

PROTEINS IN GROWTH REGULATION DURING
EARLY DEVELOPMENT OF THE CHICK EMBRYO

Progress Report

for period May 1975 to May 1976

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TABLE OF CONTENTS

Special Notice.....	i
Time and Effort Statement of Principal Investigator.....	ii
Progress Report Summary.....	iii
I Introduction.....	1
II Changes in Serum Protein Synthesis.....	3
A. Developmental Changes.....	3
B. Changes with Drug Treatment.....	4
III The Regulation of Serum Protein Synthesis.....	5
A. Cell-Free Synthesis of Serum Proteins.....	5
IV The Direct Effect of Serum Proteins on Development.....	7
A. Purification of Embryo Serum Proteins.....	7
B. Culture of Isolated Brains.....	8
V Nutrition and Yolk-Sac Function.....	9
A. Yolk-Sac Proteases and the Breakdown of Endogenous Yolk-Sac Proteins.....	9
B. Yolk-Sac Cell Cultures.....	10

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TIME AND EFFORT STATEMENT OF PRINCIPAL INVESTIGATOR

The principal investigator, Norman W. Klein, has devoted approximately 50% of his time and effort to this project during the past contract year and is expected to continue at this level during the subsequent (76-77) contract year.

PROGRESS REPORT SUMMARY

Previous studies have shown that the growth of embryo regions and the synthesis of specific proteins in embryo regions were altered when early chick embryos were cultured on various nutrient proteins. Because nutrient proteins were degraded to constituent amino acids within the yolk-sac and did not reach the embryo proper as intact macromolecules, we have been evaluating the role of the yolk-sac in the regulation of early embryonic growth and development.

The synthesis of serum proteins by the yolk-sac was observed to start as early as the primitive streak stage of development. The relative synthesis of the individual serum proteins was found to change with development, with changes in protein nutrition, and with drug induced developmental abnormalities.

Organ cultures of early embryo brains are being used to study the direct effect of individual embryonic serum proteins on developmental processes.

The complete synthesis of serum proteins by isolated yolk-sac polyribosomes has been achieved and this system will be used to study the regulation of serum protein synthesis.

We are continuing studies of the basis for the highly specific nutrient protein requirement of the early embryo. This requirement appears related to the ability of a particular nutrient protein to serve as a substrate for yolk-sac proteases and to an effect of nutrient proteins on the breakdown of endogenous yolk-sac proteins.

I INTRODUCTION

The present report covering the period May 1975 to May 1976 consists of an abstract of a paper presented at the November 1975 meeting of the Society of Cell Biology, two abstracts of papers presented at the Northeast Regional Developmental Biology Conference in February 1976, an invited paper presented at the International Symposium on Nuclear Techniques in Animal Production and Health as Related to the Soil-Plant System held February 1976 in Vienna, an abstract of a paper to be presented at the Teratology Society meeting to be held June 1976, and this report. It should also be noted that a preprint submitted with the progress report of last year, "Serum Protein Synthesis in the Early Chick Embryo," has been accepted for publication by Developmental Biology and two students associated with this project, Drs. Darrell Carney and David Kram, completed their Ph.D. work during this past year.

Our previous studies on protein nutrition and growth regulation with the intact early chick embryo directed our attention to the yolk-sac as the central organ in nutrition as well as in the regulation of growth and development. Changes in protein nutrition were shown to alter the growth of embryo regions and the synthesis of specific proteins in embryo regions. However, intact nutrient proteins as such did not reach the embryo proper but were degraded to constituent amino acids within the yolk-sac. The yolk-sac was found to be the exclusive site of serum protein synthesis and the relative synthesis of these serum proteins was dramatically altered by changes in protein nutrition. That the responses of the embryo to nutrition could be mediated by changes in the relative concentrations of circulating serum proteins became our working hypothesis.

In line with this hypothesis our foremost objective has been to demonstrate a direct effect of individual serum proteins on growth and development. For this purpose during the past year, we worked on procedures to isolate individual serum proteins and have

developed methods for culturing isolated brains of early embryos for use as the test system. We would prefer to use cultures of intact embryo explants but feel that serum proteins placed in the culture medium would only be degraded by yolk-sac proteases.

During the past year several aspects of serum protein synthesis were studied. A complete developmental sequence of serum protein synthesis by the yolk-sac and liver is presently being completed. In line with our interest in teratology and the effects of various environmental factors on development, studies on the synthesis of serum proteins by yolk-sacs of embryos cultured on various anti-cancer drugs were initiated. In addition to providing some insights into the possible role of serum proteins in teratology, we also hope to consider the possible value of the chick embryo culture system in anti-cancer drug screening.

