PROTEINS IN GROWTH REGULATION DURING
EARLY DEVELOPMENT OF THE CHICK EMBRYO

Progress Report

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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 (74-75) contract year.

PROGRESS REPORT SUMMARY

Cultures of early chick embryos are being used to investigate the role of nutrient proteins in growth regulation. The studies are involved with the mechanism of nutrient protein utilization as well as the impact of nutrition on the regulation of cellular activity during early development. During the past year we have, for the most part, concentrated on the "regulation of cellular activity". The following is a summary of our major findings of the last year.

Polysome activity, rather than the number of ribosomes as polysomes or ribosomes per cell, was found to be the major factor in the regulation of "normal" differences in regional growth. Starvation does not effect protein synthesis in a regional specific manner but does reduce protein synthetic activity. This appears due to reductions in polysome activity, number of ribosomes as polysomes, and ribosomes per cell. Polyacrylamide gel electrophoretic analyses have shown that the synthesis of proteins can be both quantitatively and qualitatively influenced by protein nutrition. The brain of the early embryo appeared particular susceptible to this influence. The synthesis of serum proteins by the yolk-sac membrane is also susceptible to alteration through protein nutrition. The activity of DNA dependent DNA polymerase was found to be directly proportional to the "net" synthesis of DNA.

INTRODUCTION

The present report covering the period May 1973 to May 1974 consists of a preprint of a paper which will appear in the July 1974 edition of *Developmental Biology*, a preprint of a chapter that was invited for Vol. III of "The Biochemistry of Animal Development", a copy of a manuscript being prepared on our work with polysomes, a copy of a manuscript that we have been working on concerning our work on the isolation of yolk proteins, an abstract of a paper that has been submitted for presentation at the July 1974 meeting of the Teratology Society, and this report. In view of the fact that this progress report is being submitted in conjunction with a three year comprehensive report, the presentation will be relatively brief. The reader is referred to the comprehensive report for additional details.

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. At this stage of our research, we have concentrated on the synthesis of serum proteins, and the qualitative and quantitative regulation of protein synthesis in regions of the embryo. The serum protein studies relate directly to nutrient protein utilization while the studies of the protein synthetic activity of embryo regions relates more to the problem of growth regulation.

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.

I. Polysome activity in relation to growth and protein starvation in
brains and hearts of cultured early chick embryos

(A manuscript with the title of this section is included with this progress report.)

We developed the technique of culturing chick embryos on protein starvation medium in order to drastically alter embryo growth in a reproducible manner. We felt that the protein starved embryo could be used as a tool to accentuate the breakdown of macromolecules and thereby provide some insights into this aspect of the growth process. We have routinely compared embryos cultured on whole egg growth and protein starvation media.

In our initial studies we found that the protein and RNA of the brain were rapidly degraded (lost radioactivity) during culture on protein starvation medium while such embryo regions as the heart were resistant (radioactivity was not lost from protein or RNA) [Develop. Biol. 24, 178, 1971]. On growth medium a loss of radioactivity was not detected in the brain or other embryo regions. In our next study we analyzed various sub-cellular fractions in order to determine their contribution to the loss of protein and RNA radioactivity during starvation. For those regions sensitive to starvation (mainly brain but also the neural tube) up to 45% of the protein label and 60 to 70% of the RNA label were derived from the ribosome fraction (Biochem. Biophys. Acta 294, 297, 1973).

During the course of these studies we became concerned with the possibility that the sensitivity of the brain to starvation was not simply the result of regional differences in protein (or RNA) breakdown. It appeared feasible that the breakdown of protein could be occurring equally

in all embryo regions and that starvation had a regional specific effect on synthesis. Or, to restate, proteins could be degraded to amino acids in all regions and our observations actually reflected regional differences in the ability of a particular region to reutilize the liberated amino acids. Thus, we felt that it was essential to determine the effects of protein starvation on regional protein synthesis.

