PROTEINS IN GROWTH REGULATION DURING
EARLY DEVELOPMENT OF THE CHICK EMBRYO

Progress Report

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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 (75-76) contract year.

PROGRESS REPORT SUMMARY

Cultures of intact early chick embryos are being used to investigate the role of nutrient proteins in growth regulation. The studies are involved on the one hand with mechanisms of protein utilization and on the other with the impact of nutrition on the regulation of cellular activity. During the past year, extensive progress has been made in both of these areas.

Nutrient proteins are broken down in the yolk-sac and utilized in the synthesis of serum proteins. Changes in nutrition alter the relative synthesis of the various serum proteins. In so far as isolated embryo regions do not sequester specific serum proteins, it is the relative synthesis that is important in growth and development. The embryo requires a precise mixture of two nutrient proteins. A possible basis for this requirement appears to be related to the proteolytic enzymes of the yolk-sac. Thus, in the presence of both proteins, lysosome preparations liberate higher levels of amino acids. Progress is being made in the study of mechanisms regulating serum protein synthesis through the use of cell-free protein synthesizing systems.

Polyribosomes isolated from brain synthesize protein at a higher rate than those of heart. This difference was achieved with myosin precipitated polyribosomes but not by polyribosomes isolated by alternative procedures. High speed supernatant factors as well as elongation factor I may be involved in polyribosome differences. The low level of microtubule protein in brains of starved embryos appears related to decreased synthesis rather than increased breakdown. The sensitivity of the brain to protein starvation appears to be characteristic of the brain in relation to the embryo rather than to a unique sensitivity of the developing nervous system.

I INTRODUCTION

The present report covering the period May 1974 to May 1975 consists of two papers that have appeared in press during the past year, two abstracts of papers presented at the Society of Cell Biology meetings last year, a copy of a manuscript related to our work on serum protein synthesis, ~~a copy of a manuscript related to our work on serum protein synthesis~~, a copy of a manuscript related to our work on regional patterns of protein synthesis, an abstract of a paper to be presented at the Teratology Society Meetings, and this report.

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 hypothesis as stated may appear conceptually narrow, however, in practice it is quite broad as it includes all aspects of nutrient protein utilization as well as many aspects of the cellular response to the influx of amino acids. Thus, we do not simply deal with the passage of free amino acids from a nutrient medium into the cells of the embryo. The early chick embryo which we employ as a model system, requires nutrient protein and this requirement appears rather specific. Nutrient proteins appear to be taken up by the yolk-sac, broken down to amino acids in the yolk-sac, and converted to serum proteins. We believe that 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 (protein accumulation) may be regulated by polysome activity. Finally, growth itself does not simply involve the accumulation of proteins (or 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 (or total accumulation of proteins). Thus, we are interested in how the nutrients (amino acids) reach the cells of the embryo and in how the cell responds in terms of the quantitative and qualitative regulation of protein synthesis. During the past year we have made considerable progress with both nutrition and growth regulation. The importance of the yolk-sac as a mediator between the nutrient medium and embryo proper has been clearly defined. The complexity of the many factors involved in regional growth regulation appears amenable to experimentation.

Cultures of intact early chick embryos have been used in these studies. The embryos are removed from the eggs after approximately 40 hours of preincubation, and those containing 11 to 13 pairs of somites are selected for experimentation. These embryos can then be cultured under conditions of precise nutritional control for periods up to three days during which time growth and development proceed at rates comparable to those observed in ovo. This experimental system is therefore uniquely suited for these investigations.

II NUTRIENT PROTEIN UTILIZATION

For some time we have been concerned with an apparent conflict in certain observations from our previous studies. Changes in the quantity as well as the type of protein contained in the culture medium were shown to have an affect on the relative growth of embryo regions as well as on the synthesis of specific proteins in embryo regions. However, our studies on protein utilization showed that most of the protein contained in the nutrient medium did not reach the embryo but rather were broken down to amino acids in the extraembryonic yolk-sac membrane. It appeared inconsistent for a nutrient protein to have an affect on embryo growth and development but not reach the embryo as such. With this in mind, we have devoted considerable time to the possible role of the yolk-sac in mediating the embryo response to protein nutrition.

A. Serum Protein Synthesis. Our initial studies involved an attempt to determine what if anything did the yolk-sac do with a pool of amino acids derived from the breakdown of nutrient proteins. For this work a new, unique, and simple technique was developed. Isolated - intact yolk-sacs were incubated in Ringer's in the presence of radioactive amino acids and were found to synthesize and release serum proteins into the Ringer's incubation medium. The basic studies on serum protein synthesis by the isolated yolk-sac have been completed. Details of this study are included with this Progress Report as a preliminary draft of a manuscript. The major findings can be summarized as follows. The yolk-sac synthesizes all of the serum proteins, as verified by comparisons with proteins contained in embryo serum. Serum proteins are exclusively synthesized by the yolk-sac in the early chick embryo and synthesis commences as early as the head fold stage. The relative synthesis of the various serum proteins changes with development and of particular importance, the relative synthesis also changes with alterations in protein nutrition (see included manuscript).

