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PROTEINS IN GROWTH REGULATION DURING
EARLY DEVELOPMENT OF THE CHICK EMBRYO

Progress Report

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May 1972 - May 1973

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

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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 (73-74) contract year.

PROGRESS REPORT SUMMARY

Cultures of early embryos are being used to investigate the role of nutrient protein in growth regulation. The studies are involved with (1) elucidating mechanisms of nutrient protein utilization and (2) determining the impact of nutrient proteins on the regulation of molecular events with the cells of the embryo. Last year we mainly worked on ^{the} ~~the~~ latter part. We previously demonstrated that the nervous system and particularly the brain region is the most sensitive region of an early embryo to protein starvation. We have attempted to determine the basis for this unique sensitivity by examining the in vitro stability of ribosomes and polysomes isolated from regions of embryos cultured under growth and protein starvation conditions. The degradation of ribosomes was studied with trypsin, ribonuclease and without added enzyme (autodegradation). In addition, the autodegradation of polysomes and the activity of ribosomal ribonuclease were studied. Several experiments have been completed with double label acrylamide gels comparing regional patterns of protein synthesis and the effect of protein starvation on these patterns. Finally, we have worked out details for determining absolute protein synthetic rates and have initiated studies with affinity chromatography for the detection of nutrient protein membrane receptors.

INTRODUCTION

The present progress report covering the period May 1972 to May 1973 consists of a reprint that appeared during the last contract period; an abstract of a paper that has been accepted for presentation at the Thirteenth Annual Meeting of the Teratology Society (June 13-16, 1973, Quebec, Canada); a preprint of a Chapter based on the past work of this laboratory which has been accepted for publication in "The Biochemistry of Animal Development", Vol. III, Academic Press, edited by R. Weber; a preprint of a paper submitted for publication to the journal, Developmental Biology; a draft of a manuscript being prepared for the journal, Arch Biochem. Biophys.; 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 acids leads to an adaptive response in a particular embryo region which involves increases either in the synthesis of ribosomes, the activity of polysomes (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

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.

The previously stated hypothesis can be conveniently divided into three areas of experimentation. First, studies dealing with the transfer of proteins synthesized in the extraembryonic membrane (serum proteins) to regions of the embryo proper. Second, investigations concerned with nutrient protein utilization (uptake, breakdown, specificity of nutrient protein requirement) and the influence of nutrient protein utilization on the synthesis of serum proteins. Finally, experiments concerned with elucidating the impact of amino acid availability on embryo regions as studied through nutrient protein starvation (protein and RNA breakdown and synthesis, ribosome synthesis, polysome activity, polysome formation, the synthesis and breakdown of specific proteins and RNA, electron microscopic analysis).

During the last contract period (May 1972 to May 1973) we have mainly worked on the last area stated above, namely, the elucidation of the impact of amino acid availability on embryo regions. Following from our past studies which have shown the brain region to be particularly sensitive to nutrient protein starvation in terms of protein and RNA breakdown, we have attempted to demonstrate that the ribosomes and polysomes isolated from the brain are particularly susceptible to breakdown in vitro. That is to say, we have attempted to relate the apparent instability of polysomes and

ribosomes in the intact brain with their stability or instability in isolation. In addition, we have made precise measurements of the quantities of polysomes in the different embryo regions and have evaluated the effect of starvation on these measurements. The influence of nutrient protein starvation (amino acid availability) on the synthesis of specific proteins has been studied by double label acrylamide gel coelectrophoretic procedures. The determination of absolute regional protein synthetic rates and the effect of starvation on these rates has finally reached a point in which the studies can be made. The procedure involves the determination of amino acid specific activity of the amino acid attached to transfer RNA. The difficulties of isolating charged transfer RNA from small embryo regions have now been overcome and the actual experimentation should start this month. Electron microscopic studies are continuing but will not be considered in this years report.

Although most of our effort has been directed along the above lines, some interesting observations have been made on nutrient protein utilization. In brief, it appears that the carbohydrate moiety of the nutrient protein may play an important role in nutrient protein utilization and it appears that we have been able to isolate a cell membrane fraction from the extra-embryonic membrane which selectively binds to a nutrient protein. Recent advances in affinity chromatography has made it possible to isolate this binding fraction. We have been so pleased with this technique that we plan to continue its use in the study of mechanisms regulating nutrient protein uptake which influence serum protein synthesis and to extend its use to provide some insights into the possible basis for the distribution of serum

proteins (or amino acids) in the embryo proper.