The changes that we have observed in serum protein synthesis with development, nutrition and now drug treatment have directed our attention to a consideration of the mechanisms regulating their synthesis. Last year we were able to achieve for the first time the complete synthesis of the major serum proteins in a cell-free system derived from the yolk-sac. The possible analysis of this system for transcriptional and translational control mechanisms regulating the changing patterns of serum protein synthesis has become possible.

Finally, we have continued studies on the nutrient protein requirements of the early embryo. Our previous studies showed that the embryo required at least two nutrient proteins for growth and development. For the most part we have employed a mixture of highly purified ovalbumin and conalbumin in these studies. Our major effort has been concerned with the interaction of these two proteins in maintaining high levels of amino acids in the yolk-sac and their role in the turnover of yolk-sac proteins. Recently, we have also started working with yolk-sac cell cultures in order to determine if these cells might be responsive to the same nutrient proteins as the intact embryo. For

example, would these cells in culture synthesize serum proteins when provided with ovalbumin and conalbumin.

II CHANGES IN SERUM PROTEIN SYNTHESIS

A. Developmental Changes. Last year a preprint of a paper was submitted with the progress report describing a new procedure to examine the synthesis of serum proteins by the yolk-sac of the chick embryo. In brief, isolated yolk-sacs incubated in chick Ringer's containing radioactive amino acids, secreted radioactive serum proteins. These proteins were separated by acrylamide gel electrophoresis and the amount of radioactivity in each protein was measured. The valine content of each serum protein was determined so that the incorporation of radioactive valine could be corrected for the valine content of the protein and provide values for relative synthesis. Yolk-sacs secreted serum transferrin, two embryo specific alpha globulins, an embryo specific prealbumin and serum albumin. Serum proteins were found to be synthesized as early as the primitive streak stage and exclusively by the yolk-sac in the early embryo. Culturing chick embryo explants on various nutrient proteins altered the pattern of serum protein synthesis and changes in the pattern were also noted with development during early stages.

At present we are completing a study of serum protein synthesis by the yolk-sac and liver throughout development and have also quantified the serum protein changes in circulating blood. The synthesis studies involved the incubation of yolk-sacs and livers with ^{14}C or ^3H valine and the coelectrophoresis of their secreted proteins. The circulating serum protein changes were quantified by staining acrylamide gels with Fast Green. Preliminary results indicated that at 9 days incubation both liver and yolk-sac synthesized transferrin, albumin, and prealbumin but the synthesis of alpha globulins

was restricted to the yolk-sac. At 10 days of development alpha globulin-a constituted 40% of the total serum protein and then declined. Alpha globulin-b comprised 14% of the serum protein on day 8 and was not detectable after day 10. The concentration of pre-albumin declined from 12% on day 6 to 1% at hatching.

B. Changes with Drug Treatment. In view of our present concerns with serum proteins in the regulation of growth and development and our interests in the teratological mechanism by which various drugs and environmental pollutants act, it was appropriate to consider the possibility that developmental abnormalities could be mediated through modifications in the synthesis of serum proteins. We turned to Dr. Florence R. White of the Drug Evaluation Branch of the National Cancer Institute who sent us a large variety of anti-cancer drugs. It seemed appropriate at this time to consider anti-cancer drugs because the National Cancer Institute was seeking model systems for drug screening. We felt with the present interest in the production of fetal antigens by various cancers that our system could be of some value. Indeed, it appeared possible that anti-cancer action of a drug could be related to the ability of the drug to inhibit the synthesis of embryo specific serum proteins.

Our procedure with these drugs has involved the establishment of a teratological dosage, evaluation of the teratological response in terms of embryo growth and morphological development, and finally the determination of serum protein synthesis. Embryo explants of 11 to 13 somites were cultured for 48 hours on whole egg homogenate medium containing the drug. Yolk-sacs were then removed and incubated for three hours in the presence of radioactive valine. The secreted serum proteins were then resolved on acrylamide gels and the amounts of radioactivity with each serum protein was determined. Preliminary results have been obtained with four drugs (Table 1). We were particularly interested to find that two drugs, Daunomycin and Melphalan, both caused rumplessness

and also showed reduced synthesis of alpha globulin-a when compared to control values. Procarbazine caused abnormalities of the eyes, brains, somites and reduced circulatory system development. The yolk-sacs from these explants synthesized reduced levels of serum transferrin and alpha globulin-b. Cyclophosphamide caused general growth retardation and did not appreciably alter the synthetic pattern of serum proteins relative to controls. These results, although preliminary, were encouraging.