B. The Specific Uptake of Serum Proteins. Presently, we are attempting to determine if the changes in relative serum protein synthesis observed with alterations in nutrition and with developmental age can be related to changes in the growth and development of the embryo. Our initial attempt to deal with this question involved determinations of serum protein uptake by isolated-intact brains and hearts. Thus, we reasoned, that the response to different serum proteins might involve differences between cells in the uptake of specific serum proteins. For these studies we first developed procedures for the culture of isolated-intact brains and hearts. Quite simply, we observed that hearts and brains could be cultured on either Millipore filters or glass surfaces in the presence of a nutrient mixture. By determining growth parameters, continuation of heart beat, and the maintenance of brain morphology, it was possible to select favorable culture conditions.

For the uptake studies, hearts and brains were isolated from chick embryo explants that had been cultured for 24 hours on whole egg homogenate medium. They were cultured on ¹⁴C-labeled embryo serum proteins in combination with F-10 tissue culture medium for two days. The medium was then recovered and after extensive dialysis it was run on acrylamide gel electrophoresis. Two experiments have been completed (Table 1). The results of the first experiment indicated a selective uptake of the embryo specific prealbumin by the brains but in the second experiment both the brain and the heart took up comparable amounts of this protein. We plan to repeat this experiment but have also considered an alternative approach. Namely, serum will be isolated from yolk-sacs of embryo explants cultured on various protein media or from yolk-sacs of various developmental ages. The brains and hearts will then be cultured in the presence of these various sera and then the brains and hearts will be labeled with radioactive amino acids. The brain and heart proteins will then be resolved on

acrylamide gels. In this way we hope to determine if the various sera can directly influence the type and quantity of proteins synthesized by these isolated regions.

Table I
The Uptake of Serum Proteins by Isolated Hearts and Brains

	Transferrin (%)	Alpha Globulins (%)	Prealbumin (%)
Experiment I			
Controls	14.9	16.7	56.2
Brains	19.8	21.6	43.7
Hearts	12.5	17.8	58.4
Experiment II			
Controls	17.9	11.5	59.1
Brains	19.9	14.1	49.9
Hearts	19.5	14.1	51.1

C. Cell-Free Serum Protein Synthesis. During the past year we initiated studies on the use of cell-free protein synthesizing system to study the regulation of serum protein synthesis. As noted above as well as in the included manuscript, the relative rates of synthesis of the various serum proteins as well as the quantities present in the serum vary with protein nutrition of the embryo and developmental age. In order to determine the factors regulating these changes we have turned to cell-free systems. It should be noted that with most embryonic tissue, the amounts of material which can be obtained are usually limiting. With the yolk-sac, however, sufficient tissue is available for such in vitro studies.

In our first experiments, we attempted to work with polyribosome preparations from yolk-sacs but failed to find serum proteins in the reaction products. Thus, we have gone back to basic steps and are presently attempting to define conditions for the synthesis of protein by various preparations of yolk-sac material. For example, we have examined crude homogenates and post-mitochondrial supernatants. Crude homogenates of yolk-sacs provided high levels of incorporation but to

our dismay incorporation declined sharply with time. We have considered the possibility that this decline was due to the presence of proteolytic enzymes and are presently working with inhibitors of proteolytic enzymes such as p-toluenesulfonyl fluoride and phenylmethylsulfonyl fluoride. Both of these inhibitors are serine protease inhibitors.

To date, we have found that these inhibitors of proteolysis can block the sharp decline in protein synthesis. These inhibitors can also reduce protein synthesis at high concentrations. Concentrations which block proteolysis without inhibiting synthesis were found to be in the range 6×10^{-3} to 6×10^{-4} $\mu\text{g/ml}$. By increasing the total protein synthesized in these cell-free preparations from yolk-sacs, we hope to detect the synthesis of serum proteins and to study the mechanisms regulating their synthesis.

