PROGRESS REPORT
Of Research Conducted
on a Proposal Entitled:

MASTER

PROTEINS IN GROWTH REGULATION DURING
EARLY DEVELOPMENT OF THE CHICK EMBRYO

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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 (72-73) contract year.

INTRODUCTION

The present progress report covering the period May 1971 to May 1972 consists of two publications that appeared during the last contract period, a preprint of a paper submitted for publication, *Remond* and this report.

The ultimate goal of these studies is to elucidate basic mechanisms involved in the regulation of regional growth during early embryonic development. The specific aim is to evaluate a possible hypothesis of regional growth regulation based on the availability of amino acids or amino acids contained in aminoacyl transfer RNA at the cellular sites for protein synthesis. The availability of amino acids is regulated through the transfer of serum proteins from the extraembryonic membrane to the embryo proper. The actual synthesis of serum proteins is in turn regulated through nutrient proteins. The availability of amino acid leads to an adaptive response in a particular embryo region which involves increases either in the synthesis of ribosomes, the activity of polyribosomes (protein synthesis), or the proportion of ribosomes in polysomes.

Cultures of intact early chick embryos have been used in these studies. The embryos are removed from the eggs after approximately forty hours of pre-incubation, 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.

During the last contract period our major effort was directed at the completion of the studies on the breakdown of RNA and protein in subcellular fractions of the protein starved chick embryo. This work is described in detail in the enclosed preprint. In addition we have worked on quantitation of polysomes in regions of growing and starved embryos, initiated studies on the synthesis of specific proteins in regions of embryos, worked out the methodology for the determination of absolute protein synthetic rates and have recently tried out a method for the determination of acetylcholinesterase. Finally, I have included two electronmicrographs of neural ectoderm from the brain region of a protein starved and growing embryo.

The effect of protein starvation on RNA and protein breakdown in subcellular fractions

During protein starvation, embryos prelabeled with radioactive amino acids or uridine, lost radioactivity from protein and RNA in a regional specific manner. Thus, there are losses from the brain, neural tube and extraembryonic membrane but not from the heart or somite regions. Most of the RNA and protein radioactivity lost from the sensitive regions is derived from the ribosomal fraction. As part of this study, determinations were also made of the amounts of ribosomal RNA per cell (DNA). These determinations showed that in the most rapidly growing regions (brain and neural tube) there is a doubling in the ribosomes per cell while in the slower growing regions

(heart and somites) the ribosomes per cell remains constant. During protein starvation, the ribosome per cell ratio remains constant in all regions. The rapid breakdown of ribosomes in conjunction with the constancy of ribosomes per cell suggests that under starvation conditions ribosome breakdown may be a mechanism modulating the activity of cells when they change from a state of rapid growth to a state of limited growth. Furthermore, regional differences under conditions of "normal" rapid growth appears related directly to differences in the ribosomes per cell ratio. It is also of interest that the ribosome per cell ratio may be correlated with the state of differentiation in that the nervous tissue may be more poorly differentiated at the time of our studies than either the heart or somites.

It should be noted that the preprint containing the details of this study is included with this report.

Polysome quantitation in regions of embryos cultured under conditions of rapid growth and protein starvation

Polysome profiles have been prepared from the brain, heart, and extraembryonic membrane regions of embryos cultured for 48 hours under growth and protein starvation conditions. Although the membrane produced some interesting observations, I will limit this brief discussion to the brain and heart regions as they are parts of the embryo proper and represent the greatest divergence of regional responses to nutrition. Polysomes were prepared according to the Triton X-100 procedure of Morse et al. (Biochim. Biophys. Acta 232:

403, 1971). A gradient of 15% to 40% sucrose was used with an NH_4Cl_2 containing buffer and the gradient was ~~run~~^{run} at 36,000 RPM for about 50 minutes. The gradients were analyzed with an ISCO gradient collecting system. The areas under the polysome tracings were determined and divided into ribosomal subunits, monosomes, and polysomes. The results from a typical run are presented below.

The distribution of ribosomes as subunits, monosomes, and polysomes in regions of chick embryos cultured for 48 hours under conditions of growth and protein starvation

	% subunits	% monosomes	% polysomes
Brain			
growing	15	29	56
starved	28	38	37
Heart			
growing	22	34	44
starved	19	38	43

From the above data several points should be noted. First, the distribution of ribosomes in the brain but not the heart can be altered by changes in nutrition. This correlates precisely with our previous observations on differences in regional sensitivity to starvation. Second, there are relatively more polysomes in the brain than in the heart of growing embryos which would be expected from the differences between these two regions in growth rates. Finally, the relative proportion of polysomes in the protein starved brain are reduced to such an extent that the number of ribosomes as polysomes (together with the number of ribosomes per cell) may well serve as a modulator of cellular activity as well as growth during early embryonic development.