I. The Effect of Protein Starvation on Embryo Regions

We have previously reported that during protein starvation there is a selective loss of radioactivity from the protein and RNA of the brain and neural tube of the early cultured chick embryo. Such regions as the somites and heart do not exhibit a comparable loss. Subsequently, (see enclosed reprint of Hickey and Klein, *Biochim. Biophys. Acta* 294: 297, 1973) we demonstrated that most of the radioactivity that was lost from the sensitive regions was mainly derived from the ribosome fraction. In addition, in this same publication, we presented data for the ribosomal RNA to DNA ratio for the various embryo regions. These data showed that under growth conditions this ratio doubled during 36 hours of culture but during starvation this ratio did not increase. Thus, amino acid availability acting either through ribosome breakdown (and possibly through ribosome synthesis to some extent) may regulate ribosome content and ultimately protein synthesis.

A. Polysome profiles for different regions and the effect of protein starvation

Our previous studies on the relative amounts of polysomes, monosomes, and ribosome subunits were based on optical density tracings. This procedure did not appear very accurate particularly because of the uncertainties of base line tracings. For this reason we repeated these studies using ^{14}C -uridine to label the ribosomes. Chick embryos of 11 to 13 somites were exposed for three hours to ^{14}C -uridine and then cultured for 48 hours in the presence of ^{14}C -uridine on either growth or protein starvation media.

Postmitochondrial supernatants were obtained from the isolated regions and placed on a 15-40% linear sucrose gradient following the procedure of Hosick and Strohman (J. Cell. Physiol. 77: 145, 1971), and Heywood et al. (Proc. Nat. Acad. Sci. U.S.A. 57: 1002, 1967). The percentage distribution of radioactivity was as follows:

TABLE I
Radioactivity Distribution in Polysome Profiles

	<u>Growth Medium</u>		<u>Protein Starvation Medium</u>	
	Brain (%)	Heart (%)	Brain (%)	Heart (%)
Polysomes	48.3	39.3	33.6	36.4
Monosomes + Subunits	31.5	41.1	38.9	41.8
Light RNA	20.2	19.7	27.5	21.8

From the above Table it can be seen that during growth, the rapidly growing brain regions contain relatively more ribosomes as polysomes than does the slowly growing heart. During protein starvation there is a sharp reduction in polysomes in the brain region while the polysome profile of the heart is not appreciably influenced by this treatment. Although the percentage distribution obtained by optical density differed quantitatively from these data obtained with radioactivity, the qualitative changes were comparable.

B. The in vitro stability of ribosomes and polysomes

On the basis of (I) the changes in polysome profiles with starvation and (II) the previously published studies showing that in the intact embryo

the major loss of radioactivity from protein-starvation sensitive regions occurred in the ribosome fraction, it was felt that the ribosomes or polysomes in the various regions might have different stabilities due to structural factors. A regional difference in ribosome or polysome stability could provide a basis for the regional differences in sensitivity to protein starvation. For this reason we have studied the effect of trypsin and ribonuclease on ribosomes and autodegradation of ribosomes and polysomes.

Our initial studies were made with trypsin. Chick embryos of 11 to 13 somites were pulsed for 3 hours with ^{14}C -valine and then cultured for 48 hours in the presence of ^{14}C -valine. Ribosomes were isolated from whole embryos or regions according to the procedure we previously used (Hickey and Klein, *Biochim. Biophys. Acta* 294: 297, 1973). An extensive series of studies were made with ribosomes from whole embryos in order to work out the basic parameters of the enzyme reactions such as enzyme and substrate concentrations, temperature, time, etc. However, in general we followed the reported studies of Chang and Flaks (*J. Mol. Biol.* 61: 387, 1971; *Proc. Nat. Acad. Sci. U.S.A.* 67: 1321, 1970) who worked on the structure of bacterial ribosomes. The in vitro breakdown of ribosomes was followed either by measuring the radioactivity released into trichloroacetic acid or alternatively the reaction mixture was passed through a Millipore filter and the amount of radioactivity on the filter was taken as a measure of intact ribosomes. Following all the preliminary studies we compared ribosomes from the various embryo regions, working mainly with the brain and heart as these two regions exhibit the largest difference in sensitivity to protein starvation. In brief, ribosomes isolated from the heart or brain, from embryos cultured under growth or starvation conditions did not exhibit differences in

