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PROTEINS IN GROWTH REGULATION DURING EARLY DEVELOPMENT

Comprehensive Three Year Report
for Period 1974 to 1977

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TABLE OF CONTENTS

PAGE

Research Accomplishments 1

I Introduction. 1

II Background 2

A. Introduction 2

B. The protein requirement 3

C. The response of embryo regions to nutrition 5

D. The rationale for examining yolk-sac function 9

E. The synthesis of serum proteins by the yolk-sac 10

III Research Accomplishments of the Last Three Years 11

A. Serum protein synthesis and protein nutrition 11

B. Serum protein synthesis and protease secretion 12

C. Serum protein synthesis and development 13

D. Serum protein synthesis and teratogenic agents 14

E. Serum protein synthesis and genetic abnormalities 16

F. Serum protein synthesis and yolk-sac cell cultures 19

G. Serum protein synthesis and cell-free systems 21

H. Response of chick embryos to protein nutrition: patterns of synthesized proteins 23

I. Response of rat embryos to serum proteins 24

J. Response of chick embryos without yolk-sacs to serum proteins 26

K. Response of isolated brains to serum proteins 27

L. Protein starvation 30

M. The two protein requirement 32

PAGE

Proposed Studies 33

I Introduction 33

II. The regulation of serum protein synthesis 33

A. Yolk-sac cell cultures 33

B. Cell-free synthesis of serum proteins 37

III The regulation of growth and development by serum proteins 41

A. The rat embryo 42

B. Chick embryos without yolk-sacs 45

C. Isolated brains 47

Significance 49

Literature Cited 53

Graduate Students and Post-doctorals 57

Bibliography (Bibliography of Principal Investigator and
Publications Associated with this Contract) 59

Federal Support 63

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RESEARCH ACCOMPLISHMENTS

I. Introduction.

To start, the original objective of this contract as stated in the title has been and will continue to be the determination of the role of protein nutrition in the regulation of growth and development of the early chick embryo. Progress over the last three years as well as the fact that both avian and mammalian embryos are being used suggests refinements as well as greater precision in our objective. We are attempting to determine the role of serum proteins (serum factors) in the regulation of embryonic growth and development and the role of nutrient proteins in the regulation of serum protein synthesis by the yolk-sac. Our present working hypothesis is that serum proteins (or factors) be they circulating in the embryo or present in the uterine environment represent signals that regulate (or modulate) developmental processes and that the synthesis of serum proteins is in turn regulated by the proteins of the nutrient medium.

Clearly, we are presently involved with serum proteins, a subject of intensive and massive interest as attested to by the overwhelming literature on the subject. Without attempting to be exhaustive one finds interest in such areas as the physical and chemical identification and isolation of serum components, changes in serum with pathology as possible regulatory factors or simply used for diagnostic purposes, serum factors in cell and embryo culture, the metabolism of serum components by the body, and finally the use of liver and yolk-sac serum protein synthesis as a model for studies of the regulation of protein synthesis. Thus, it would appear that we have succumbed to the call from a giant band wagon. This is not the case. I feel that our research has led us in this direction and hope that the reader will find the following in support of this contention.

(Note, the word "modulation" in place of "regulation" appears to have

entered the jargon of the trade in dealing with substances that act on cells but not necessarily directly on DNA. For the purpose of the following discussion, I will use the terms interchangeably as a reflection of the fact that at present we cannot make the distinction.)

II. Background

A. Introduction

During the past three years, one observation was of particular significance to our present research objective. The relative synthesis of the individual serum proteins by the yolk-sac of the cultured chick embryo was susceptible to modulation by the protein composition of the nutrient medium. Although it is difficult to precisely define "background" studies from those "accomplishments of the last three years", I will use this single observation for that distinction.

Starting in 1960, we have worked extensively with cultures of intact chick embryos. The technique first developed by Waddington in 1932 (1), has been used by such researchers as Spratt (2) and Herrmann (3) in the U.S.A., and New (4) in England. Our own modifications have involved the use of embryos selected for 11 to 13 pairs of somites (approximately 40 hours of preincubation), yolk-sacs trimmed just peripheral to the sinus terminalis, culture with yolk-sac endoderm in direct contact with the nutrient medium and the use of precisely controlled levels of oxygen (5). It should be clearly noted that the cultured chick embryo system has consisted of the embryo proper and the extraembryonic yolk-sac with both area pellicida and area opaca. Under our conditions with a nutrient medium of fresh egg yolk and white, embryos survived for periods up to three days during which time extensive morphological changes occurred and the accumulations for protein, RNA, and DNA by the embryo were approximately 20 fold. Our justifications for use of this system have been the selection of large numbers of uniform embryos

for experiments (in one morning we can set up 100-150 cultures) and the dependence of the embryo on the nutrient medium for growth and development. For these reasons the cultured chick embryo has proveded a unique system in which to study developmental nutrition as well as the more general topic of environmental affects on embryos (6).

Because our early work has been reviewed repeatedly, I will attempt to be brief in the following discussion of background studies. The reader is referred to an extensive review (9) of both this research and related studies from other laboratories.

B. The protein requirement

Our first studies with chick embryo cultures involved attempts to grow them on a protein free chemically defined medium (7). Although we were able to demonstrate reduced embryo growth when single essential amino acids were omitted, growth of embryos on the complete defined medium was considerably less than on whole egg medium consisting of egg yolk and white. Thus, after 48 hours of culture the mean protein nitrogen contents were 15.9 μg/embryo for chemically defined and 53.3 μg/embryo on whole egg. Various attempts to improve embryo growth on defined medium were unsuccessful. For example, increased levels of amino acids were toxic. In another study we found that diluting the whole egg medium to a nitrogen level comparable to the defined medium led to similar growth on the two media (8). These observations together with several reports in the literature (see 9 for review) led us to the conclusion that the early chick embryo required proteins in the nutrient medium. Although this conclusion has yet to be disproved, a second conclusion made at this time was subsequently found to be inaccurate. We believed that proteins simply provided a concentrated source of amino acids in a tolerable form and that egg proteins could be easily replaced by other proteins.