D. Proteolytic Enzyme Substrate Specificity. Previous studies in our laboratory have demonstrated that only certain proteins will support the growth and development of the chick embryo. For example, casein and bovine serum albumin would not support embryo growth but embryo growth was supported by ovalbumin and conalbumin. Furthermore, no single protein would support embryo growth. To date we have found that the embryo requires a transferrin either in the form of conalbumin or yolk transferrin in combination with another protein, either ovalbumin or lipovitellin. The basis for the specificity or the required combination of proteins has not been clarified. One approach that we have been considering involves a possible specific requirement by the proteolytic-lysosomal enzymes of the yolk-sac. The yolk-sac contains high levels of proteolytic enzyme activity and we have demonstrated the breakdown of nutrient proteins within the yolk-sac.

In one study, yolk-sacs were homogenized and centrifuged first at 800 x g for 10 minutes and then 10,000 x g for 20 minutes. Both the pellet

and supernatant contained lysosomes and proteolytic activity. They were tested with ovalbumin alone or ovalbumin plus conalbumin as substrates. As previously noted, embryo explants can be cultured on ovalbumin plus conalbumin but not on either ovalbumin or conalbumin alone. The reaction mixtures were prepared according to a published procedure and the trichloroacetic acid soluble fraction was tested for amino acids by the ninhydrin reaction. The results showed that a mixture of ovalbumin and conalbumin was more readily broken down than ovalbumin alone (Table 2). This observation provided the first insight into a possible basis for the requirement of these two proteins.

Table II
The Breakdown of Proteins by Yolk-Sac Proteolytic Enzymes

Substrate	Percentage of Zero time			
	0 time (%)	3 hrs. (%)	7hrs. (%)	16 hrs. (%)
2 mg ovalbumin	100	230	390	560
1 mg ovalbumin + 1 mg conalbumin	100	340	520	700
Blank	100	230	280	320

In a second experiment the breakdown of non-substrate proteins was evaluated. Lysosomal preparations were made from yolk-sacs that had been previously incubated with ¹⁴C-amino acids. It was interesting and possibly significant to note that the breakdown of endogenous protein was less in the presence of both ovalbumin and conalbumin than when the exogenous substrate was ovalbumin alone.

III REGULATION OF CELLULAR ACTIVITY

This section of the report is concerned with our studies designed to identify those cellular activities of the embryo which are responsive to nutrition and hopefully to gain insights into mechanisms regulating growth and differentiation. We have previously demonstrated that changes in the quantity and type of protein contained in the nutrient medium have striking effects on regional growth. For example, the presence of high levels of ovalbumin in the medium leads to increased accumulation of DNA, RNA, and protein in the brain relative to the trunk. When the total protein in the medium was reduced, regional differences in DNA, RNA, and protein accumulation were lost. This loss of regional differences for protein accumulation, was the result of increased protein degradation rather than decreased synthesis. More recently, we have employed the double labeled acrylamide gel technique to demonstrate that the synthesis of individual proteins in regions of the embryo was sensitive to both the quantity and type of protein contained in the nutrient medium. A preliminary draft of a manuscript concerned with these studies is included with this Progress Report.

A. Polyribosome Activity. In the Progress Report of last year, we reported on our studies of polyribosome activity. The most pertinent observation showed that the polyribosomes of brain were more active in the synthesis of protein in a cell-free system than the polyribosomes of heart. This difference in polyribosome activity correlated precisely with the differences in protein accumulation between brain and heart and provided the first possible mechanisms of regional growth regulation. These studies were done with embryo explants cultured on growth medium. We also examined the brain and heart polyribosomes of embryo explants cultured on protein starvation medium. Again, brain polyribosomes were more active than those of heart but starvation reduced the protein synthetic activity

of both brain and heart polyribosomes relative to those obtained from growing embryos. These data were important in confirming our previous observations which suggested that protein synthesis was not reduced in starved embryos in a regional specific manner. Rather, these data together with other observations suggested that the loss in regional growth differences with embryos cultured on protein starvation medium was due to regional differences in protein breakdown.

The data discussed above were based on polyribosomes that had been isolated by a single procedure; myosin precipitation. Because of this, last year we isolated polyribosomes by two other procedures. Polyribosome were isolated by pelleting through a 5% sucrose cushion and by pelleting after polyribosomes were first collected by sucrose density gradient centrifugation. Both procedures gave results which were more variable than the myosin precipitation procedure but the results in general did not confirm those obtained through myosin precipitation. Indeed, the differences in protein synthesis between the brain and the heart were essentially lost. This suggested the possibility that myosin precipitated polyribosomes could be contaminated with cytoplasmic factors. Thus, we next compared protein synthetic rates of crude homogenates derived from brain and heart. Again, the heart and brain homogenates provided similar results. At this point, we plan to examine the polyribosomes isolated by these various procedures (for example, acrylamide gel analysis of the proteins associated with the ribosome) with the hope of finding the basis for this difference.