Table 1

Relative synthesis of serum proteins by yolk-sacs isolated from embryo explants cultured for 48 hours on whole egg medium containing various drugs

Drug	Trans.	α Glob-a	α Glob-b	Alb.	Prealb.
	%	%	%	%	%
Control	23.9	28.6	15.4	4.6	27.7
Cyclophosphamide	26.8	24.5	13.6	9.9	25.2
Daunomycin	25.2	13.2	21.0	14.7	26.0
Melphalan	29.2	12.5	16.8	14.9	26.4
Procarbazine	8.4	29.0	9.7	8.8	44.2

III REGULATION OF SERUM PROTEIN SYNTHESIS

A. Cell-Free Synthesis of Serum Proteins. The changes in relative synthesis of serum proteins with development, nutrition, and teratology have directed our attention to the obvious question of the possible mechanisms regulating their synthesis. Approximately one and one-half years ago we initiated studies on the cell-free synthesis of serum proteins to approach this regulatory problem. Our first studies involved crude

homogenates and post-mitochondrial supernatants of yolk-sacs. These preparations incorporated radioactive amino acids into protein. They contained high levels of proteases and we worked with some protease inhibitors to overcome this problem. However, last year we turned to purified polyribosome preparations because the products synthesized by the crude preparations could not be resolved into definitive proteins.

During this past year yolk-sac polyribosomes were purified and the products synthesized in vitro were identified as serum proteins. Yolk-sacs were homogenized and centrifuged at 10,000 x g. The supernatant was placed on a 2 M to 0.5 M discontinuous sucrose gradient to separate free polyribosomes (pelleted) and a fraction at the interphase considered membrane-bound polyribosomes I. By treating the 10,000 x g pellet with sodium deoxycholate and Triton X-100 and passing this material through the discontinuous sucrose gradient a fraction referred to as membrane-bound polyribosomes II was pelleted. Membrane-bound polyribosomes I fraction was also treated with detergent and pelleted through sucrose.

Employing standard ingredients for cell-free protein synthesis, the membrane-bound polyribosomes I were three times and the membrane-bound polyribosomes II were four times more active than the free polyribosomes in the synthesis of protein. The reaction period was 60 minutes and the data were based on ribosomal RNA. The reaction products were precipitated with acetone and run on SDS (sodium dodecyl sulfate) polyacrylamide gels. They were coelectrophoresed with serum proteins produced by intact yolk-sacs. The cell-free products electrophoresed closely but not precisely with the intact yolk-sac products. We believe that this was due to post-translational modifications (for example, the addition of carbohydrate moieties) that occurred in the intact yolk-sac products but not in the cell-free products. Nevertheless, the close proximity of the two groups of products was most encouraging. In this study, six serum proteins were

tentatively identified as products synthesized by free polyribosomes, five from membrane-bound polyribosomes I, while only two were synthesized by the membrane-bound polyribosome II fraction.

We have been working on the identification of the cell-free products. Our original studies on serum protein synthesis involved standard non-detergent gels. To identify these same proteins in SDS gels it was necessary to isolate individual serum proteins from standard gels and then determine their mobility following acetone precipitation and electrophoresis on SDS acrylamide gels. This work will soon be completed.

IV THE DIRECT EFFECT OF SERUM PROTEINS ON DEVELOPMENT

A. Purification of Embryo Serum Proteins. Purified embryo serum proteins are essential to study the direct effect of individual serum proteins on developmental processes. Purified serum proteins would also be of considerable value in studies such as cell-free serum protein synthesis where antibodies against specific serum proteins could be used for both identification and quantification. During the past year we have worked on several different methods to collect embryo serum and to separate the individual proteins through column chromatographic techniques. For the collection of serum we have found that large quantities of serum can be obtained from 8-day chick embryos by simply removing them from the egg and allowing them to bleed while rocking in an Earlenmyer flask for one hour at 37.5°C. For fractionation we have tried ammonium sulfate precipitation and Sephadex G-100 and G-200 gel filtration. To date we have been most successful with Sephadex QAE-A50 anion exchanger with a step wise sodium chloride elution. Based on acrylamide analysis we have obtained reasonably pure separation of transferrin, alpha globulin-a, and prealbumin. With some modification we hope to also obtain alpha globulin-b and serum albumin.

B. Culture of Isolated Brains. Once we have purified sufficient quantities of the individual serum proteins we will attempt to culture embryo explants on these proteins. However, the proteases of the yolk-sac will probably make this approach impossible. For this reason we have been working on procedures to culture isolated brains and to measure their response to different conditions in quantitative terms.

For brain cultures we have been using media consisting of whole serum from 8-day embryos, ovalbumin plus conalbumin and whole egg. We have tried culturing brains on semi-solid agar media, on glass, on plastic, on Millipore filters and shaking in small Earlenmyer flasks. At this time the Falcon organ culture dishes in which the brains were placed directly onto the plastic surface appeared to support growth and development of the brains and were convenient to use.