In our original study, we simply "pulse" labeled embryo explants with radioactive amino acids, dissected the regions, and determined incorporation into protein. The incorporation values were corrected for regional differences in precursor uptake. The results indicated that starvation did not have a regional specific effect on starvation. However, we felt this study was inadequate because precursor pool specific activity was not determined. Therefore, we turned to the technique of Regier and Kafatos (J. Biol. Chem. 246, 6480, 1971) for the determination of precursor specific activity. We added an additional dimension to this by attempting to determine the specific activity of the amino acid attached to transfer RNA. Although the technique appeared to be working quite well, at the same time we were also working with isolated polysomes in a cell-free protein synthesizing system. The cell-free system provided excellent results, the approach seemed quite direct, and the system was clearly amenable to analysis. For these reasons, we set aside the precursor specific activity studies and turned to isolated polysomes.

In our most recent study we considered both of the points raised above. First, the protein synthetic activity of isolated polysomes and second the in vitro stability of ribosomes. (Recalling that a large percentage of the radioactivity lost during starvation was derived from

ribosomes.) This study was limited to brain and heart as these two regions exhibited the greatest difference in sensitivity to starvation.

Polysomes isolated from brain were more active on a per polysome basis in the synthesis of protein than those of the heart. 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 proportionally for both the brain and heart. Thus, these results confirmed our previous studies which suggested that starvation resulted in regional differences in protein breakdown but not in regional differences in protein synthesis. As interesting as these results were on the starved embryo, we were particularly moved by the observations with the growing embryo in that during growth the brain polysomes were more active than the heart polysomes. This difference followed the difference in rates of protein accumulation and provided, to this author's knowledge, the first insight into the basis for regional growth differences.

In addition to the above, we also examined polysome profiles of brains and hearts of growing and starved embryos. Starvation reduced the number of ribosomes as polysomes by 6% in both the brain and heart. We also considered that the difference in synthesis could result from differences in the stability of the messenger RNA-ribosome complex. In general, polysome stability did not appear to be a factor in this protein synthetic activity difference. In addition, we examined ribosome-associated ribonuclease activity and again this did not appear to be a factor.

From the above and a previous study on regional ribosomal RNA/DNA determinations (Biochem. Biophys. Acta. 294, 297, 1973) we have concluded the following. The synthesis and accumulation of protein by embryo

regions during rapid growth is mainly regulated by regional differences in polysome activity. The reduction of protein synthesis during starvation occurs in all regions of the embryo and is the result of reductions in polysome activity (probably the major factor), the number of ribosomes as polysomes, and in the number of ribosomes per cell.

In the second series of studies, a direct attempt was made to determine a possible basis for the sensitivity of brain and insensitivity of the heart to starvation. Ribosomes labeled with ^{14}C -amino acids or ^{14}C -uridine were treated either with trypsin, ribonuclease, or no enzyme (autodegradation) and the release of radioactivity was followed. The differences between the two sources of ribosomes with added enzyme were small and the opposite of what might be expected (heart ribosomes were more readily degraded). However, measurements of autodegradation were generally in agreement with regional differences in sensitivity to starvation. The significance of this observation was difficult to interpret in view of the conflict between autodegradation and the added enzyme.

II. Nutrition and the synthesis of specific proteins - gel electrophoresis

We are in the process of completing a rather extensive study of the synthesis of protein in embryo regions analyzed by polyacrylamide gel electrophoresis. For the most part we have used the double label technique (^3H and ^{14}C amino acids) and coelectrophoresis of high speed supernatant proteins. Several different comparisons have now been completed and I will try to summarize our findings.

First, employing embryo explants cultured for 45 hours on whole egg growth medium and pulse labeling between 45-48 hours, we ran control comparisons between the various regions (brain, heart, neural tube,

somites, yolk-sac membrane). The differences between regions were clearly greater than the controls (brain against brain, heart against heart, etc.). This indicated that although each region consisted of numerous cell types, they synthesized proteins which were qualitatively and quantitatively distinct. In comparing growing and protein starved embryos, the largest difference was noted for the brain and neural tube while the somites and heart did not differ. This was in agreement with our previous observations on regional differences in sensitivity to starvation. In considering specific proteins, during 45 hours of culture on growth medium four new proteins appeared in the brain region. During 45 hours of culture on starvation medium only three of these appeared. This suggested that the synthesis of some proteins was time or developmental stage dependent while that of others was growth dependent.