The growth (protein and DNA accumulation) of cultured chick embryos on a variety of proteins and sources of protein as well as a variety of non-protein

supplements was compared (8). The list of substances tested included whole egg, yolk alone, white alone, ovalbumin, casein, bovine serum albumin, sub-blastodermal fluid, bovine colostrum, chick embryo extract and serum from horse, hen, rooster and rabbit. A clear distinction could be drawn between the superior growth of embryos on media containing egg proteins (yolk, white, ovalbumin) and the poor growth on non-egg proteins. It was of interest that two very similar proteins, ovalbumin and bovine serum albumin showed a striking difference in their ability to support embryo growth (bovine serum albumin failed to support the slightest growth even after purification of the commercially available product). The results with serum should be stressed because of the importance placed on serum in this proposal. Regardless of the source, serum failed to support embryo growth. However, the accumulation of protein by the yolk-sacs of serum cultured embryos was high relative to the other materials tested. Furthermore, the growth of embryos was greatly improved when serum was combined with ovalbumin over that achieved with either alone.

By attempting to grow embryos on highly purified ovalbumin and highly purified yolk proteins we learned that no single protein would support the growth of the early chick embryo (10). A mixture of two proteins was found to be essential. A transferrin was required and either conalbumin or yolk transferrin could be used. The transferrin requirement could not be replaced by iron and the removal of iron from transferrin (conalbumin) was not detrimental. Either ovalbumin or lipovitellin, two rather dissimilar proteins could serve as the second protein. It should be noted that with either transferrin, ovalbumin, or lipovitellin alone embryo growth was equally poor. However, like whole serum, transferrin alone supported high levels of protein accumulation by the yolk-sac. This latter observation together with the complimentation between ovalbumin and serum previously mentioned suggested that the active component in serum was transferrin. (The recent importance being placed on transferrin as

a cell culture medium component should be noted, 11, 12, 13). Some insights into the basis for the two protein requirements and particularly the transferrin requirement will be considered in the section on Research Accomplishments During the Last Three Years.

The ability to culture embryos on purified ovalbumin (in combination with a small amount of conalbumin) as the primary source of amino acid nitrogen, permitted us to carry out a series of quantitative experiments on protein utilization (14). Radioactive ovalbumin was isolated from eggs following the injection of ^{14}C-amino acids into a hen and rabbit anti-ovalbumin serum was prepared (15). We followed the path taken by intact ovalbumin, sites of ovalbumin breakdown (trichloroacetic acid soluble ^{14}C), and the synthesis of embryo proteins from ovalbumin. To summarize a rather involved series of experiments, we found that essentially all of the ovalbumin was taken up by the yolk-sac and degraded to amino acids within the area opaca of the yolk-sac. Only trace amounts of ovalbumin reached the embryo proper as intact molecules.

C. The response of embryo regions to nutrition

In working out the initial problems of early chick embryo nutrition we simply separated the embryo proper from the yolk-sac and determined growth in terms of total DNA, RNA and protein content. We also examined embryos morphologically and did some histological sectioning. Because some of our nutrition work as well as our teratological studies (for example, actinomycin D, see 16 and 17) showed differences between regions in responsiveness, we turned to embryo dissections (brain, heart, somites, neural tube, limb buds, area opaca, area pellucida) prior to growth analysis. In addition, we sought meaningful biochemical markers to measure with the hope of gaining some insights into the developmental state of these early embryonic tissues with changes in nutrition.

As previously noted, serum and transferrins (conalbumin) supported high levels of protein accumulation by the yolk-sac but the embryo proper did not grow appreciably. Ovalbumin did the opposite, supporting the embryo but not the yolk-sac. This initially observed difference between embryo and yolk-sac in response to nutrition was followed by analysis of regions of the embryo proper. Based on morphology, we could predict that the trunk regions and the brains were responding differently to ovalbumin and serum and determinations of DNA, RNA and protein on isolated brains and trunks were in support of the morphology (9). Ovalbumin supported high levels of DNA, RNA and protein accumulation by the brains relative to trunks while with serum the trunks were large relative to brains. (In the section on Research Accomplishments During the Past Three Years this subject will be continued with reference to the patterns of proteins synthesized in embryo regions.)

Because of the need for a control medium in our studies of protein nutrition, we formulated two conditions by which it was possible to maintain embryos but starve them of protein. In one condition embryos were cultured on Dextran that had been allowed to equilibrate across a dialysis membrane with whole egg (egg yolk-plus white). In the second, embryos were simply cultured on dialysis membranes which in turn were placed on whole egg medium. Comparable results have been obtained with both conditions. The protein starved embryos not only served as a nutrition control in these early studies, but they have been used repeatedly in more recent studies to compare with other conditions of nutrition. Furthermore, the protein starved embryos have been of interest in their own right, because in these embryos regional growth differences were lost. For example, when embryos were cultured on whole egg growth medium the accumulation of DNA, RNA and protein in the brain exceeded that of the heart but in the starved the accumulation of these macromolecules was comparable for brain and heart. Thus, we have used the protein starved embryo to gain insights into the regulation of growth and particularly regional growth by nutrient proteins.

When embryos were cultured on protein starvation medium after being exposed to radioactive amino acids, the greatest loss of radioactivity from protein occurred in the brain while the least was lost from the heart (18). The loss of radioactivity from RNA (uridine labeling) followed protein. Of the total radioactivity lost from the brain, 40 to 45% of the protein label and 60% to 70% of the RNA label were derived from the ribosome fraction (19).

