became particularly apparent when studies were made of the breakdown of protein and RNA. Thus, in one of our earlier studies we determined for the individual regions total ribonuclease as well as cathepsin (proteolytic) activity (employing the methods of R. Weber, 40).

(It should be noted that we did determine optimal pH, ionic strength, substrate concentration and enzyme concentration.) In general, the differences between regions for the activities of both enzymes was relatively small and the differences in enzyme specific activities did not relate to regional differences in sensitivity to starvation. Thus, we have tentatively concluded that the effects of starvation on the early embryo were not related to these two enzymes.

It should be noted that the above studies were distinct from those on ribosomal associated ribonuclease activity that were previously discussed and presented in the preprint included with this proposal (22a).

II. SUMMARY OF RESEARCH ACCOMPLISHMENTS OF THE LAST THREE YEARS

In the previous discussion an attempt was made to present in some depth our research accomplishments of the last three years in the context of research activities that preceded this period. I will now attempt to summarize only those activities of the last three years.

1. Yolk Proteins. Much of the work discussed on yolk proteins had been completed prior to the current three year period. However, one of the major observations was made during the current period. We had observed that no single yolk protein would support embryo growth. However, when two proteins were combined, lipovitellin and livetin, embryo growth was excellent. Lipovitellin was relatively pure but the livetin fraction consisted of some six to ten different proteins. During the last three years the livetin fraction was resolved into a series of subfractions by column chromatography and each was tested in combination with lipovitellin. Of the several fractions, the major growth promoting activity was associated with yolk transferrin. Thus, it was possible to demonstrate a high degree of specificity in the embryo nutrient protein requirement. Furthermore, it is now possible to study in rather precise terms the basis for this requirement. This work has been accepted for publication (preprint included with the proposal, 11) and will appear in the July 1974 edition of Developmental Biology. A second paper on the yolk fractionation methods developed (modifications of published procedures) for these studies is almost ready for submission.

2. Nutrient Protein Starvation. During the last three

years we have worked extensively on the protein starved embryo. Indeed, prior to this period we had only completed our rather gross observations on regional differences in protein and RNA breakdown. During the last three years we first studied losses from subcellular fractions. This investigation showed that 40 to 45% of the protein label and 60 to 70% of the RNA label that was lost during starvation from sensitive regions were derived from the ribosome fraction (2). In addition, we studied rather extensively the effects of starvation on regional polysome activity (22A). Thus, during starvation there was a reduction in the protein synthetic activity of isolated polysomes. This reduction occurred to the same extent in the brain which was highly sensitive to starvation as well as in the heart which was highly resistant. Furthermore, during starvation there was a reduction in the number of ribosomes as polysomes as well as in the number of ribosomes per cell. Again, these reductions occurred to approximately the same extent in brain and heart. From this we have been able to conclude (A) regional differences in sensitivity to protein starvation did not involve regional differences in the inhibition of protein synthesis and (B) during protein starvation protein synthesis is reduced and this is due to reductions in the protein synthetic activity of the polysomes themselves (probably the major factor), as well as the number of ribosomes as polysomes, and the number of ribosomes per cell.

In the above studies reductions were observed to occur in the brain as well as heart. However, we also observed that the protein

synthetic activity of polysomes isolated from the brain was considerably greater than that for heart polysome. This was true for embryo explants cultured on growth as well as protein starvation media. Now, as previously mentioned starvation results not only in a reduction in polysome activity but also in reductions of number of ribosomes as polysomes and ribosomes per cell. But, all three reductions were approximately proportional for brain and heart. If one considers only one culture condition, namely growth, the difference between brain and heart was restricted to polysome activity. This is to say, the brain and heart of growing chick embryo explants have approximately the same number of ribosomes as polysomes as well as the same number of ribosomes per cell. Thus, the regulation of "normal" regional growth at least for protein synthesis appears primarily to involve differences in the activity of the polysomes themselves. This observation, to the knowledge of this investigator, provides the first clearly defined mechanism for the regulation of regional growth during early embryonic development. Indeed, polysome activity can be precisely related to the differences between brain and heart in the rate of protein accumulation during the period of development studied.

In addition to the above, several pertinent aspects of the cell-free protein synthesizing system used in the study were evaluated. For example, ribosome associated ribonuclease activity, the stability of the ribosome-messenger RNA complex (polysome), and the stability of the ribosomes themselves. All gave negative results with regard to

a correlation between regions and their differences in protein synthesis or between regions and their differences in protein starvation sensitivity.

The results of the studies on polysomes are contained in one of the manuscripts presented with this proposal (22A). The manuscript was originally prepared with Biochem. Biophys. Res. Comm. in mind but we may have too much data we wish to present for this particular journal. An abstract of some of this material has been submitted for presentation at a meeting and is enclosed.

3. Nutrition and the Synthesis of Specific Proteins -

Gel Electrophoresis. This work dealing with the effects of nutrition on the synthesis of proteins by the early chick embryo was first initiated during the past three years. These studies involve the use of acrylamide gel disc electrophoresis in combination with "double" label and co-electrophoresis techniques. In double label co-electrophoresis, two groups of protein mixtures can be compared to provide a single value expressing the degree of similarity. For example, in comparing the brain proteins from protein starved and growing embryo explants, one group was "pulse" labeled with ^3H -valine and the other with ^{14}C -valine. The proteins from both were combined for electrophoresis which makes direct comparison possible.

In the first study we showed that the regions we routinely study (brain, heart, somites, neural tube and extraembryonic yolk-sac) synthesized a mixture of proteins which were distinctive both quantitatively and qualitatively. Turning next to the effects of

nutrition on the synthesis of proteins, we first examined the embryo explant cultured on protein starvation medium. In comparing regions of starved and growing embryo explants, the largest difference was found for the brain and second for the neural tube. These two regions were previously found to be the most sensitive to starvation. We next compared zero-time (11 to 13 somites) with embryos cultured on growth and starvation medium for 45 hours. The results with the starved embryo explants were particularly interesting in that the heart which was shown to be resistant to starvation differed to the greatest extent of the various regions from the zero-time pattern. On the other hand, the starvation sensitive brain and neural tube differed least from their zero-time patterns. From this type of study, we have tentatively concluded that protein starvation restricted the onset of "new" protein synthesis. Starvation, however, not only alters the nutrition of the embryo but also severely restricts growth. Thus, we have turned to comparisons between explants cultured on conalbumin-ovalbumin with those cultured on our standard whole egg growth medium. In this comparison, nutrition was changed without greatly altering growth (net accumulation of protein).