One of the most interesting comparisons that we made last year was that of embryo explants cultured on ovalbumin-conalbumin medium with that of whole egg cultured and zero-time embryos. Protein starvation does not only involve a change in nutrition but also a severe restriction of growth (or total protein accumulation). With ovalbumin-conalbumin growth is not restricted but the nutrient proteins are quite distinct from whole egg medium. In this comparison, the brains again showed the largest difference in protein pattern. However, this difference was quite distinct from that of starved brains. Indeed, when compared with zero-time brains, the brains of ovalbumin-conalbumin embryos appeared more advanced in development than those of whole egg. Thus, we feel that we have demonstrated that protein nutrition can effect the patterns of protein synthesis and that the brain is particularly susceptible to changes in protein nutrition.

We have also worked on protein turnover in growing and starved embryos. In the previous studies embryos received a three hour pulse of radioactive amino acids and were then immediately sacrificed. In these studies of turnover, embryo explants were labeled at zero-time, cultured for periods up to 45 hours, and then sacrificed. From these studies, we feel that we have, for the first time, demonstrated protein turnover in a growing embryo. Our studies with starved explants have not been completed but preliminary observations suggested that breakdown involves specific proteins.

III. Nutrition and the synthesis of specific proteins - serum proteins

The possible role of serum in the regulation of early embryonic growth has been a major component of our working hypothesis. This was derived from the observations that nutrient protein was taken up by the yolk-sac and degraded to amino acids within the membrane (Develop. Biol. 26, 380, 1971). Free amino acids did not appear to be transported as such to the embryo but were, we felt, used in the synthesis of serum proteins which were in turn transported to the cells of the embryo proper. We have for some time attempted to find an experimental approach 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 contract year, we were quite fortunate to find an approach for the study of serum protein synthesis. In brief, yolk-sac membranes were simply placed in buffered chick Ringer's and gently shaken. Serum proteins were released into the chick Ringer's and could be analyzed by polyacrylamide gel electrophoresis. When radioactive amino acids were

used, three major radioactive serum protein bands appeared. That these were indeed serum proteins was verified by comparisons with actual serum samples.

We have been able to run several studies during the last year which can be summarized as follows. First, the synthesis of serum proteins was found to be restricted to the yolk-sac membrane. Yolk-sac membranes of only 24 hours of preincubation (2-5 somites) do not have a functional circulatory system. However, these very young membranes synthesize all three serum proteins. More recently we have started to investigate the effects of nutrition on the synthesis of serum proteins. Yolk-sac membranes from embryo explants cultured for 48 hours on protein starvation medium synthesized the three serum proteins in the same relative proportions as those from embryo explants cultured on whole egg growth medium. However, with yolk-sacs from embryo explants cultured on ovalbumin-conalbumin medium, the relative synthesis of the three serum proteins was quite distinct. This observation provided the first indication that protein nutrition could effect the synthesis of serum proteins.

IV. DNA and DNA dependent DNA polymerase activity

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

In previous studies we had followed the loss of radioactivity from protein and RNA during starvation as well as growth. Although radioactivity was lost in a regional specific manner during starvation, radioactivity

was not lost during growth. Repeating these experiments with DNA and radioactive thymidine presented a different picture. Radioactivity was lost during growth from DNA, but was restricted to the brain and neural tube. Naturally, under the condition of protein starvation radioactivity was also lost. We are not certain at this time if the losses during rapid growth are the result of "normal" cell death or possibly some form of DNA repair. This observation also suggested the possibility that our previous failure to detect losses of radioactivity from protein and RNA during growth could have been the result of reutilization. Thus, unlike free amino acids or uridine, free thymidine may have been rapidly degraded.

DNA dependent DNA polymerase activity was determined for the various regions for embryo explants cultured on growth and protein starvation medium. The procedure of Margolis was used (*J. Neurochem.* 16, 447, 1969) with heat denatured calf thymus DNA as substrate. The various controls have been run and the first experiments have been completed. DNA polymerase activity was found to be directly proportional to the "net" rate of DNA synthesis for the regions of both growing and starved embryo explants. The value for "net" synthesis was derived by combining DNA accumulation values with those obtained from DNA degradation. This close relationship between DNA synthesis and DNA polymerase activity has suggested that this enzyme plays a central role in the regulation of regional differences in cellularity.