Although these observations suggested that the loss of regional differences in growth in the starved embryo was the result of increased breakdown, we were concerned that a differential effect on synthesis could be involved. For example, both the sensitive brain and insensitive heart could be degrading protein and RNA to the same extent but the ability to reutilize (synthesis) the breakdown products could differ. Thus, we started to examine the effect of starvation on synthesis. Our first attempt simply involved incorporation kinetics of amino acids into protein with intact embryos and showed that starvation did not selectively inhibit synthesis in starvation sensitive regions (18). In these initial studies incorporations into embryo protein were corrected only for precursor uptake and not precursor specific activity. To make this correction, we turned to the elegant technique of Regier and Kafatos (20) and attempted to determine the specific activity of the amino acid precursor attached to transfer RNA. Ultimately, we found this approach difficult to apply to our system and turned to working with isolated polysome in cell free protein synthesizing systems. In addition, for the most part we limited ourselves to comparisons between the brain and heart because these two organs (or regions) showed the greatest difference in growth and sensitivity to starvation.

Our studies with isolated polysomes and related experiments were completed approximately 2 to 3 years ago but were just recently submitted for publication to Biochimica Biophysica Acta. (A preprint is enclosed with this proposal.) The delay was due in part to our hope of obtaining additional information which could

be added to the paper and a desire to repeat some of the studies. The following is a brief summary.

Polysomes isolated from the brains of cultured chick embryos were found to be more active (on a polysomal RNA basis) than those from the heart (21). This was true for growing as well as protein starved embryos. However, starvation reduced polysome activity for both brain and heart but reduced then in proportion to their relative activities in the growing embryo. Thus, in confirmation with our initial studies (18) protein starvation did not selectively inhibit synthesis in the sensitive region (brain). This also supported our contention that the loss of regional growth differences in the starved embryo was the result of increased degradation. In addition to the question of starvation, these studies on polysomes provided to this authors knowledge the first molecular basis for regional growth differences in so far as the brain polysomes were more active than those isolated from the heart.

In addition to the polysome protein synthesis kinetics we also examined polysome profiles, polysome stability, ribosome associated ribonuclease, and the sensitivity of ribosomes to degradation (21) (see attached preprint). The percentage of ribosomes as polysomes (greater than dimers) differed by only 1% between brains and hearts for both growing and protein starved embryos but there were 6% fewer polysomes in the starved as compared to the growing regions. Thus, a difference in polysomes may have contributed to the lower activity of starved polysomes but not for the brain-heart difference. Polysome stability was tested by incubating polysomes for 60 minutes at 37.5°C. The starved hearts showed that lowest stability and also the lowest synthetic activity but the polysome stability of the other regions (fed heart, starved brain, fed brain) did not follow synthetic activity. Ribosomal ribonuclease (^{14}C-polyuridylic acid was used as the substrate) activity followed polysome stability but in so far as polysome stability did not follow the kinetics of synthesis these results did not appear pertinent.

Because, so much of the radioactivity lost from the RNA and protein of starved embryos was derived from the ribosome fraction (19), we next studied the sensitivity of ribosomes to degradation. Ribosomes made radioactive by culturing embryos with either ^{14}C-uridine or ^{14}C-valine were incubated with trypsin, ribonuclease or without added enzyme (autodegradation). The results with trypsin and ribonuclease were inconsistent with regional differences in sensitivity to starvation. However, under conditions of autodegradation brain ribosomes were generally more readily degraded than those from heart. An exception was noted when EDTA was used with fed embryo ribosomes as the heart ribosomes were slightly more readily degraded than those of brain. (For details the reader is again referred to the attached preprint).

Approximately three years ago we had started work on the patterns of proteins synthesized in embryo regions. Although most of this work should therefore appear in the Accomplishments of The Last Three Years section, I will mention these studies here for continuity purposes. In brief, embryos that had been cultured for 48 hours on media containing various proteins (as well as protein starvation) were incubated in radioactive amino acids (either ^{14}C or ^{3}H); dissected into brain, heart, somites and neural tube; homogenized and centrifuged; and finally soluble supernatant fractions were run on acrylamide gels. By employing ^{3}H and ^{14}C, it was possible to directly compare soluble supernatants in single gels. The major finding was that protein nutrition did indeed alter the patterns of proteins synthesized in embryo regions. These changes in patterns were not simply related to embryo growth and all changes of nutrition did not result in a different pattern (a preprint of a manuscript in preparation is included with this proposal).

D. The rationale for examining yolk-sac function

Although it may not have been apparent in reading the previous sections, our studies forced us into a certain dilemma. On the one hand we had observed that protein nutrition affected regional growth in the embryo proper, polysome activity in hearts and brains, and the patterns of proteins synthesized in embryo regions.

On the other hand the studies with radioactive ovalbumin indicated that nutrient proteins (assuming that ovalbumin served as a model for all proteins) did not even reach the embryo proper but were degraded in the yolk-sac. It was natural therefore for us to ask "what was going on" in the yolk-sac which could mediate the responses of the embryo to protein nutrition.

E. The synthesis of serum proteins by the yolk-sac.

(It should be noted that similar to the polysome-starvation and patterns of embryo protein synthesis studies, the work on serum protein synthesis was initiated some three years ago but a large portion of the work was done during the past three years. A major paper on serum protein synthesis appeared last year in Developmental Biology and a reprint is included with this proposal (22)).

In one of our first studies on yolk-sac function, we simply incubated with a rocking motion yolk-sac isolated from 11-13 somite stage in buffered Ringer's containing ^{14}C-valine. After several hours we collected the Ringer's solution and, after dialysis and concentration, we ran the material on polyacrylamide gels. Much to our amazement five rather distinct proteins could be detected. Although the synthesis of serum proteins by much older yolk-sacs had been suggested through immunoelectrophoretic analysis (2), we didn't expect such large quantities of such a limited number of proteins to be secreted. That these proteins were indeed serum proteins was verified by coelectrophoresis of the Ringer's incubation medium with serum isolated by micro-heartpuncture from early chick embryos. The proteins were mainly identified by electrophoretic mobilities (molecular weights) and were transferrin, alpha globulin-a (probably the so called alpha fetoprotein), alpha globulin-b, serum albumin and prealbumin. One additional point of methodology, each protein was isolated from acrylamide gels and their valine contents were determined on an amino acid analyzer. This made it possible to correct radioactive valine incorporation so that the "relative" synthesis of each protein could be determined. Referring the reader to the preprint included with this proposal for details (22) some of the

important findings were: (A) serum proteins were synthesized exclusively by the yolk-sac in the early chick embryo, (B) serum proteins were found to be synthesized as early as the primitive streak stage of development, and (C) the relative synthesis of several serum proteins by the yolk-sac was altered (modulated) by the protein composition of the medium used in chick embryo cultures. This latter point was crucial in directing our interests to the possible pivotal position of serum proteins in mediating the response of embryos to the proteins in the nutrient medium.