Similar to starvation, the brain and neural tube of ovalbumin-conalbumin cultured embryo explants differed more than the other regions ^{when compared to} ~~those~~ those cultured on standard growth medium. However, the response to ovalbumin-conalbumin was quite distinct from that of starvation. This became quite apparent by examining individual protein peaks in the gel and from zero-time comparisons. With reference to

zero-time comparisons, starved brain patterns were more comparable to zero-time than whole egg brains. However, conalbumin-ovalbumin brains were more distinct relative to zero-time than whole egg brains. This has suggested that the ovalbumin-conalbumin brains were actually precocious. In any case, we believe that we have demonstrated that nutrition can effect the synthesis of proteins both qualitatively and quantitatively. Furthermore, unlike protein starvation, the differences in proteins synthesized cannot be attributed simply to differences in growth.

In addition to the above, we have employed acrylamide gels to study protein turnover. Although these studies have not been completed, we believe that we have demonstrated, for the first time, protein turnover during rapid embryonic growth.

A preliminary report of these studies was presented at a meeting (27) and we are presently preparing some of this material for publication.

4. Nutrition and the Synthesis of Specific Proteins -

Serum Proteins. During the last three years we initiated studies on the synthesis of serum proteins. For some time we have attempted to study serum proteins because the effects of nutrition on early embryos and particularly protein nutrition may be mediated through the synthesis of serum proteins. Recently, we were fortunate to find a single and direct method to study the synthesis of these proteins and to collect them in reasonable quantities. Quite simply, we found that intact yolk-sac membranes released serum proteins into

a liquid medium. The yolk-sacs were placed in buffered chick Ringer's and gently shaken. The Ringer's was collected, centrifuged, dialyzed, and placed on acrylamide gels. In the presence of radioactive amino acids, three radioactive proteins were released into the Ringer's.

To date, we have made several interesting observations. First, within the sensitivity of the technique, serum proteins were synthesized exclusively by the yolk-sac membrane. Yolk-sacs as early as 24 hours of incubation in ovo (2-5 somites) synthesized serum proteins. The relative synthesis of the three serum proteins was found to be similar in yolk-sacs from embryo explants cultured on whole egg growth and protein starvation media. However, when embryo explants were cultured on ovalbumin-conalbumin medium the relative synthesis of the three proteins was found to be distinct. (In the previous part of this proposal, I indicated that these results had not as yet been repeated. By now, they have been repeated and provided a confirmation of the previous results.) In addition, the relative synthesis of the three proteins was found to change with developmental age.

Although we have been particularly pleased to be able to demonstrate that protein nutrition can effect serum protein synthesis, we are equally pleased to be able to collect relatively large amounts of early embryonic serum proteins. The value of this serum to our proposed research will be considered under "Proposed Research".

We hope to complete some additional studies, particularly those involving the addition of proteins to the shaking solution,

before preparing this material for publication.

5. Miscellaneous Studies. One miscellaneous study should possibly have been placed in a separate category as a major study. This work concerns DNA synthesis and breakdown and DNA dependent DNA polymerase activity. When the protein or RNA was prelabeled and embryo explants were cultured on growth medium, radioactivity was not lost from the RNA or protein of the various regions. With thymidine, however, considerable radioactivity was lost from DNA during growth. Although this may have indicated that free thymidine unlike free uridine or free amino acids was rapidly degraded (this has been demonstrated in other systems), the relatively large amounts lost were surprising. The losses occurred mainly in the brain and neural tube regions and both regions have been shown to be particularly active in DNA synthesis (as well as sensitive to starvation). This may be indicative of active DNA repair in those regions undergoing rapid cell division. This may become a subject for future study.

In addition to following the loss of radioactivity from DNA, we also examined DNA dependent DNA polymerase activity in the various regions. In brief, it was possible to directly relate, DNA polymerase activity to the "net" value for DNA synthesis for a region. This close relationship suggested that this enzyme may be of primary importance in the regulation of the cellularity of a particular region.

Histological and electron microscopic observations have been made of the growing and protein starved embryo explants. These observations have supported our biochemical findings by indicating that the

nervous system was particularly and uniquely sensitive to protein starvation. The electron micrographic observations have suggested that protein starvation may reduce the number of microtubules in the neural ectoderm.

Some time was devoted to the determination of absolute protein synthetic rates. The procedure we considered involved the determination of the specific activity of the amino acid attached to transfer RNA. Although this approach appeared feasible, our success with isolated polysomes and cell-free protein synthesis suggested a more direct and productive approach to the problem.

Preliminary studies were initiated with affinity chromatography. The objective was to relate receptor sites on the cell membranes of the yolk-sac to the ability of the early chick embryo to utilize a particular protein. To date we have not been successful.

Finally, we attempted to relate the sensitivity of a particular embryo region to protein starvation with the activity of two hydrolytic enzymes. Both ribonuclease and cathepsin (proteolytic enzymes) were studied but their activities could not be related to protein starvation sensitivity.

PROPOSED RESEARCH

For some time we have worked on the basic hypothesis that the availability of amino acids at the cellular sites for protein synthesis ultimately regulates the differences observed in the rates of growth of various regions of a developing organism. This rather simplistic hypothesis assumes greater significance when we broaden the hypothesis to include all aspects of nutrient protein utilization as well as the cellular response to the influx of amino acids. Thus, we are not simply dealing with the passage of free amino acids from a nutrient medium into the cells of the embryo. The early chick embryo requires nutrient protein and this requirement appears rather specific. Nutrient proteins appear to be taken up by the yolk-sac membrane, broken down to amino acids in the yolk-sac, and converted to serum proteins. We believe that the serum proteins are transported to the cells of the embryo where they act either as a source of amino acids or perform a more specific function. Turning to the cellular response, it appears that regional growth (at least protein accumulation) may be regulated by polysome activity. Finally, growth itself does not simply involve the accumulation of proteins (and other macromolecules) but the accumulation of a rather specific group of proteins that appear related to both the stage of development as well as the extent of growth (the total accumulation of protein).

The proposed studies represent a continuation of our ongoing research program dealing with several aspects of the above statement. The order of their presentation follows simply from the preceding

discussion of "Research Accomplishments".