response to trypsin. Examples of the results are presented in Figure 1 for embryos cultured on growth medium and Figure 2 for protein starvation. Ribosomes (1 mg/ml) were incubated with trypsin (4 µg/ml) at 37°C and the reaction mixtures were collected on Millipore filters after the reaction was stopped by the addition of trichloroacetic acid. In addition, to the above study, ribosomes were isolated from embryo regions after shorter periods of culture as well as after shorter periods of exposure to ¹⁴C-valine. These studies also failed to show a difference in the sensitivity of ribosomes to trypsin.

A second series of experiments were next conducted with pancreatic ribonuclease. These studies were not as extensive as those carried out with trypsin but again basic enzymatic parameters were determined as mentioned for trypsin. In general we followed the procedures of Pinder and Gratzer (Eur. J. Biochem. 26: 73, 1972) who worked with pancreatic ribonuclease to study structural aspects of bacterial ribosomes. Ribosomes were isolated from regions of embryos pulsed with ¹⁴C-uridine and cultured in the presence of this labeled precursor. In brief, the ribosomes of the brain and heart did not show differences in their response to degradation by ribonuclease. An example of the type of results obtained are presented in Figure 3. In this study, ribosomes labeled with ¹⁴C-uridine were isolated from the brain and heart of embryos cultured for 48 hours. The ribosomes (1 mg/ml) were incubated with pancreatic ribonuclease (4 µg/ml) at room temperature. The reaction was stopped by the addition of trichloroacetic acid and aliquots of the acid soluble material were plated out for the determination of radioactivity.

Figure 1 and Figure 2

The effect of trypsin on the degradation of ribosomes isolated from regions of growing embryos (Fig 1) and and starved embryos (Fig 2).

Figure 3

The effect of ribonuclease on the degradation of ribosomes isolated from regions of growing embryos.

We next turned to studies of autodegradation of ribosomes and polysomes following such studies as those by Gagnon and Lamirande (Arch. Biochem. Biophys. 150: 573, 1972) with ribosomes from tobacco pith tissue and by Zomzely *et al.* (J. Biol. Chem. 243: 5396, 1968) with ribosomes and polysomes from the rat cerebral cortex. These studies were conducted in a manner similar to those with ribonuclease in that ribosomes were labeled with ¹⁴C-uridine. Ribosomes were isolated from the brain and heart regions of embryos cultured for 48 hours on either growth or protein starvation media. The ribosome suspensions were incubated for various periods at 37°C and the reaction was stopped by the addition of trichloroacetic acid. The radioactivity in the acid soluble fraction was determined. Although there did not appear to be a difference between ribosomes from starved and growing embryos, there was a relatively small but consistent difference between regions. Brain ribosomes were somewhat more susceptible to autodegradation than heart ribosomes. Examples of the results obtained are presented in Figure 4 for growing embryos and Figure 5 for protein starved embryos. It should be noted that the addition of EDTA did not enhance ribosome breakdown. In other systems (for example, Gagnon and Lamirande, Arch. Biochem. Biophys. 150: 573, 1972) it appears that EDTA may activate ribosomal ribonuclease.

The stability of polysomes were examined next. Embryos were exposed to ¹⁴C-uridine for 48 hours of culture on either growth or protein starvation media. Postmitochondrial supernatants were prepared and divided in half. One half was placed on a sucrose gradient immediately after preparation and the second half was incubated at 37°C for one hour prior to placement

Figure 4 and Figure 5

Autodegradation of ribosomes isolated from regions of growing embryos (Fig4) and starved embryos (Fig5)

on a sucrose gradient. The distribution of radioactivity in the polysome profiles for the heart and brain of growing and starved embryos is presented below in Table II.