III. Research Accomplishments of the Last Three Years

A. Serum protein synthesis and protein nutrition

As previously noted, we found that isolated chick embryo yolk-sacs would synthesize and secrete serum proteins when incubated in buffered chick Ringer's (22). Our experiments showed that in the early embryo the yolk-sac was the exclusive site of serum protein synthesis and as early as the primitive streak stage (prior to the formation of a heart or circulatory system) these proteins were being synthesized. The relative synthesis of serum proteins changed with development but of greater significance to our hypothesis their relative synthesis changed with nutrition. I would like to elaborate briefly on this last point.

Chick embryos of 11-13 pairs of somites were cultured for 48 hours on various media including protein starvation, ovalbumin-conalbumin, yolk alone, and whole egg (yolk plus white). At the end of this period the isolated yolk-sacs were incubated for three hours in buffered chick Ringer's containing radioactive valine. The Ringer's was separated from the yolk-sacs, dialyzed, concentrated, and run on polyacrylamide gels. The electrophoretic gels were sliced and the amount of radioactivity associated with each serum protein was determined. The radioactivity was corrected for the valine content of each protein so that the relative synthesis could be calculated.

Each culture condition presented a unique and reproducible pattern of serum

protein synthesis. For example, the relative synthesis of serum transferrin ranged from a high of 37.6% on ovalbumin-conalbumin to a low of 22.7% on whole egg. Serum albumin synthesis had a low of 5.6% on whole egg and a high of 12.5% on yolk alone while the range for prealbumin was 24.9% on yolk alone to 35.6% on protein starvation. Unlike the other serum proteins, alpha globulin-a which we believe to be the well studied alpha fetoprotein, related directly with the ability of a particular culture condition to support embryo growth. Thus, the lowest embryo growth was on protein starvation with an alpha globulin-a level of 14.9% followed in increasing growth by ovalbumin-conalbumin, 19.7%; yolk alone, 22.2%; and finally whole egg, 26.0%. The function of alpha fetoprotein has not been established. Naturally, we have felt that this observation was of significance to this important question.

B. Serum protein synthesis and protease secretion

We have been concerned over the possibility that proteases contained in the yolk-sac could be released into the Ringer's incubation medium along with the serum protein. Conceivably the proteases could selectively degrade the serum proteins and ultimately obscure the actual pattern of serum proteins synthesized and secreted. I don't wish to suggest that the following respresents a "major accomplishment" but rather an important factor that was considered with particular reference to the method we have used to study serum protein synthesis. Serum proteins were made radioactive by incubating yolk-sacs from four-day chick embryos in ^{14}C-valine containing buffered chick Ringer's. The Ringer's containing radioactive serum proteins was dialyzed and aliquots containing approximately 10,000 cpm were incubated with intact four day yolk-sacs. Samples were taken at time periods up to five hours and the radioactivities in trichloroacetic acid soluble and precipitable materials were determined. For the first three hours there was essentially no loss of radioactivity from the precipitable nor did the soluble increase in radioactivity. After three hours, degradation could be detected and an

acrylamide gel pattern from the five hour time period indicated the following sequence from the most to the least degraded; alpha globulin-a, transferrin, prealbumin, alpha globulin-b, and serum albumin. It should be noted that in our previous studies on serum protein synthesis the incubating period was limited, fortunately, to three hours. (Actually three hours was selected because the synthesis and secretion of radioactive serum proteins was linear for this period.)

C. Serum protein synthesis and development

Ontogenetic changes in serum proteins have been studied in many organisms and the chick has not been an exception (see 24 for a brief review). However, the techniques previously used have been qualitative and limited to relatively late stages of development (23, 24, 25). Thus, we felt that the quantitative nature of our appraoch justified a developmental study. Furthermore, our developmental study has provided results which were not in complete agreement with published results. For example, we found both the liver and yolk-sac synthesized albumin at seven days of incubation while it has been reported that the synthesis of this protein was restricted to the yolk-sac at this stage (26). Our approach was to incubate, for three hours, livers and yolk-sacs from embryos between 5 and 19 days of incubation in radioactive valine and analyze the incubation fluid for serum proteins by acrylamide gel electrophoresis (gels were sliced, radioactivity determined for each protein, and corrected for protein valine content). In addition, blood samples were analyzed in an attempt to relate the liver and yolk-sac contribution to the circulating levels of serum proteins (in this case we could not use radioactivity but rather gels were scanned and the areas under the peaks were calculated).

The following were our major findings. Both yolk-sac and liver synthesized transferrin, alpha globulin-b, albumin and prealbumin. The synthesis of alpha globulin-a (alpha fetoprotein), in agreement with Gitlin and Kitzes (23), was restricted to the yolk-sac. It was of interest that the synthesis of this protein

reached a peak on day 7 of incubation (as measured with ^{14}C-valine and isolated yolk-sacs) while the circulating level peaked on day 12. The synthesis of alpha globulin-b stopped after day 11 in both liver and yolk-sac. The electrophoretic mobility of liver and yolk-sac transferrin did not coincide suggesting possible differences in the carbohydrate moiety (see 27 for carbohydrate differences between conalbumin and transferrin). At all stages (days 5 to 19) the relative synthesis of transferrin was greater in liver than yolk-sac and while the synthesis declined in both organs during development the circulating levels remained constant. During development the synthesis of albumin increased for both liver and yolk-sac while the synthesis of prealbumin decreased in both. These changes were dramatic. For example, with the liver at day 5, albumin was 14.5% and prealbumin 23.8% while at day 19 liver albumin synthesis increased to 68.2% and prealbumin dropped to 6.2%. These changes in synthesis were reflected in the circulating levels of these two proteins. (A manuscript on these developmental changes is presently being prepared. A paper on part of this material was presented at the Cell Biology meeting in 1975 (28)).