The synthesis of specific proteins in regions of cultured chick embryos under various growth conditions

To date we have simply completed all the necessary ground work to start these studies. After various periods of culture on various media, embryos will be exposed to either ^{14}C or ^3H amino acids and dissected into the various regions. If two regions are to be directly compared double labeling procedures will be used. For example, if brains from protein starved and growing embryos are to be compared, one group will receive ^3H amino acids and the other ^{14}C amino acids. We have been using a dialyzed post ribosomal supernatant for analysis. Two acrylamide gel electrophoresis systems have been used. The method of Davis (Ann. N.Y. Acad. Sci., 121: 404, 1964) for normal discontinuous gels and that of Weber and Osbourn (J. Biol. Chem. 244: 4406, 1969) for SDS gels. Gels are stained with Amido Schwarz for densitometric tracing and they are also sliced (0.8 mm) with a Joyce-Loeble Gel Slicer for detection of radioactivity. We have worked on several variables in the way of sample preparation, electrophoresis and the counting of radioactivity. We feel that most of the problems have been worked out. At this time we are in the process of doing a double label experiment. In Figures 1 and 2 are presented radioactivity tracings from the brain and heart regions of embryos cultured for 48 hours on growth media. I present these figures to indicate mainly that we are in the process of doing this type of study and to demonstrate that there appears to be many differences between the proteins synthesized in these two regions.

Fig. 1 Radioactive tracing from acrylamide gel of the brain region of chick embryos cultured for 48 hours on growth medium prior to 3 hour pulse with ^{14}C -uridine

Fig. 2 Radioactive tracing from acrylamide gel of the heart region of chick embryos cultured for 48 hours on growth medium prior to 3 hour pulse with ^{14}C -valine

The determination of absolute protein synthetic rates in regions of cultured embryos

During the last contract year we have worked extensively on procedures for the determination of the amino acid specific activity in a manner which would allow one to correct the observed incorporation into protein. The problem one faces in determining protein synthetic activity is to evaluate the dilution factor that occurs when the radioactive amino acid enters the cell and is mixed with the endogenous pool of free amino acids. We plan to isolate charged transfer RNA and to determine the specific activity of a particular amino acid (probably valine) contained on the transfer RNA. At this time we have isolated charged transfer RNA's and have stripped the amino acids from the transfer RNA. The next step requires the separation of the amino acid. In this regard we are following the procedure of Regier and Kafatos (J. Biol. Chem. 246: 6480, 1971) which involves the formation of a dinitrophenyl derivative from ^{14}C -1-fluoro-2,4-dinitrobenzene. This procedure is extremely sensitive and is particularly adapted for use with small amounts of tissue. We have visited the laboratory of Dr. Kafatos and have been able to make and separate the derivatives. We plan to run the actual experiments in the very near future.

The determination of acetylcholinesterase

In an attempt to determine rather precise parameters for functional differentiation we have considered the formation of various proteins with enzyme activity. For example, it would be of considerable

interest to evaluate the relationship between growth in an intact organism and the synthesis of specific proteins. Thus, one may ask if the brain of a protein starved embryo continues to undergo differentiation in the absence of a net increase in protein and what controls the synthesis and the breakdown of specific proteins. With this objective in mind, we are attempting to determine acetylcholinesterase activity in the brain regions of early embryos. To date, we have found a method that is highly sensitive and does work with small amounts of tissue from chick embryos. This is the procedure of Hobbiger and Lancaster (J. Neurochem. 18: 1741, 1971) which involves the use of the substrate acetylthiocholine.

Electron microscopy of the brain region from growing and protein starved cultured chick embryos

As noted in previous progress reports we do some histological work in conjunction with our biochemical studies. Last year a student, while taking a course in electron microscopy, had the opportunity to examine the brain regions of chick embryos which had been starved. As previously observed, starvation appears to mainly disrupt the developing nervous system. The electron micrographs presented in Figures 3 and 4 were made of the brain ectoderm from embryos cultured for 48 hours. Figure 3 is of a growing embryo and Figure 4 of a protein starved embryo. The tissue was fixed in 4% glutaraldehyde and 2% osmium tetroxide, araldite-epon for embedding and stained with uranyl acetate and lead citrate. Sections were cut at 400-600 Å.

Fig. 3 Electron micrograph of brain ectoderm from embryo cultured for 48 hours on growth medium

Fig. 4 Electron micrograph of brain ectoderm from embryo
cultured for 48 hours on protein starvation medium

The clear region at the top of each figure represents the inner edge of the neural ectoderm suggesting that these figures were made of comparable regions. The most striking observation is the loss of the highly columnar arrangement of the neural ectoderm in the protein starved brain region. This appears to be one of the first morphological changes observed in starved embryos. Although our morphological work remains quite limited, another student plans to follow up this work in association with a course in electron microscopy.