1. The Stability of Soluble Proteins. During culture on protein starvation medium, radioactivity was lost from pre-labeled proteins in a regional specific manner. The brain lost considerable amounts of radioactivity while essentially none was lost from the heart. Approximately 40 to 45% of the radioactivity lost from the brain was found to be derived from the ribosome fraction. In a subsequent study we attempted to demonstrate that brain ribosomes were more susceptible to degradation than those of the heart. We essentially failed to demonstrate a difference for ribosomes and now propose to examine the stability of soluble proteins. The objectives are to first find a possible basis for the regional differences in susceptibility to starvation and second to characterize the proteins synthesized in a particular region in terms of their stability. It should be noted that we have examined two hydrolytic enzymes, ribonuclease and cathepsin, and failed to find regional differences. If the regions susceptible to starvation did not contain high levels of degradative enzymes, it appears logical to consider the susceptibility of the substrate to degradation.

Chick embryo explants will be cultured and labeled with radioactive amino acids according to our standard procedures. In our initial studies brains and hearts will be compared and these regions will be isolated from embryo cultured on whole egg growth and protein starvation media. Subsequently, other regions (somites, neural tube, extraembryonic yolk-sac) as well as culture media will be studied.

Isolated regions will be homogenized and post-ribosomal supernatants will be collected. The supernatants may then be dialyzed against buffer if the level of free radioactive amino acids is high and they will then be incubated either without added enzyme (autodegradation), or with degradative enzymes. Initially we will consider trypsin and chymotrypsin, and subsequently pronase and subtilisin. This selection of degradation enzymes is taken from the work of Bond (41) who showed that the susceptibility of various rat liver enzymes in vitro to trypsin and chymotrypsin degradation followed the half life of these enzymes in the intact animal. Pronase and subtilisin did not degrade the liver enzymes in a manner related to their half lives. In addition to the studies involving autodegradation and commercially obtained enzyme fractions, we will attempt to use natural sources of proteolytic enzymes. For example, the yolk-sac membrane is an excellent source of proteolytic enzymes. Various controls such as combining two batches of supernatant proteins will be studied. This control, for example, will show if the supernatant proteins themselves contain differences in proteolytic enzymes.

Two analytical techniques will be used. First, the kinetics of breakdown and quantitative differences in breakdown will be determined by following the release of radioactivity into a form soluble in 5% trichloroacetic acid. Second, in order to determine if breakdown is specific, the protein mixtures will be analyzed by polyacrylamide gel electrophoresis. For example, soluble proteins labeled with ^{14}C -amino acids and treated with enzyme will be analyzed by co-electrophoresis

with soluble proteins labeled with ^3H -amino acids and not treated with enzymes. Alternatively, two groups of soluble proteins (e.g. brain and heart) one labeled with ^3H and the other with ^{14}C will be combined before enzyme treatment and analyzed by co-electrophoresis.

It should be clearly noted that we have had considerable experience with both acrylamide gel analysis (double label) as well as with following breakdown with trichloroacetic acid.

2. Protein Synthetic Activity of Ribosomes. We have shown that the protein synthetic activity of isolated brain polysome was greater than those of the heart when derived from the early chick embryo. Insofar as this difference in polysome activity follows the difference between heart and brain in rates of protein accumulation, we feel that polysome activity may be a key factor in the regulation of growth. Logic would dictate that our next experiments should involve an examination of ribosomal factors (initiation factors, binding factors, etc.) which may be responsible for the observed differences between brain and heart. And, we will attempt to follow this logic. However, one of the limitations of the chick embryo explant systems is that it provides relatively small quantities of tissue. Thus, although we will attempt to directly approach the problem, we may be forced to consider alternatives.

Initially we will follow the approaches used by Rowley et al. (42) who studied the decline of protein synthetic activity of polysomes from maturing reticulocytes; Johnson (23), and Gilbert and Johnson (24) who have studied the decline in protein synthetic activity of polysomes

with maturation of the rat brain; and the studies from the laboratory of Heywood (43) and Gilbert (44) who have studied ribosomal factors with reference to translational specificity. (Heywood is located at the University of Connecticut and will be available for assistance.) I will not attempt to provide details, but the general approach that may be used would involve the treatment of ribosomes with KCl. KCl dissociates ribosomes into subunits and the subunits can be separated from the KCl by centrifugation. One can then separate the larger subunit from the smaller by sucrose density gradient centrifugation. Endogenous messenger RNA can be removed from the KCl by the DEAE cellulose batch procedure. The three components (large subunit, small subunit, and KCl supernatant) can then be interchanged in an attempt to determine which might be involved in regulating the activity of a particular group of polysomes. Naturally, a source of messenger RNA must be added. We would first use the synthetic message, polyuridylic acid and follow radioactive phenylalanine incorporation and next consider relative pure natural messenger RNA's such as the one for myosin or globin. Finally, we can use the polysomes themselves as a source of non-specific messenger RNA's.

An experiment would proceed in the following manner. Ribosomes will be isolated from brains and hearts of cultured chick embryos. The ribosomes will be dissociated into subunits with KCl and the KCl supernatant will be collected. To determine, for example, if the KCl contains factors which regulate the activity of polysomes, brain KCl extract will be added to brain ribosomes as well as to heart

ribosomes. Samples from the same batch of messenger RNA will be added to both reaction mixtures, and the incorporation of radioactive amino acids will be followed.

It appears quite possible that we will be unable to obtain sufficient ribosomes to prepare an adequate KCl extract. If this should be the case, we can prepare the extract from ribosomes taken from the entire embryo or from some region of an older embryo. Thus, we still could compare ribosomes from the heart and brain of the early embryo with a constant source of factors.

If we are able to work with the ribosome factors, it would be particularly interesting to compare the proteins comprising the factors. We would be interested not only in the differences between factors for two different regions but also in the possible effects that changes in the nutrition of the early chick embryo might have on these factors. Gilbert (44) has recently shown that these factors can influence the selection of messenger RNA's for synthesis employing total forebrain messenger RNA fraction. Furthermore, Rowley *et al.* (42) has demonstrated by gel electrophoresis that there are differences between KCl extract of young and mature reticulocyte ribosomes. We have shown that protein nutrition can effect the pattern of proteins synthesized in the brain of the cultured early chick embryo and in the relative synthesis of serum proteins by the yolk-sac membrane. Although clearly extending beyond the limits of the data, it is intriguing to suggest that the protein nutrition of the early embryo could effect the protein composition of the KCl soluble factors.

Although there are many uncertainties concerning the feasibility of approaching the above question, the experimental design can be outlined. The KCl soluble factor fraction will be derived from brains, hearts, and yolk-sac membranes of chick embryos cultured on various media. The factor fractions will be compared by acrylamide gel electrophoresis. Alternatively, factor fractions from various regions and embryo culture conditions will be used in a cell-free protein synthesizing system and the protein products will be analyzed by acrylamide gel electrophoresis. One could go on with the isolation of individual proteins from the factor fraction, etc. but this should be sufficient to indicate the manner in which such studies might proceed.