D. Serum protein synthesis and teratogenic agents

For several reasons we have been examining the effects of various teratogenic agents on serum protein synthesis. First, one might expect serum protein synthesis to be influenced because many agents which ultimately harm the embryo must pass through the yolk-sac. Second, in a variety of pathological conditions, including both developmental abnormalities and cancer, abnormal patterns of serum proteins have been demonstrated. Finally, we have been and continue to be concerned with the application of our rather "basic" research to applied problems. With these factors in mind we have started to test the effects of a variety of chemicals and environmental factors on serum protein synthesis. Our objectives have been to provide a developmental test system for

possible screening and hopefully to be able to relate a particular change in serum protein synthesis to a specific abnormality (and ultimately to demonstrate a causal relationship). With these lofty objectives, I am sorry to say that, as yet, we have not been terribly successful. Our first approach was to contact the National Cancer Institute and to test several compounds with anti-cancer activity which were of particular interest to the N.C.I.. The reasoning for this was based on some suggestions in the literature that the regression of certain cancers by chemotherapy was related to a reduction in the circulatory levels of embryo specific serum proteins (for example, see 29).

In our first experimental approach, chick embryos of 11 to 13 somites were cultured on whole egg growth medium containing various levels of drug to establish a toxicity-teratogenicity curve. Selecting a level that would allow at least 50% survival after 48 hours of culture, yolk-sacs were isolated from drug treated embryos and incubated for 3 hours in buffered chick Ringer's containing radioactive valine. Following our standard procedures (22), acrylamide gel electrophoretic of the Ringer's provided estimates of relative synthesis for the serum proteins. To date, we have worked with two compounds which cause degeneration of the posterior trunk region of the embryo, daunomycin and adriamycin (abnormalities like actinomycin D, see 16), and two compounds which cause general embryo growth retardation, cyclophosphamide and dibromomannitol. We have observed differences between the relative synthesis of serum proteins with these various treatments. And these differences have been reasonably reproducible. The major difficulty with the data were the magnitudes of the differences which were small. For example, the relative synthesis of transferrin was: control, 23%; adriamycin, 18%; daunomycin, 19%; cyclophosphamide, 21%; and dibromomannitol, 22%. The largest difference observed was for prealbumin which ranged from a low of 29% for control to a high of 39% for cyclophosphamide. The higher synthesis of prealbumin for

cyclophosphamide was reflected in a reduced synthesis in the alpha globulins (control, 43% and cyclophosphamide, 34%). Again, these differences between control and cyclophosphamide were the largest differences that we have observed. (The reader is referred to the Progress Report 1976-1977 included with this report for a complete table of these data.)

In the second experimental approach, yolk-sacs of 11 to 13 somite chick embryos were rinsed and then placed into buffered Ringer's containing not only radioactive valine but the compound under consideration. Again a dosage curve was run during three hour incubation periods and acrylamide gels were used to estimate relative serum protein synthesis. Using this method we have tested adriamycin, cyclophosphamide, and actinomycin D. Similar to the first experimental appraoch, we were more impressed by the constancy of the relative serum protein synthesis than by the differences. (Again, see Progress Report 1976-1977 for the complete data.)

Clearly, we have not tested a large number of compounds. Nevertheless, in comparison to the large changes in relative serum protein synthesis observed with altered protein nutrition, the small changes observed with the various drugs becomes striking. After our nutrition studies we became concerned that the synthesis of serum proteins could be altered by any variety of treatments. The drug study, if nothing else, has lessened this concern.

It was recently learned that at least one other laboratory has been doing work on drugs and serum protein synthesis in the chick embryo. Dr. E.M. Weller working with 12 day chick embryos has injected puromycin and cycloheximide into the circulatory system (30). Employing immunoelectropherograms he reported that puromycin reduced the concentration of all serum proteins but cycloheximide specifically reduced the concentration of albumin.

E. Serum protein synthesis and genetic abnormalities

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largely through the extensive work of Dr. Walter Landauer, has been noted for studies on the genetics of chickens and for maintaining flocks with various mutations. One of the most studied has been the Creeper gene which was recognized in 1926 to be autosomal and dominant (31). In the homozygous state it was found to be lethal with most of the embryos dying during the fourth day while a few survived until hatching. Studies of the heterozygous Creeper have suggested a defect associated with cartilage and ossification of the long bones. Examination of homozygous embryos at three or four days of incubation, however, showed retardation of embryo growth and development and poor development of the yolk-sac. Blood islands could be recognized in the area opaca of the yolk-sac, but a functional circulatory system was not formed (32). Thus, it appeared reasonable to expect that the synthesis of serum proteins by the yolk-sac of the homozygous Creeper could be defective. Our interest was encouraged by the recent observation of a deficiency in plasma protein synthesis associated with the X-ray induced lethal mutation at the albino locus in the mouse (33).

Following our standard procedures of yolk-sac incubation in radioactive valine and acrylamide gel analysis (22), the relative synthesis of serum proteins by control and homozygous Creeper were compared. At three days of incubation except for slightly elevated synthesis of prealbumin and slight reductions in alpha globulin-a and transferrin in the Creeper, the relative synthesis of the serum proteins in the Creeper and control were quite similar (see Progress Report 1976-1977 included with this report for the complete data). By incubating large numbers of eggs, we were able to isolate yolk-sacs from homozygous embryos after seven days of incubation. The difference between Creeper and control was even less at seven than was observed on day three. Next, we ran some double labeled (^{3}H and ^{14}C valine) acrylamide gels with sodium dodecylsulfate of serum proteins with the hope of increasing the resolution and finding some difference between

Creeper and control. (In this double label procedure the Creeper and control serum samples could be run on a single gel because one was prepared by incubating yolk-sacs with ^{14}C and the other with ^{3}H.) Once again, only minor differences were detected.