TABLE II
Changes in Polysome Profiles After One Hour Incubation at 37°C

Region	Treatment	Polysome (%)	Monosomes + Subunits (%)	Light RNA (%)
Brain	Fed Control	48.3	31.5	20.2
	37°C - 1 hr	25.3	57.7	17.1
	Difference	23.0	26.2	3.1
	Starve Control	33.6	38.9	27.5
	37°C - 1 hr	24.1	54.0	21.9
	Difference	9.5	15.1	5.6
	Fed Control	39.3	41.1	19.7
	37°C - 1 hr	18.1	68.1	13.8
Heart	Difference	21.2	27.0	5.9
	Starve Control	36.4	41.8	21.8
	37°C - 1 hr	20.7	64.4	14.9
	Difference	15.7	22.6	6.9

In examining the values for polysomes in Table II, it would appear that starvation greatly enhances the stability of brain polysomes and to a lesser extent starvation also enhances the stability of heart ribosomes. However, this does not appear reasonable. At present we would suggest two possibilities. First, there exists two distinct populations of polysomes. One population is highly susceptible to autodegradation in vitro and to protein

starvation in the intact animal, and a second population resistant to autodegradation in vitro and to starvation. Alternatively, these results may indicate differences in endogenous ribonuclease activity. During protein starvation cultivation, proteins are broken down in the embryo. It would not be surprising to find that ribonuclease is one of the proteins destroyed during starvation.

From Table II it is also possible to compare regions. Thus, the stability of polysomes appears comparable for the heart and brain of embryos cultured under growth (fed) conditions. However, in the starved embryos the brain polysomes appear more stable than those of the heart. We would suggest that this result may be explained by either of the two possibilities stated above. Thus, starvation being less drastic for the heart has allowed for the preservation of a polysome population highly susceptible to in vitro autodegradation. Or, the level of ribonuclease activity is greater in the polysomes of the starved heart than in the starved brain.

C. Endogenous ribonuclease activity

In view of the above results on autodegradation, we have initiated studies on ribonuclease activity. To date, we have looked at the activity of this enzyme in preparations of ribosomes employing ^{14}C -labeled polyuridylic acid (poly U) as a substrate. We are in the process of extending these studies to polysomes and also plan to do time course studies during the 48 hour embryo culture period.

In an initial series of experiments, ribosomes were isolated from regions of embryos cultured for 48 hours on either growth or protein starvation media. The isolated ribosomes were incubated with ^{14}C -poly U at 37°C . At the

desired time periods, the reaction was stopped by the addition of tri-chloroacetic acid and the mixture was filtered through a Millipore filter. The undegraded poly U was collected on the filter and the amount of radio-activity was determined. The comparison of the brain and heart regions of embryos cultured on growth medium is presented in Figure 6 and for starvation culture in Figure 7. Under growth conditions, ribosomal ribonuclease appears higher in the brain than heart while under starvation conditions the activity of heart ribosomes appears somewhat higher than brain ribosomes. This observation is compatible with the differences in polysome stability previously considered. Thus, under growth conditions the "stability" of brain polysomes was less than those of the heart while under starvation conditions the brain polysomes were more stable than those of the heart. On the other hand, the studies on autodegradation of ribosomes previously considered do not agree with the poly U studies. Thus, under starvation conditions, autodegradation was greater for brain ribosomes than for heart ribosomes while the poly U studies would suggest that the heart ribosomes have more ribonuclease activity than the ribosomes of the brain. Although the actual differences between regions in both the autodegradation and poly U experiments may be too small to account for the differences in polysome stability, additional experimentation is clearly indicated.

D. An acrylamide gel electrophoretic analysis of the patterns of protein synthesis and breakdown

In the progress report of last year, I indicated that we were in process of working out all the procedures for doing double label (^{14}C and

Figure 6 and Figure 7

The effect of ribosomes isolated from regions of growing embryos (Fig 6) and starved embryos (Fig 7) on the breakdown of Poly U.

³H) acrylamide gel electrophoretic analysis of the proteins in embryo regions. All of the many details (labeling procedure, tissue fractionation, type of acrylamide gel, counting cocktail for liquid scintillation, detection of radioactivity, gel slicing, counting vial, etc.) were worked out last summer and since that time we have been able to run several successful and interesting experiments. The results will be presented at the meeting of the Teratology Society in June of this year (see enclosed abstract).