3. Nutrition and the Synthesis of Specific Protein - Gel

Electrophoresis (completion of study). Most of the work on this problem has been completed and part of it is being prepared for publication. We would like to propose a few additional experiments to complete this study. In view of the extensive discussion that has been given, the experimental details will be omitted.

First, we have observed by acrylamide gel electrophoresis that the synthesis of proteins particularly in the brain can be influenced by protein nutrition. To date, we have studied embryo explants cultured on whole egg, protein starvation, and conalbumin-ovalbumin media. We propose to extend this study to other nutrient media and particularly to a medium containing only egg yolk or the lipovitellin and livetin fractions of yolk. Second, we would like to complete our studies on the specificity of protein breakdown in the intact embryo.

Chick embryo explants are labeled at zero-time (11-13 somites) with radioactive valine and cultured on protein starvation medium. The soluble proteins are extracted from the various regions after periods of culture and analyzed by polyacrylamide gel electrophoresis.

4. The Synthesis and Breakdown of Microtubule Protein.

The brain of the developing chick embryo has become an organ of major interest. The brain accumulates DNA, protein, and RNA at a faster rate than other regions during the embryo explant culture period, brain polysomes in vitro have the highest protein synthetic rate, and the brain is particularly sensitive to starvation. Thus, we have been trying to study a protein that might be specific for brain which could be followed in terms of synthesis and breakdown and provide some insights into the regulation of brain growth. We have, for example, considered the brain specific S-100 protein but this protein first starts to appear at the end of the period when embryo explants can be cultured (45). More recently we have started to consider microtubule protein (tubulin). Although this protein is not brain specific, the brain of the 5-day chick embryo contains 20% microtubule protein (46) and as previously discussed microtubules may be essential for nuclear migration in the neural ectoderm. In addition, there is some evidence that this protein has a relatively short half life (47) and may change in stability with development (46). Thus, we propose first to quantitate microtubule protein in the brains of chick embryos cultured under various conditions of protein nutrition. Second, we propose to isolate microtubule protein so that its stability can be followed in

the intact embryo as well as in vitro.

Several methods for the quantitation of microtubule protein have been reported. For example, there is the technique of radioactive colchicine binding coupled with time-decay analysis (46). Another method involves polyacrylamide gel analysis and staining with Fast Green (46). We are presently working with both methods.

The experimental design involves simply the quantitative analysis of brain microtubule protein in embryos cultured under various conditions as well as zero-time embryos. For example, we will determine if the "precocious" brains of ovalbumin-conalbumin cultured embryos contain particularly high levels of microtubule protein and if the level of this protein in the brains of protein starved embryos is particularly low. Furthermore, in the starved embryo we will follow the concentration of microtubule protein during the entire 48 hour culture period in order to determine if a decrease in this protein precedes the general breakdown of brain proteins.

There are several methods for the isolation of microtubule protein. For example, vinblastine precipitation (47), acetone precipitation (48), and ammonium sulfate precipitation followed by DEAE Saphadex chromatography (49). By isolating microtubule protein from embryo explants labeled with radioactive amino acids, it will be possible to follow the kinetics of synthesis and breakdown of this protein. In brief, the kinetics of microtubule protein synthesis and breakdown will be followed in the brains of chick embryo explants cultured on various nutrient media. These media containing various

nutrient proteins have been described above.

In addition to studies of microtubule protein stability in the intact embryo, we also propose to study the stability of this protein in vitro. We propose to follow the same procedures for microtubule protein as were previously outlined under "The Stability of Soluble Proteins". In brief, radioactively labeled microtubule protein will be incubated with various proteolytic enzymes and the release of radioactivity from the protein will be determined. The release of radioactivity from this protein will be compared to that of the total soluble proteins.

5. Nutrition and the Synthesis of Specific Proteins -

Serum Proteins (completion of study). As previously discussed, we have developed a new procedure to study serum protein synthesis by the yolk-sac membrane. To date, we have found that the three major serum proteins are exclusively synthesized by the yolk-sac and that serum proteins are synthesized quite early in development. In addition, we have found that protein nutrition can effect the relative synthesis of the serum proteins. We propose several additional experiments to complete our initial studies of serum protein synthesis.

First, we have shown that the relative synthesis of serum proteins by embryo explants cultured on ovalbumin-conalbumin medium is different from that of explants cultured on whole egg medium. We now propose to study serum protein synthesis by embryo explants cultured on yolk alone or on the yolk fractions, lipovitellin and livetin. As previously discussed, intact yolk-sac membranes will be isolated from

embryo explants cultured for 48 hours on yolk or yolk protein fractions. The yolk-sacs are then placed in chick Ringer's containing radioactive amino acids and gently shaken for 3 hours. At the end of this period, the Ringer's is centrifuged, dialyzed, and analyzed by polyacrylamide gel electrophoresis. The relative synthesis of the three serum proteins will be compared with that of embryo explants cultured on whole egg (a mixture of egg white and yolk) and ovalbumin-conalbumin media.

Another aspect of this work that has just recently been initiated, involves the addition of various nutrient proteins directly to the chick Ringer's solution. Thus, after a period of embryo explant culture, yolk-sac membranes are placed in chick Ringer's containing various nutrient proteins and radioactive amino acids. The relative synthesis of the three major serum proteins is then determined as outlined above. We propose to study the effects of conalbumin, ovalbumin, lipovitellin, livetins and bovine serum albumin.

6. The Selective Uptake of Serum Proteins. In our basic hypothesis (see the introduction to "Proposed Research") the serum proteins play a major role in regional growth regulation either by supplying amino acids or in some more specific and unique manner. Central to the hypothesis is the suggestion therefore that there are regional differences in the cellular uptake of serum proteins. We now propose to test rather directly this aspect of the hypothesis.

Single cell suspensions will be prepared from the various regions of the early chick embryo. We will start with cells from the brain and heart. These cells will next be incubated with radioactive

serum proteins for various periods. The cells will be filtered and rinsed on Millipore filters and the amount of radioactivity on the filter will be analyzed. We have had considerable experience with preparing cell suspension from early embryos as well as Millipore filtering of cells. We will employ the same techniques that we used in an earlier study (50). In addition to studies of serum protein uptake by cells from various regions, we also propose to compare uptake by cells of embryos cultured on various media. For example, we would be particularly interested in the possibility that cells from embryos cultured on protein starvation medium alter their capacity to take up serum proteins.