Realizing the difficulty of attempting quantitative studies on serum protein synthesis, we followed with time the secretion of radioactive proteins by control and homozygous Creeper yolk-sacs at day three of incubation. The Creeper secreted radioactive proteins at a much greater rate than controls. We suspected that this was the result of a much greater pool of free valine in control than in Creeper. Checking this out with ninhydrin determinations showed on the basis of DNA that the pool of ninhydrin positive material (trichloroacetic acid soluble) was actually higher per cell in the Creeper. Now we suspect that the control may secrete more free valine into the incubation medium than Creeper which could be the reason for the lower incorporation kinetics by the control relative to the Creeper.

Frustrations with attempting quantitative studies of protein synthesis in an intact tissue have led us to examine polysome profiles and the Creeper contained 12% fewer polysomes (greater than dimers) than the control. Soon we will determine the protein synthesis kinetics of these polysomes and eventually compare their protein products.

Although I do not wish to be repetitious, I would like to point out to the reader that similar to the drugs our attempt to find an alteration in serum protein synthesis with a mutation was largely negative. True, only a single mutation has been studied but once again the modulation of serum protein synthesis to protein nutrition and lack of responsiveness to this mutation was striking.

F. Serum protein synthesis and yolk-sac cell cultures

The previous sections of Research Accomplishments were concerned for the most part with conditions effecting serum protein synthesis. In this section and in the next our progress in working on systems to study the regulation of serum protein synthesis will be considered.

Establishing cells in culture which produce specific gene products in the form of proteins offers certain advantages over the intact tissue or organism studies of regulatory mechanisms. One has the potential of working with a single cell type under carefully controlled conditions which can be maintained and readily duplicated. In addition one can be certain with cell cultures that any substance added to the culture medium acts directly on the cells rather than through some other cell type or organ. On the other hand, studies with cell cultures may lack the reality of an intact organism or organ and thus represent a questionable model. With all of this well in mind, we have initiated work on the regulation of serum protein synthesis by cells of the yolk-sac. It should be noted that we are not the first to study yolk-sac cell cultures (34). Furthermore, the synthesis of serum proteins by chick embryo liver cell cultures has been reported (35). However, to this author's knowledge we may be the first to study serum protien synthesis in yolk-sac cell culture.

We started this work with cell cultures derived from the total yolk-sac and at present are attempting to set up cell cultures of the specific cell types that constitute the yolk-sac; endoderm, splanchnic mesoderm, and somatopleure (ectoderm plus mesoderm). I will discuss our results to date with the total yolk-sac in this section and the cell type isolation under "Proposed Studies".

Although Moscovici and Moscovici (34) worked with twelve to fourteen day chick embryo yolk-sacs their procedure was used with four day yolk-sacs. Yolk-sacs were cut into small pieces and dissociated with 0.25% trypsin. After brief centrifugation the cells were placed into plastic culture dishes and after approximately eight hours,

fibroblast type cells settled out and the cells in suspension were transferred to a fresh dish. The culture medium consisted of 80% minimal essentials (salts, amino acids, vitamins, etc.), 10% tryptose phosphate broth, 5% fetal calf serum and 5% adult chicken serum.

In our first experiment cells were cultured for 24 hours in the presence of radioactive valine and the secretion of trichloroacetic acid precipitable material into the medium was followed. An increase was observed with time to 20 hours and a sample at 24 hours was analyzed by acrylamide gel electrophoresis. Although several peaks of radioactivity appeared on the gel, the large total amount of protein in the medium from the serum component relative to the small amount of secreted radioactive protein made gel analysis difficult. We therefore turned to immunological procedures and made rabbit antibody against 8 day chick embryo serum proteins. The antibody was found to react against both chick embryo and adult chick serum but not against fetal calf serum. A second time course study with radioactive valine showed that at 20 hours 34% of the radioactivity secreted by yolk-sac cells in culture could be precipitated by the antiserum. This strongly suggested that yolk-sac cells were indeed synthesizing and secreting serum proteins but when the antibody-antigen precipitate was analyzed by acrylamide gels, radioactive serum proteins were not detected. Although the procedure worked with radioactive serum proteins derived from intact yolk-sacs, it appeared that the total amount of serum proteins secreted by the cells was too small for the total protein (antibody plus carrier) placed on the acrylamide gel. Seeking a still more sensitive technique, we recently turned to the crossed immunoelectrophoresis technique of Clarke and Freeman as described by Grieninger and Granick (35) in their studies of serum protein synthesis by chick embryo liver cell cultures. (This procedure provides a highly sensitive quantitative estimation of specific proteins combining the specificity of immunology with electrophoretic separation.) Again yolk-sac cells were cultured in the presence of radioactive valine and the medium

was used for crossed immunoelectrophoresis analysis employing the antibody we had prepared previously against chick embryo serum proteins. Staining the gel showed the presence of several serum proteins in the cell culture medium but the autoradiogram of the gel was negative. We have not as yet attempted to repeat this experiment. We could not determine if the positive results with the staining was the result of serum protein contamination or the negative autoradiogram was the result of insufficient exposure time. At this point we turned to separating the cell types of the yolk-sac so that the synthesis of serum proteins by a single cell type could be studied. This will be discussed under "Proposed Studies".