To date, we have attempted to answer the following questions: (I) do the different regions of an early embryo (brain, neural tube, heart, somites and extraembryonic membrane) exhibit distinct patterns of protein synthesis, (II) does the pattern of protein synthesis for a particular region change during 48 hours of in vitro embryo culture, and (III) do the regions of embryos cultured under conditions of rapid growth synthesize the same proteins as regions of embryos cultured under conditions of protein starvation? There are two additional questions which we hope to consider in the very near future. (I) During culture on protein starvation medium are specific proteins broken down, and closely related, can protein turnover be detected by gel electrophoretic analysis in rapidly growing embryos? (II) Does the nutrient protein provided in the medium influence the pattern of protein synthesis in the various embryo regions with particular concern for the synthesis of serum proteins in the extraembryonic membrane?

In these studies, high speed supernatant fractions derived from embryos labeled with either ³H-valine or ¹⁴C-valine are combined and coelectrophoresed on acrylamide gels. The gels are sliced (usually 0.7 mm slices with about

130 slices per gel); dissolved in liquid cocktail; and the radioactivity from ^3H and ^{14}C is determined. One can then plot the distribution of ^3H and ^{14}C and examine these patterns for differences. However, these patterns are often hard to evaluate. Thus, we have also attempted to "quantitate" the total differences. We use a procedure similar to that published by Becker and Stanners (J. Cell. Physiol. 80: 51, 1972). The counts per minute for each slice is converted to disintegrations per minute (dpm) and expressed as a percentage of the total ^{14}C or ^3H radioactivity in the gel. In this way the total area under each curve is the same and differences in the percent of ^{14}C or ^3H in a slice represents quantitative and qualitative differences in the pattern of protein synthesis at that point on the gel. The absolute difference between percent of ^3H and ^{14}C for each slice is then totalled to give a value representing the degree of difference between the two patterns. Thus, by examining these patterns qualitative and quantitative differences can be detected and the computation of the "absolute total of the percent difference" provides a quantitative estimate of the total difference.

In comparing the various regions qualitatively after 48 hours of culture on growth medium many differences were observed but it was particularly striking to note a protein of approximate 25,000 molecular weight (Fig. 8). This protein was quite prominent in the brain region, present at very low concentrations in other regions, and essentially absent from the neural tube region. The quantitative comparisons were not predictable. Thus, the difference between the brain and heart was less than for the brain and any of the other regions (Table III).

Figure 8

Acrylamide gel patterns of brain and extraembryonic membrane. Embryos pulsed with ^{14}C or ^3H valine for 3 hours after 45 hours of culture on growth medium.

^3H Brain - - - - 1% = 898 dpms

^{14}C Extraembryonic
membrane ——— 1% = 114 dpms

TABLE III

Total Percent Difference for Proteins Synthesized in the Brain
in Comparison to Other Embryo Regions*

Regions Compared	Total Percent Difference (%)
Brain/Brain	5.84
Brain/Heart	9.4
Brain/Somites	12.16
Brain/Neural tube	13.47
Brain/Extraembryonic membrane	14.47

* In this study embryos of 11 to 13 somites were cultured for 48 hours on growth medium and then divided into two groups. One group was pulsed for 3 hours with ^{14}C -valine and the second for 3 hours with ^3H -valine.

Next, a comparison was made between zero time embryos (11 to 13 somites) and those cultured for 48 hours on growth medium. Each group was pulsed for three hours with either ^{14}C - or ^3H -valine at zero time or after 48 hours of culture. Various qualitative changes were observed but particularly striking were two proteins present in the brain at 48 hours but absent at zero time (Fig. 9). The quantitative changes are presented in Table IV. The largest difference occurred in the extraembryonic membrane which may reflect the "turning on" of serum protein synthesis. The various other regions, however, also exhibited considerable change over the 48 hours culture period.

Figure 9

Acrylamide gel patterns of brain regions from embryos pulsed for 3 hours with ^3H or ^{14}C valine at zero time (11-13 somites) and after 45 hours of culture on growth medium.