Another aspect to this study, concerns the possibility of qualitative specificity of serum protein uptake. For example, do cells from different regions selectively take-up specific serum proteins. To answer this question, the medium containing the serum proteins will simply be analyzed by polyacrylamide gel electrophoresis following the removal of the cells.

Another direction being considered involved the use of affinity chromatography. If differences between cells in the uptake of serum proteins can be demonstrated, it would be pertinent as a follow-up experiment to demonstrate differences in surface membrane receptors. Thus, Sepharose derivatives containing serum proteins could be used to detect possible differences in cell membrane receptors. The methods published by Guatrecasas (39) would be used for preparing the Sepharose column and for the preparation of membrane receptors.

7. The Specificity of the Nutrient Protein Requirement.

The requirement of the chick embryo for nutrient proteins is rather specific. For example, the chick embryo utilized ovalbumin but not bovine serum albumin which is quite similar to ovalbumin. At various times we have worked at attempts to investigate the basis for this specificity. These attempts have involved rather specific modifications of a particular nutrient protein followed by an evaluation of the ability of the modified protein to support embryo explant growth. To date we have not been very successful but would still like to propose some continuation of this work.

We have been able to culture embryo explants quite successfully on a mixture of two high purified proteins, ovalbumin and conalbumin. Both of these proteins have received considerable attention by protein chemists and are well characterized. At this time, we simply propose to study the carbohydrate moieties. Conalbumin and ovalbumin will be treated either with mannosidase or N-acetylglucosamine. After the particular enzyme fraction is removed through column chromatography, the ability of the altered conalbumin and ovalbumin to support embryo growth will be determined. We have made some preliminary attempts to isolate these two enzymes by published procedures (51, 52).

STATE OF KNOWLEDGE, SIGNIFICANCE, AND NEEDS FOR FUTURE STUDIES
ON PROTEIN NUTRITION AND GROWTH REGULATION

The studies that have been supported during the last three years by contract with the U.S.A.E.C. as well as those investigations proposed for continued support are mainly related to the general area of developmental nutrition. More specifically, we are concerned with the determination of the role that nutrient proteins play in the regulation of early embryonic growth and development. Our research encompasses not only the mechanisms of nutrient protein utilization and the basis for the specificity of the nutrient proteins requirement but also the growth response of the embryo to nutrient proteins in terms of both the quantitative and qualitative regulation of macromolecular synthesis (particularly proteins). The system that we employ for these investigations provides a unique opportunity to study developmental nutrition and the regulation of growth. Cultures of early chick embryos represent the only available system in which the nutrition of an intact embryo can be precisely controlled. Furthermore, the ability to select embryos of uniform size and developmental stage provides the investigator with the opportunity to make precise quantitative biochemical analyses.

Studies of early embryonic protein nutrition and the regulation of early embryonic growth can clearly be related to a multitude of active areas of research in both biology and medicine. Thus, our research appears relevant not only to developmental nutrition but also to such central issues in developmental biology as the relationship of growth to

differentiation and the control of differentiation in terms of the regulation of the synthesis of embryo specific proteins. From a medical standpoint our work appears relevant to such areas as protein nutrition and brain development, teratology (for example, the effects of teratogens on embryonic growth), and carcinogenesis as a problem of growth regulation. Without attempting to cover these many subjects, I will briefly consider those areas which I consider particularly relevant to our research activities.

Studies of dietary protein restriction during development have been mainly conducted on the pregnant rat. If the restriction of protein is severe and early in pregnancy, embryos are resorbed (18). Furthermore, this does not appear to be the direct result of protein starvation of the embryo but rather an indirect response to maternal hormonal insufficiency. Thus, the cultured chick embryo provides a most unique opportunity to study the direct effects of protein starvation on an embryo.

Our studies on early embryonic protein starvation have clearly demonstrated that the brain is the most sensitive region of an early embryo to protein starvation. Studies on protein starvation in the pregnant animal have also directed attention to the developing brain. However, this attention is based more on the importance of the brain to the subsequent behavior of the organism than to the sensitivity of the brain to starvation in terms of macromolecular synthesis. Indeed, in the newborn rat the accumulation of DNA, RNA, and protein appears less sensitive to protein starvation than that of any other organ (53).

And, the resistance of the adult brain to starvation has been well documented (54). An extrapolation from the early chick embryo to the rat may be unrealistic. However, if our results on the sensitivity of the brain of the early chick embryo to starvation represents a general phenomenon for embryos, the basis for this sensitivity as well as the apparent change in sensitivity during development may be of considerable importance to our understanding of brain development. The studies that we have proposed represent a unique approach to this problem and hopefully will provide some new insights into this subject area.

We have demonstrated that the early chick embryo requires nutrient protein and that this requirement is somewhat specific. Furthermore, it appears that the embryo may require more than a single nutrient protein. We have not been able to culture embryo explants on single purified nutrient proteins but certain mixtures of two purified proteins will support embryo growth and development. In older organism, such nutrient protein complementation has been demonstrated but appears to simply involve a complementation in terms of amino acid composition. For example, one protein may lack a certain essential amino acid which is present at high concentrations in a second protein. This does not appear to be the case in our studies. Furthermore, we have obtained data on the synthesis of embryo specific proteins and serum proteins which suggest that nutrient proteins can have an effect on these synthetic processes. Therefore, we feel that the specificity of the nutrient protein requirement as well as the specificity of the nutrient protein complementation requirement are of developmental significance.

One can cite the nerve growth factor, blastokinin, or the "gastrulation" factor of Briggs as examples in which proteins appear to have rather specific effects on developmental processes. However, in distinction from our studies, these examples involve relatively small quantities of very unique proteins. Our observations may relate more to the many studies involving cell cultures with various fractions of serum or embryo extract. Such fractions have been implicated in the control of cell proliferation (55, 56, 57), migration (57), survival (57, 58) and the maintenance of the differentiated state (59, 60, 61). Although the active components have not been rigorously defined several observations do suggest that a protein(s) is involved (see 10 for review). These studies with cell cultures may also be quite unrelated to the observations that we have made. Studies with cultured mammalian embryos which could be relevant to our studies remain rather controversial on this subject (10). However, Hsu (8) in his highly successful culture of mammalian embryos found that it was essential to change the source of serum in the culture medium at rather specific stages of development. Again, proteins may be involved. In any case, at this time our observations on nutrient protein complementation and specificity appear quite unique. The possibility that this nutritional requirement is not restricted to the chick embryo will require studies with embryos of other organism. However, the basis for this requirement may be central to our understanding of protein nutrition during development.