B. Ribosomal and Supernatant Factors. In the course of our studies of protein synthetic patterns and protein nutrition in which double labeled acrylamide gels were used (see included manuscript), a single brain protein appeared particularly susceptible to changes in protein nutrition. Various cellular fractions were isolated to determine if this particular protein was concentrated in one cellular component. The protein was particularly high in a

high salt (1 M KCl) wash of ribosomes. By various fractionation procedures and molecular weight determinations the protein has been tentatively identified as elongation factor 1. It should be noted that others have also identified elongation factor one as a major variable with nutritional changes.

Although it would have been desirable to compare the high salt washes of ribosomes isolated from various embryo regions in cell-free protein synthesis systems, we felt that it would be impossible to harvest sufficient quantities of ribosomes for this type of study. In view of this difficulty and the polyribosome studies which were previous discussed, we next examined high speed (2000,000 xg) supernatants of brains and hearts from embryos cultured on growth and protein starvation media. These high speed supernatants were combined with a single source of ribosomes; those from the brain of four day embryos. The results (Fig. 1) showed that the high speed supernatant from brains of growing embryos was more active than those from protein starved embryos while the hearts of both groups showed lower activity.

The possibility that the differences observed with these high speed supernatants was related to the differences in myosin precipitated polyribosomes is presently under consideration. We plan to compare protein patterns of myosin precipitated polyribosomes with the high speed supernatants in order to determine if they contain any proteins in common.

C. Microtubule Protein. Because nervous tissue has been shown to contain large numbers of microtubules and because we have shown the nervous system to be highly responsive in the early embryo to changes in protein nutrition, we have attempted to examine the synthesis and breakdown of microtubule protein. Two methods of quantitation were used; colchicine binding and vinblastine precipitation in conjunction with SDS acrylamide gels. In comparing the brains of growing and protein starved embryos, the colchicine binding assay indicated that the starved

Figure 1. Ability of high speed supernatants isolated from cultured embryo regions to support Cell Free incorporation into trichloroacetic acid precipitable material.

Each point represents the average of two experiments where 0.1 ml aliquots were taken from 0.5 ml reaction mixtures containing 115 μ g of high salt (1M KCl) washed brain ribosomes, 320 μ g of high speed supernatant from the various regions, plus energy, cold amino acids and luc of 14 C amino acid mixture.

brains had approximately 40% of the microtubule protein of the fed while the value with vinblastine precipitation was 53%. The loss of radioactive label as well as incorporation of radioactive label into microtubule protein was next examined. A loss of radioactivity was not detected. However, incorporation into the microtubule proteins of brains of starved embryos was reduced by 54% relative to brains of growing embryos. Thus, it would appear that the level of microtubule protein was regulated by synthesis. This observation appeared quite distinct from our previous studies which indicated an extensive breakdown of brain proteins during culture of embryos on protein starvation medium. However, in these previous studies, total brain proteins were examined rather than specific proteins.

D. Isolated Brains and Hearts and Protein Starvation. In previous studies, we showed that the brain of the early chick embryo was, unlike other regions, highly sensitive to protein starvation. Thus, during culture on protein starvation medium, the protein, RNA and the ribosomes of the brain were rapidly degraded. The heart on the other hand was the most resistant region in terms of degradation of macromolecules and the continuation of growth. It was initially felt that this unique sensitivity of the brain had a cellular basis and we attempted to demonstrate this by studies of such possibilities as the sensitivity of isolated brain ribosomes to degradation. This approach was not successful.

Last year we developed procedures for the culture of isolated brains and hearts. The success of these cultures enabled us to determine if the unique sensitivity of the brain was a characteristic of the developing nervous system or if the sensitivity was more a characteristic of the brain in relation to the whole embryo. Brains and hearts were taken from embryos which had previously been incubated with ^{14}C -valine. The isolated regions were then cultured for periods up to 36 hours on either growth or protein starvation media. The radioactivity

in the protein of the regions as well as the protein content were followed. Although the data for the brains and hearts on growth medium were of interest, the losses of radioactivity for these regions in the protein starved group were particularly pertinent (Table III). Thus, the starved hearts actually lost more radioactivity from protein than the starved brains. This would suggest that the unique sensitivity of the brain to starvation in the intact embryos was the result of features characteristic of the brain in relation to the whole embryo. These results did not support the alternative possibility that cells of the developing nervous system were particularly sensitive to starvation.

TABLE III
Radioactivity in Proteins of Isolated Hearts
and Brains Cultured on Starvation Medium

	Zero time	12 hours	24 hours	36 hours
Brain	673	661 (98) ¹	507 (75)	467 (69)
Heart	114	94 (83)	50 (44)	56 (49)

¹The data presented represents the actual counts per minute. The figures in parentheses are percentages of zero-time.