We have used morphological development and quantitative protein determinations to measure the growth response. However, most of our effort has been devoted to the use of double labeled acrylamide gels. Such gels permitted a quantitative analysis through computer of the degree to which two mixtures of radioactive proteins were similar. Thus, in one particular study brains were removed from embryo explants that had been cultured for 24 hours and then they were cultured as isolated brains for 24 hours on whole egg medium. The control group consisted of brains removed from embryo explants after 48 hours of culture. Both groups were exposed to radioactive valine (one to ^{14}C the other to ^3H) for three hours. They were then homogenized and the proteins were co-electrophoresed on SDS acrylamide gels. A control comparison involved two groups of brains both isolated from embryo explants after 48 hours fo culture. The results were expressed as a single value reflecting the overall percentage difference between two mixtures. From three determinations the average control value for the percentage difference was 5.63 while comparing cultured brains with brains from intact embryos gave

a mean from three determinations of 11.51. We feel that this difference of 5.88 was sufficiently reproducible and of sufficient magnitude to be able to employ this procedure in evaluating the response of cultured brains to individual serum proteins.

V NUTRITION AND YOLK-SAC FUNCTION

A. Yolk-Sac Proteases and the Breakdown of Endogenous Yolk-Sac Proteins. Previous studies in our laboratory have shown that the cultured early chick embryo required two nutrient proteins. One of the required proteins was a transferrin and conalbumin, yolk transferrin, or serum transferrin could be used. The second protein required was less specific in that either ovalbumin or lipovitellin would support growth and development when combined with a transferrin. It was also observed that nutrient proteins were taken up by the yolk-sac and degraded to constituent amino acids within this structure. Thus, in seeking a possible basis for the two nutrient protein requirement we have focused on the yolk-sac. During the past year we considered yolk-sac proteases in regard to substrate specificity and have started also to examine the breakdown of yolk-sac endogenous proteins in response to nutrient proteins. We have made use of highly purified ovalbumin and conalbumin in these studies.

Employing published procedures crude preparations of yolk-sac proteases were obtained by homogenization of yolk-sacs in 0.25 M sucrose and 1 mM EDTA and centrifugation at 500 x G. The supernatant was used as the enzyme preparation. The substrate consisted of equal amounts by weight of ovalbumin and conalbumin. The pH optimum was found to be approximately 4.6 for enzyme activity. The activity was increased by the addition of NaCl and by the addition of Triton-X-100. These observations strongly suggested that the yolk-sac proteases are lysosomal.

After establishing these optimum conditions, ovalbumin alone, conalbumin alone, and a mixture of ovalbumin and conalbumin were used as substrates. Conalbumin was found

to be a preferred substrate to ovalbumin in that over a 6 hour period 97% more ninhydrin positive material was released from conalbumin. During shorter incubation periods (2 hours) the mixture of ovalbumin and conalbumin gave slightly higher levels of ninhydrin positive material than conalbumin alone.

Because the protease enzymes were contained in a crude preparation, we next considered the influence of the substrate proteins on the degradation of proteins (endogenous proteins) contained in the enzyme preparation. For this purpose, intact yolk-sacs were incubated for three hours in the presence of ^{14}C -valine prior to homogenization. The release of ^{14}C -valine from the enzyme preparation was followed in the material soluble in 5% trichloroacetic acid. The breakdown of yolk-sac proteins was greater in the presence of ovalbumin alone than with conalbumin alone. The mixture of the two proteins was intermediate. Thus, the higher level of ninhydrin positive material observed in the previous study with the two proteins combined may have been due to two factors. Conalbumin may have served as the preferred substrate for yolk-sac proteases while ovalbumin enhanced the breakdown of endogenous proteins.

In a second study, the labeling period for the endogenous proteins was extended by culturing yolk-sacs for 6 hours after the 3 hour exposure period to ^{14}C -valine. Under these conditions less endogenous yolk-sac proteins in the enzyme preparation were degraded in the presence of ovalbumin than with conalbumin. Thus, there appeared to be a possible relationship between substrate protein, endogenous turnover rate, and susceptibility to degradation. At present we are attempting to determine if this same relationship exists within the intact yolk-sac. In order to follow breakdown in the absence of reutilization we are employing the protein synthesis inhibitor, cycloheximide.

B. Yolk-Sac Cell Cultures. Employing published procedures we have been attempting to culture cells isolated from the yolk-sac. The objectives were to determine if

at this level of organization these cells would respond to such nutrient proteins as ovalbumin and conalbumin and if these cells would produce serum proteins. To date we have found that it was possible to set up monolayer cultures of yolk-sac cells employing trypsinization to dissociate the tissue and a culture medium of Eagle's minimal nutrients supplemented with 5% fetal calf serum and 5% conalbumin. In these preliminary studies, 6 day yolk-sacs were used.

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