Our studies with the protein starved embryo have focused our attention not only on the basis for the sensitivity of the developing nervous system to nutrition but also to the question of the relationship

of growth to differentiation. This relationship has received considerable interest in the developmental biology literature but has for the most part been restricted to cell cultures. In our studies we employ an intact embryo and have recently been able to show that the synthesis of certain proteins particularly in the brain is growth dependent while that of others is time dependent. In earlier studies of total protein and RNA we were able to show that during starvation the proteins and RNA of the brain are more readily degraded than that of the other regions. We proposed that this degradation was due to the fact that the brain relative to other regions was undifferentiated. And, the breakdown of proteins in such regions as the brain was essential to provide the necessary precursors for the continuation of differentiation. From our current studies this does not appear to be entirely the case. The brain begins to synthesize at least four new proteins during our culture period on growth medium and at least one of these "new" proteins is not synthesized in the starved brain. This suggests a degree of specificity in the synthesis of proteins during starvation. Some preliminary studies have also suggested that the protein breakdown process is also rather specific. It is hoped that the proposed studies may provide some insight into the basis for the selective synthesis as well as the selective breakdown of brain proteins during development.

Finally, the intriguing question of regional growth regulation during early embryonic development should be considered. Years ago investigators such as Spratt (62) demonstrated regional differences in early embryos. These differences were for the most part demonstrated

histologically or through the use of inhibitors and involved general metabolic enzymes or enzymes involved in some pathway of energy metabolism. I feel, that these studies did not contribute significantly to our understanding of growth regulation. Indeed, Gross stated in his book on growth that embryonic growth regulation is simply not understood. Our recent observations that the polysomes of brain in an early embryo are more active in vitro than those of heart is, I believe, the first precise demonstration of a basis for early embryonic growth regulation. The factors involved in this polysome difference may be central to our understanding of embryonic growth regulations and to the many areas of research (cancer, teratology, etc.) which deal with normal as well as abnormal growth. We have proposed several lines of investigation which should provide important insight into this pertinent issue.

Although this section has not been treated in depth, the rather lengthy preceding sections and the preprints of a review chapter and original publications presented with this proposal provide a more detailed treatment of these various subjects. The reader is referred to these supplementary materials for additional information.

LITERATURE CITED

1. Schimke, R.T. (1969) In: "Current Topics in Cellular Regulation" (B.L. Horecker and E.R. Stadtman, eds.) Academic Press, New York.
2. Enwonwu, C.O. and V. Clover (1973) J. Nutr. 103, 61.
3. Munro, R.N. (1970) Fed. Proc. 29, 1490.
4. Henshaw, E.C., C.A. Hirsch, B.E. Morton and H. Hiatt (1971) J. Biol. Chem. 246, 436.
5. Baril, E.P., V.R. Potter and H.P. Morris (1969) Cancer Res. 29, 2101.
6. Klein, N.W., E. McConnell and B.J. Buckingham (1962) Develop. Biol. 5, 296.
7. Klein, N.W., E. McConnell and D.J. Riquier (1964) Develop. Biol. 10, 17.
8. Hsu, Y.-C. (1973) Develop. Biol. 33, 403.
9. Klein, N.W. (1968) J. Exp. Zool. 168, 239.
10. Klein, N.W. (1974) In: "The Biochemistry of Animal Development" Vol. III (R. Weber, ed.) Academic Press, New York. (The galley proofs have been returned so I expect this chapter to appear soon. A copy of this chapter is included with the proposal.)
11. Yager, J.D. and N.W. Klein (1974) Develop. Biol. (This will appear in the July 1974 edition. A copy is included with the proposal.)
12. Yager, J.D. and N.W. Klein (1974) (We plan to submit this paper to Arch. Biochem. Biophys. A copy of the manuscript is included.)
13. Williams, J. (1962) Biochem. J. 83, 355.
14. Nace, G.W. (1953) J. Exp. Zool. 122, 423.

15. Lambson, R.O. (1970) Amer. J. Anat. 129, 1.
16. Hassell, J. and N.W. Klein (1971) Develop. Biol. 26, 380.
17. New, D.A.T. (1971) Advances Biosci. 6, 367.
18. Zamenhof, S., E. Van Marthens and L. Grauel (1971) J. Nutr. 101, 1265.
19. Klein, N.W., J.D. Yager, K. Hagedorn (1971) Develop. Biol. 24, 178.
20. Klein, N.W., J.D. Yager, E.D. Hickey and S.J. Amott (1974)
(In preparation.)
21. Hickey, E.D. and N.W. Klein (1973) Biochem. Biophys. Acta 294, 297.
- 22a. Lynch, A.G. and N.W. Klein (1974) (A copy of this manuscript is included with this proposal.)
- 22b. Regier, J.C. and F.C. Kafatos (1971) J. Biol. Chem. 246, 6480.
23. Gilbert, B.E. and T.C. Johnson (1973) Brain Res. 63, 313.
24. Johnson, T.C. (1973) Texas Rep. Biol. Med. 31, 331.
25. Yund, M.A., F.C. Kafatos and J.C. Regier (1973) Develop. Biol. 33, 362; also Yund, M.A., W.F. Yund and F.C. Kafatos (1971) Biochem. Biophys. Res. Comm. 43, 717.
26. Becker, H. and C.P. Stanners (1972) J. Cell. Physiol. 80, 51.
27. Carney, D.H. and N.W. Klein (1973) Teratology 7, A-13.
28. Nace, G.W. (1953) J. Exp. Zool. 122, 423. (same as 14).
29. Schechtman, A.M. and H. Hoffman (1952) J. Exp. Zool. 120, 375.
30. Gorovsky, M.A., K. Carlson and J.L. Rosenbaum (1970) Anal. Biochem. 35, 359.
31. Weller, E.M. (1971) Anat. Rec. 169, 452. (see also 28 for consideration of this point).
32. Webb, J.M. and H.V. Lindstrom (1965) Arch. Biochem. Biophys. 112, 273.