G. Serum Protein Synthesis and Cell-Free Systems

Protein nutrition and developmental stages of the embryos have been shown to influence the relative synthesis of serum proteins by the yolk-sac of the chick embryo. As an initial approach to the question of regulatory mechanisms. we have been attempting to study serum protein synthesis by isolated yolk-sac polysomes. We would like to know if the changes we find with the intact yolk-sac would be reflected in the relative synthesis of serum proteins by isolated polysomes. If the intact yolk-sac and polysomes followed similar patterns with changing nutrition and development, it would suggest that regulation was at the level of "functional" messenger RNA availability (i.e. transcription). If the polysomes and intact yolk-sac were not in agreement, one would look for regulation at the levels of either translation or post-translation.

Working for the most part with yolk-sacs from four day chick embryos, we have been perfecting a system for the cell-free synthesis of serum proteins. We have tried a variety of approaches and have observed the synthesis of some of the serum proteins (36). The basic reaction mixture was similar to that used in our work with starvation on brains and hearts (21, see attached preprint). Yolk-sac polysomes isolated after passage through 1.46 M sucrose were combined with a 200,000 x g enzyme fraction from four day chick embryos (without yolk-sacs), ATP, GTP, creatine

phosphate, creatine kinase, and radioactive amino acids. (See Progress Report 1976-1977, included with this report for details of reaction kinetics). Following incubation, the polysomes were separated by centrifugation and the proteins in the supernatant were analyzed by sodium dodecyl sulfate acrylamide gel electrophoresis (gels were sliced and radioactivity in each slice determined). We have done this type of study several times and although we feel rather certain that several of the serum proteins were synthesized, the major products were the low molecular weight serum proteins (particularly prealbumin at a M.W. of 23,000).

At present, we have stepped back and reexamined the problem. First, we have found that the relatively large amount of yolk fat associated with the yolk-sac may have created a unique problem. We have found that the amount of deoxycholate used to free the polysomes was critical and only by careful titration of this detergent could we produce excellent polysome profiles (see Progress Report 1976-1977 for the profile). Second, we found through orcinol RNA determinations that a large amount of RNA (approximately 45% of the total RNA) was being discarded with a floating fat fraction that we produced in the initial centrifugation after homogenization. During the last several weeks we have been working on the recovery of this RNA and have felt that a large proportion of the polysomes coding for the higher molecular weight serum proteins were located in this floating fat fraction. In the next couple of weeks we should have the answer to this question. We feel confident that after the total recovery of yolk-sac polysomes we will be able to evaluate the crucial transcription-translation question.

Two additional points should be noted. First, at present we are working on procedures to separate the yolk-sac cell types. We will determine just which type is actually synthesizing the serum proteins. Clearly, there would be considerable advantage to study the polysomes from just those cells involved in serum protein synthesis. Second, an additional procedure to study the transcription-translation question will be proposed. Procedures are available to isolate polyadenylated messenger RNA molecules employing so-called oligo dT cellulose columns. Messenger RNA can be isolated and the

messenger RNA can be combined with highly effecient protein synthesizing system such as the one derived from wheat germ. Thus, one can determine if cellular messenger RNA's for a particular set of proteins corresponds to the actual cellular synthesis of this set of proteins.

H. Response of Chick Embryos to Protein Nutrition: Patterns of Synthesized Proteins.

During the course of the last three years a study was completed on the effects of nutrition on the patterns of proteins synthesized in regions of the cultured chick embryo. (A Manuscript in preparation is included with this report and this work provided the basis for a completed Ph.D. dissertation). The objective of this study was to determine if changes in protein nutrition could influence the patterns of proteins synthesized by the early embryo. We were careful to take into consideration the possibility that any differences that might be observed could be simply associated with differences in total embryo growth.

Our approach was to culture 11 to 13 somite chick embryos on various media for 45 hours and then expose the whole preparation to either ^{3}H or ^{14}C-valine in buffered chick Ringer's. Next, the embryos were dissected into brain, heart, somite and neural tube regions. Regions were homogenized, centrifuged, dialyzed and the soluble proteins were placed onto acrylamide gels (either with or without sodium dodecyl sulfate). The gels were sliced and the amounts of ^{14}C and/or ^{3}H determined. A computer program was developed for the analysis of the data. For example, when two samples were compared on a single gel (using ^{3}H and ^{14}C) the program provided a single number for the degree of difference between the two samples. The distribution of radioactivity was always plotted for specific differences but the estimation of overall difference proved to be of interest.

The most important finding was that changing protein nutrition did indeed alter the patterns of proteins synthesized. The brain was found to be the most responsive region of the embryo. Embryos cultured on yolk alone or a mixture of ovalbumin plus

conalbumin accumulated comparable amounts of total protein but the patterns were quite distinct. With yolk alone the pattern more closely resembled that observed with whole egg (yolk plus white) cultured embryos. Culture on protein starvation medium appeared to inhibit pattern development. Thus, the total gel difference between zero time (11 to 13 somites) and 45 hour starved was much smaller than that between zero time and 45 hour whole egg cultured embryos. Recalling that ovalbmin was previously found to enhance brain growth relative to the embryo trunk, it was interesting to find that zero time versus 45 hours on ovalbumin plus conalbumin provided a greater difference in pattern than zero time versus 45 hours on whole egg.

The possibility that some of these differences could have been the result of serum protein distribution differences was also considered. For example, during the three hours of exposure to radioactivity, serum proteins synthesized by the yolk-sac could have been transferred to the embryo. Serum proteins were identified in the soluble supernatants of embryo regions. Furthermore, differences in distribution were observed but correcting for the serum proteins did not alter our basic conclusions from this study (37).

I. Response of Rat Embryos to Serum Proteins.

From the start of Graduate School some twenty years ago, this principal investigator has worked in one way or another almost exclusively with the chick embryo. Thus, with the thrill and excitement that only accompanies much apprehension, we started to work last year with cultures of rat embryos. Actually, we started working with rat embryos in the hope that they would be responsive to serum proteins. To recall, chick embryos could not be cultured on serum and because we would like to determine if indeed serum proteins represent developmental regulatory signals we were forced to consider various other systems. New (38) some years ago demonstrated that rat embryos could be cultured on rat serum so we have turned to the rat and, I might add, with great success. In addition, two other systems are being considered for studies on developmental responses to serum proteins; chick embryos without yolk-sacs and isolated brains. They will be considered in the next two sections.