TABLE IV

Total Percent Difference for Proteins Synthesized in Various Regions
for Zero Time Versus 48 Hours culture on Growth Medium and for
48 Hours culture on Growth Medium Versus Protein Starvation Medium

Region	Zero time versus 48 hours culture on growth medium (%)	Growth medium versus starvation medium. 48 hours of culture	
		Regular Gel (%)	SDS Gel (%)
Brain	12.5	12.37	12.18
Heart	17.64	8.14	7.70
Somites	12.45	7.80	6.37
Neural tube	17.63	9.69	10.27
Extraembryonic membrane	25.52	12.08	12.64

The effect of protein starvation culture was next studied by comparing the proteins synthesized in regions after 48 hours of culture on either growth or starvation medium. Similar to the preceeding experiments embryos were pulsed with either ^{14}C - or ^3H -valine for 3 hours following 48 hours of culture. During starvation certain proteins specific for various regions are either altered, broken down, or not synthesized. This was most apparent in the brain region (Fig. 10). The largest quantitative differences were observed in the brain region and extraembryonic membrane (Table IV) while the heart and somites are quite similar. Gels which were run in the presence of sodium dodecyl sulfate (to overcome protein charge differences) showed similar differences.

In attempting to determine if the synthesis of new proteins occurs with time during culture on protein starvation medium (molecular differentiation

Figure 10

Acrylamide gel patterns of brain regions from embryos cultured for 45 hours on growth or protein starvation media. Pulse was for 3 hours with ^3H or ^{14}C valine.

in the absence of protein accumulation), a comparison can be made for the results with zero time versus 48 hours and starvation versus growth. Thus, the differences between zero time and 48 hours in all regions except the brain were much greater than for starvation versus growth. This would suggest molecular differentiation in the absence of growth for those regions. For the extraembryonic membrane, there is still a large difference between starve versus growth which may indicate a direct influence of nutrition on the synthesis of serum proteins. The large difference for the brain may indicate that certain proteins are not synthesized under starvation conditions or alternatively that certain proteins are selectively degraded. These alternatives are presently under investigation.

E. Absolute protein synthetic rates

In a previous publication, we attempted to determine the rate of protein synthesis in various embryo regions and to measure the effect of protein starvation on this process (Klein, et al., Develop. Biol. 24: 178, 1971). We measured the incorporation of radioactive amino acids into protein and attempted to correct for difference in amino acid pools by determining radioactivity present in the trichloroacetic acid soluble fraction. We felt that this approach was inadequate because we failed to correct for the actual specific activity of the precursor. A method was reported by Regier and Kafatos (J. Biol. Chem. 246: 6480, 1971) which appeared applicable to our problem in that the specific activity of an amino acid could be determined at very low concentrations. In this procedure, one provides a pulse of ³H-amino acid and determines incorporation into protein. The cell pool of amino acids is isolated (for example the trichloroacetic acid soluble

material) containing both the ^3H -amino acid and the unlabeled amino acids. The total pool is reacted with ^{14}C -2,4-dinitrofluorobenzene and derivatives are formed from both the ^3H and unlabeled amino acids. The amino acid derivatives are then separated by thin layer chromatography and the spot containing the ^3H -amino acid which you provided in the pulse is isolated. The amount of ^{14}C present in the spot provides an estimate of the total pool and the ratio of ^3H to ^{14}C provides the specific activity. On the basis of the specific activity, the incorporation into protein can be corrected to provide the absolute rate of protein synthesis.

Although this approach appears quite direct, we have become increasingly concerned with the problem of cellular compartments. Thus, for our correction of "pool dilution factors" we felt that we must attempt to determine the amino acid dilution in the most immediate precursor to the newly synthesized protein. For this reason, we have attempted to isolate transfer RNA and to determine the specific activity of the amino acid attached to this molecule.

Last year we felt that most of the problems were solved. However, the stripping of the amino acid from the Transfer RNA did not appear complete and was not reproducible. Thus, we compared six different methods for the isolation of transfer RNA and several methods for the stripping step. At this time, we feel most confident with the recently published method of Judes and Jacob (*Anal. Biochem.* 50: 586, 1972) with the use of 0.1 M Na_2CO_3 , pH 10.0, 60 minutes incubation time for amino acid stripping. This method has given both reproducible and linear extractions of transfer RNA and of the attached amino acids.