33. Giles, K.W. and D.A. Myers (1964) *Nature (London)* 206, 93.
34. Levi-Montalcini, R. (1963) In: "The Nature of Biological Diversity" (J.M. Allen, ed.) McGraw-Hill Book Co., Inc., New York.
35. Margolis, F.L. (1969) *J. Neurochem.* 16, 447.
36. Shimada, H. and H. Terayama (1972) *Biochim. Biophys. Acta.* 287, 415.
37. Messier, P.-E. and C. Auclair (1973) *J. Embryol. Exp. Morph.* 30, 661.
38. Regier, J.C. and P.C. Kafatos (1971) *J. Biol. Chem.* 246, 6480.
(same as 22b.)
39. Curatrecasas, P. (1972) *Proc. Nat. Acad. Sci. U.S.* 69, 1277.
40. Weber, R. (1963) *Physiol. Pharmacol. Acta* 21, 277.
41. Bund, J.S. (1971) *Biochem. Biophys. Res. Comm.* 43, 333.
42. Rowley, P.T., R.A. Midthun and M.H. Adams (1971) *Arch. Biochem. Biophys.* 145, 6.
43. Heywood, S.M. (1970) *Proc. Nat. Acad. Sci. U.S.* 67, 1782; and
Rourke, A.W. and S.M. Heywood (1972) *Biochem.* 11, 2061.
44. Gilbert, J.M. (1974) *Biochim. Biophys. Acta* 340, 140.
45. Friedman, H.P. and B.S. Wenger (1970) *J. Embryol. Exp. Morph.* 23, 289.
46. Hamburg, J.R., E.M. Shooter and L. Wilson (1973) *Biochem.* 12, 1476.
47. Hemminki, K. (1973) *Biochim. Biophys. Acta* 310, 285.
48. Renaud, F., A. Rowe and I. Gibbons (1968) *J. Cell Biol.* 36, 79.
49. Weisenberg, R.C., G.C. Borisy and E.W. Taylor (1968) *Biochem.* 7,
4466.
50. Piper, J.A. and N.W. Klein (1969) *J. Cell Biol.* 40, 209.

51. Snaith, S.N. and G.A. Levy (1968) *Biochem. J.* 110, 663.
52. Li, Y.-T. and S.-C. Li (1968) *J. Biol. Chem.* 243, 3994.
53. Winick, M. and A. Noble (1966) *J. Nutr.* 89, 300.
54. Munro, H.N. (1964) In: "Mammalian Protein Metabolism" (H.N. Munro and J.B. Allison, eds.) Vol. I, Academic Press, New York.
55. Holley, R.W. and J.A. Kiernian (1968) *Proc. Nat. Acad. Sci. U.S.* 60, 300.
56. Scher, C.D. and G.J. Totaro (1971) *Exp. Cell Res.* 68, 479.
57. Paul, D., A. Lipton and I. Klinger (1971) *Proc. Nat. Acad. Sci. U.S.* 68, 645.
58. Lipton, A., I. Klinger, D. Paul and R.W. Holley (1971) *Proc. Nat. Acad. Sci. U.S.* 68, 2799.
59. Coon, H.G. (1966) *Proc. Nat. Acad. Sci. U.S.* 55, 66.
60. Cahn, R.D. and M.B. Cahn (1966) *Proc. Nat. Acad. Sci. U.S.* 55, 106.
61. Spooner, B.S. and S.R. Hilfer (1971) *J. Cell. Biol.* 48, 225.
62. Spratt, N.T., Jr. (1952) *Ann. New York Acad. Sci.* 55, 40.

GRADUATE STUDENTS AND POST-DOCTORALS

(One asterisk indicates individuals associated with this contract over the last six years and two asterisks for individuals associated with this project during the last three years.)

Graduate Students

Eileen D. Hickey-Weber, Master's Degree, Marquette University 1967. Dr. Hickey-Weber received her Ph.D. with Dr. R. Weber (she married a different Weber not related to R. Weber) at the University of Bern, Switzerland in 1970. Dr. Hickey-Weber rejoined the laboratory now at the University of Connecticut as a Post-doctoral -Research Assistant II. In September of 1972 she accepted the position of Assistant Professor at Russell Sage College, Troy, New York.

*Judith Smith, Master's Degree, Marquette University 1968. I believe that she is presently teaching biology in Milwaukee, Wisconsin.

*Judith Piper-Airhardt, Ph.D. Degree, Marquette University 1969. Dr. Piper-Airhardt holds a research associate position with Dr. E.A. Khairallah of the Biochemistry and Biophysics Section, Biology Division, University of Connecticut.

**James D. Yager, Jr., Ph.D. Degree, University of Connecticut, November, 1971. Dr. Yager has been an Assistant Scientist with Dr. Van R. Potter of the McArdle Laboratory for Cancer Research at the University of Wisconsin. In September of this year (1974) he will become Assistant Professor of Biology at Dartmouth College, New Hampshire.

**John Hassell, Ph.D. Degree, University of Connecticut, February 1972. Dr. Hassell is presently at the National Institutes of Health where he is a Senior Staff Fellow with the National Institute of Dental Research.

****Audrey G. Sawyer-Lynch, Master's Degree, University of Connecticut 1972.** After she received her degree she remained associated with this laboratory as a Research Assistant. Mrs. Sawyer-Lynch will probably stay with this laboratory another year.

****Darrell H. Carney, Ph.D. candidate.** Mr. Carney holds a PHS Traineeship from the Institute of Cellular Biology. He should receive his Ph.D. Degree by June of 1975. He has been working in this laboratory since 1970.

****David Kram, Ph.D. candidate.** Mr. Kram holds a PHS Traineeship from the Institute of Cellular Biology. He should receive his Ph.D. Degree in 1975. He has been with this laboratory since 1971.

****Four graduate students have expressed interest in joining this laboratory for their Ph.D. studies.** Mr. Frank Longo, Ms. Nancy Janul, Mr. Jeffrey Rothstein, and Mr. Philip Minghetti have communicated their interest. At this time I am only certain of Mr. Minghetti as he is planning to start work in another week.

Post Doctorals

Robert C. Fitzsimmons, Ph.D., University of Minnesota 1965. He was a Post-doctoral while this laboratory was at Marquette University between 1965 and 1967. He is presently a member of the faculty of the University of British Columbia.

****Eileen D. Hickey-Weber, Ph.D., University of Bern, Switzerland.** Dr. Hickey-Weber was a Post-doctoral with this laboratory from 1970 to 1972. She is presently an Assistant Professor of Biology at Russell-Sage College, Troy, New York.

BIBLIOGRAPHY

Bibliography of Principal Investigator and Publications Associated with this Contract

(One asterisk indicates publications associated with this contract over the last six years and two asterisks for those publications from the last three years of this contract.)

- Grau, C.R., N.W. Klein and T.L. Lau 1957 Total replacement of the yolk of chick embryos. J. Embryol. Exp. Morph 5: 210-214.
- Grau, C.R. and N.W. Klein 1957 Sewage-grown algae as a feedstuff for chicks. Poultry Sci. 36: 1046-1051.
- Klein, N.W., C.R. Grau and N.J. Green 1958 Yolk-sac perfusion of chick embryos: Effects of various media on survival. Proc. Soc. Exp. Biol. Med. 97: 425-428.
- Grau, C.R., N.W. Klein and H.I. Fritz 1959 Nutrition of chick embryos studied by replacement of yolk and albumen by nutrient media. Fed. Proc. 18: 527 (abstract).
- Klein, N.W., E. McConnell and B.J. Buckingham 1962 Growth of explanted chick embryos on a chemically defined medium and effects of specific amino acid deficiencies. Develop. Biol. 5: 296-308.
- Klein, N.W., E. McConnell and D.J. Riquier 1962 Effects of high oxygen levels on the growth and survival of explanted chick embryos. Amer. Zool. 2: 35 (abstract).
- Grau, C.R., H.I. Fritz, N.E. Walker and N.W. Klein 1962 Nutrition Studies with chick embryos deprived of yolk. J. Exp. Zool. 150: 185-195.

- Klein, N.W. and L.J. Pierro 1963 Actinomycin D: Specific inhibitory effects on the explanted chick embryo. *Science* 142: 967-969.
- Roth, J.S., B. Buccino and N.W. Klein 1963 Inhibition of growth of chick embryos by inhibition of deoxycytidylate deaminase. *Science* 142: 1473-1474.
- Klein, N.W., E. McConnell and D.J. Riquier 1964 Enhanced growth and survival of explanted chick embryos cultured under high levels of oxygen. *Develop. Biol.* 10: 17-44.
- Herrmann, H., N.W. Klein and O. Albers 1964 In vitro culture of muscle tissue from normal, heterozygous and dystrophic chicks. *J. Cell Biol.* 22: 391-401.
- Klein, N.W. 1965 Teratological studies with explanted chick embryos. IN: *Teratology: "Principles and Techniques"*. (J.G. Wilson and J. Markany, eds.). The University of Chicago Press, Chicago, Illinois, pp. 131-144.
- Klein, N.W. 1966 The relation of growth and differentiation in explanted chick embryos. *Amer. Zool.* 6: 328 (abstract).
- Hickey, E.D. and N.W. Klein 1966 Sequences of changes in DNA and protein content of regions of the explanted chick embryo following insulin treatment. *Amer. Zool.* 6: 552 (abstract).
- Fitzsimmons, R.C. and N.W. Klein 1966 Utilization of proteins by explanted chick embryos grown on horse serum. *Amer. Zool.* 6: 523 (abstract).
- Piper, J.A. and N.W. Klein 1969 Differential sensitivity of chick mesodermal cells to actinomycin D. *J. Cell Biol.* 40: 209-215.

- *Klein, N.W., J.D. Yager and K. Hagedorn 1969 Regional patterns of protein degradation in the explanted chick embryo. Amer. Zool. 9: 606 (abstract).
- *Yager, James D. and N.W. Klein 1969 Growth of explanted chick embryos on yolk protein fractions. Amer. Zool. 9: 609 (abstract).
- *Hassell, J. and N.W. Klein 1969 The quantitative uptake, distribution, and breakdown of ^{14}C -labeled ovalbumin by the explanted chick embryo. Amer. Zool. 9: 609 (abstract).
- *Hassell, J., J.D. Yager and N.W. Klein 1970 Incorporation of ^{14}C -amino acids into egg proteins by the laying hen. Poult. Sci. 49: 1121-1125.
- *Klein, N.W., J.D. Yager and K. Hagedorn 1971 The direct effect of protein starvation on protein breakdown and synthesis in regions of the cultured early chick embryo. Develop. Biol. 24: 178-197.
- *Hickey, E.D. and N.W. Klein 1971 Changes in DNA, RNA and protein content of regions of explanted chicken embryos following insulin treatment. Teratology 4: 453-460.
- *Hassell, J. and N.W. Klein 1971 A quantitative analysis of ovalbumin utilization and its relationship to growth regulation during development. Develop. Biol. 26: 380-392.
- *Hickey, E.D. and N.W. Klein 1971 The effect of protein starvation on RNA and protein breakdown in subcellular fractions of the cultured chick embryo. Teratology 4: 231 (abstract).
- **Yager, James D. 1971 The role of various yolk protein fractions in the nutrition of the cultured early chick embryo. Ph.D. Thesis, University of Connecticut.

- **Hassell, John R. 1972** An analysis of protein utilization by the cultured chick embryo and its relationship to growth regulation during development. Ph.D. Thesis, University of Connecticut.
- **Hickey, E.D. and N.W. Klein 1973** The effect of protein deprivation on RNA and protein breakdown in subcellular fractions of the cultured chick embryo. *Biochem. Biophys. Acta* 294: 297-308.
- **Carney, D.H. and N.W. Klein 1973** The effect of protein starvation on the patterns of protein synthesis in the cultured chick embryo. *Teratology* 7: 13 (abstract).
- **Klein, N.W. 1974** Protein Nutrition in Growth Regulation. A chapter in "The Biochemistry of Animal Development", Vol. III, (R. Weber, ed.), Academic Press Inc., New York (in press).
- **Yager, J.D., Jr. and N.W. Klein 1974** The growth and development of early chick embryos cultured on several purified proteins. *Develop. Biol.* 39: No. 1, July, 1974 (in press).

Research Completed - Manuscripts in Preparation

- **Klein, N.W. and L.J. Pierro 1974** A quantitative and histological analysis of regional growth in chick embryo explants cultured at various rate of growth.
- **Hassell, J. 1974** The interaction of ovalbumin and conalbumin in the growth and development of the cultured chick embryo.
- **Klein, N.W., J.D. Yager, E.D. Hickey and S.J. Amott 1974** The effect of protein starvation on the degradation and synthesis of RNA in regions of the cultured early chick embryo.

- **Yager, J.D., Jr. and H.W. Klein 1974 The isolation and partial physical-chemical characterization of several egg yolk proteins.**
- **Lynch, A.G. and H.W. Klein 1974 Polysome activity in relation to growth and protein starvation in brains and hearts of cultured chick embryos.**
- **Carney, D.H. and H.W. Klein 1974 The effects of nutrition on the patterns of proteins synthesized in regions of the early cultured chick embryo.**

FEDERAL SUPPORT

The contract with the United States Atomic Energy Commission has been the main source of federal support for our overall research program. In addition, approximately 30% of the principal investigators academic year salary and approximately \$500 per year for supplies are provided through Hatch Funds administered by the Storrs Agricultural Experiment Station.