It should be noted that we have had no difficulty in making the 2,4,dinitrofluorobenzene derivative or separating the amino acids by thin layer chromatography. This week we are running an experiment in which embryos will be pulsed with ^3H -valine for the determination of absolute protein synthetic rates. We feel fairly confident that the experiment will be a success.

From these experiments we hope to determine if regional growth differences are related to differences in absolute protein synthetic rates. And, if these differences are related to polysome activity, ribosomes per cell, or the amount of ribosomes present as polysomes. In addition, we hope to determine the effect of protein starvation on these processes. For example, does protein starvation increase protein degradation without influencing protein synthesis?

II. The Requirement for Specific Nutrient Proteins

As previously noted, most of our effort has been directed toward evaluating the response of the embryo to amino acid availability. However, we are still quite interested in the other aspects of our basic hypothesis. Thus, how are nutrient proteins utilized and how do they influence the synthesis of serum proteins. In previous studies (Hassell and Klein, Develop. Biol. 26: 380, 1971) we showed that proteins are taken up from the nutrient medium by the extraembryonic yolk-sac membrane and are broken down to amino acids in this structure. In addition, we showed that not all proteins will support embryo growth. For example, a mixture of ovalbumin and conalbumin as well as lipovitellin and livetin will support embryo growth but bovine serum albumin alone or in combination with other proteins as well

as serum will not support embryo growth (Klein, J. Exptl. Zool. 168: 239, 1968). Recently, we have initiated some experiments which we hope will provide some insights into the basis for these differences.

A. The role of the carbohydrate moiety

Ovalbumin and conalbumin are glycoproteins while serum albumin does not contain carbohydrate. Ovalbumin contains five molecules of N-acetylglucosamine and three molecules of mannose per molecule of protein. Conalbumin contains six of the former and four of the latter per molecule. In view of the possibility that these carbohydrate moieties play a role in the process of protein uptake, embryos were cultured on medium containing ovalbumin and conalbumin (actually egg white) in the presence of free glucosamine and mannose. These sugars inhibited embryo growth. However, when egg yolk was used as a source of protein, these sugars did not inhibit embryo growth.

In an attempt to evaluate the importance of the carbohydrate moiety more directly, we are isolating enzymes which will specifically cleave the sugar from the protein. Mannosidase is being isolated from the Jack Bean according to the method of Snaith and Levy (Biochem. J. 110: 663, 1968) and N-acetylglucosaminidase from Jack Bean and boar epididymis (J. Biol. Chem. 243: 3994, 1968). To date, we have incubated ovalbumin and conalbumin with purified glucosaminidase and measured the release of glucosamine. We have not as yet cultured embryos on the ovalbumin and conalbumin after enzyme treatment.

B. Receptors for nutrient protein uptake - affinity chromatography

The recent advances with affinity chromatography which have led to the demonstration of cell membrane receptors, have suggested an alternative approach to the problem of nutrient protein uptake. Following the procedure

of Cuatrecasas (Proc. Nat. Acad. Sci. U.S. 69: 1277, 1972) we have attempted to determine if there are cell membrane receptors for nutrient proteins in the cells of the extraembryonic yolk-sac membrane. We have only run one experiment to date but we were so pleased with the results that it is included in this report.

Working with cyanogen bromide activated -Sepharose 4B the derivative containing the nutrient protein, conalbumin, was prepared. Chick embryos were cultured on protein starvation medium (with the hope of keeping the receptors free) in the presence of ^{14}C -amino acids. A membrane fraction was isolated from the yolk-sac membrane and placed on the column. Most of the radioactivity passed through the column. After sufficient buffer was passed through the column to reduce the effluent to background, the ionic strength of the buffer was increased. Two small peaks of radioactivity appeared with the high ionic strength buffer. Although it may be premature, we are eager to compare the various other nutrient proteins with the hope of relating nutrient protein uptake with receptor concentration. In addition, we hope to use affinity chromatography to study possible relationships of receptors to serum protein distribution and to regional growth.