Following for the most part published procedures from the laboratory of D. A. T. New (39, 40, 41) we learned quite a bit of rat husbandry and how to culture rat embryos. We were intrigued by the observation that so called "true" serum supported superior embryo growth and improved heart development in comparison to regular serum (39, 42). True serum was prepared by immediate centrifugation of blood after removal from the dorsal aorta of a rat and has been referred to as I.C. or immediately centrifuged blood. Regular or standard serum was prepared by allowing blood to clot overnight before removal of the serum. Again, we were very interested in the fact that such a "small" modification in serum preparation could lead to some striking affects on embryo growth and development. A second point of interest was the observation that superior embryo growth could be achieved by either frequent changes of the serum medium (24 hour intervals) or reducing the number of embryos cultured per volume of serum (our observation as well as New (40)). The large amount of protein contained in the serum relative to embryo growth suggested that the problem was not simply depletion of protein. Rather, we considered that either a minor serum component was depleted or inhibitory substances rapidly accumulated in the medium.

Our experimental approach was quite simple but the results were of considerable interest. In fact, we have submitted a paper to Nature on this material. (A copy of this manuscript is included with this proposal (43).) Rat embryos of 10 days gestation (head fold) were cultured by the rolling tube method (see 43 for design of our culture tube) for 48 hours. The I.C. serum was not changed. This depleted I.C. serum was compared with I.C. serum rolled for 48 hours without embryos and D.C. (regular) serum on sodium dodecyl sulfate acrylamide gels. The major finding was that a particular "protein" band was essentially absent from the depleted I.C. serum and the staining intensity of this same band was reduced in D.C. serum relative to the unused I.C. serum. This

observation was repeated. Estimations of molecular weight placed this band at approximately 125,000. Although no "functional" serum protein has been identified at a M.W. of 125,000, a 126,000 M.W. alpha globulin has been identified as one of two major human serum DNA-binding proteins (44). Naturally, we have immediate plans to follow up our work on the depleted "protein" band. This will be discussed under Proposed Studies.

I would like to mention some additional work on rat embryos that we have initiated recently. We have developed a very simple procedure which allows us to obtain rather large amounts of embryo serum from 8-day chick embryos. We simply allow the embryos to "bleed out" and we find that the serum obtained in this manner to be essentaal free of non-serum proteins. This serum contains only transferrin, alpha globulin-a, alpha globulin-b, albumin and prealbumin. Employing various column chromatographic procedures we have been fractionating the embryo serum into the protein components. Recently, we added some "purified" alpha globulin-a (alpha fetoprotein) and the prealbumin (this is also an embryo specific protein) to I.C. serum for the culture of 10-day rat embryos. Both proteins appeared to stimulate the growth of rat embryos relative to that achieved on I.C. serum alone.

J. Response of chick embryos without yolk-sacs to serum proteins

As previously noted in this report, chick embryos could not be cultured on serum alone (8). Sera from a variety of animals were tested as well as a number of different concentrations and conditions. Serum enhanced embryo growth when combined with ovalbumin. We attributed this to the transferrin of serum because serum could be replaced by conalbumin (egg white transferrin) and both serum and conalbumin enhanced growth and development of the yolk-sac but not the embryo proper. We have felt that serum failed to support embryo growth either because of the serum proteolytic enzyme inhibitors or because most of the serum protein could not be taken up by the yolk-sac. Furthermore, if serum proteins were taken up by the

yolk-sac and degraded by yolk-sac proteases (like nutrient proteins) we would be unable to study their direct effect on the embryo proper. Embryo size has precluded any attempt to by-pass the yolk-sac by direct circulatory system perfusion with serum proteins so we have been culturing chick embryos without their yolk-sacs. We have tried a variety of culture conditions (Millipore filters, stainless steel mesh, solid medium, liquid medium, oxygen levels, embryos of various developmental stages, etc.) and have settled on the roller tube method used for our rat embryo culture. We have used the same culture tube that we designed for the rat and a rotation of 60 rpm with the apparatus we built for our rat work.

Although we have run a number of experiments, I would like to describe only one in some detail. Yolk-sacs were removed from embryos containing 8 to 10 pairs of somites. The embryos were placed in glass culture vessels containing various media, gassed with 100% oxygen and rotated at 60 rpm for 24 hours. The media consisted of various supplements added to a mixture of salts and vitamins developed for the chick embryo (45). These supplements were; a mixture of amino acids, ovalbumin, conalbumin, and a mixture of equal amounts of ovalbumin and conalbumin. One additional medium consisted of 8-day chick embryo serum. The embryo serum contained approximately 16 mg/ml protein and the protein supplements were used at a total of 20 mg/ml. The results showed that under these conditions the growth of the embryon on serum was more than double that achieved on the other media. It was particularly interesting that one medium that supported excellent embryo growth with an intact yolk-sac, ovalbumin plus conalbumin, was inadequate in the absence of a yolk-sac. On the other hand embryo serum which supported the highest growth in the absence of a yolk-sac would not support growth in the presence of a yolk-sac. We feel that this study supported our basic hypothesis that serum proteins are the essential mediators between the proteins of the medium and the embryo proper. The reader is referred to the Progress Report 1976-1977 for a pictorial presentation of these experimental results.

K. <u>Response of isolated brains to serum proteins</u>

The inability to culture intact chick embryos with yolk-sacs on serum

has led us to consider several different model systems. I have discussed our work with rat embryos and chick embryos without yolk-sacs. Cultures of isolated chick embryo brains have been still another system that we have been using for this purpose. I have decided to save this system for last because to some extent it has not been in line with our primary objective. Many have shown the importance of serum proteins in cell culture and many have been studying the mechansim by which serum proteins regulate cells in culture. Our rather unique objectives have been to demonstrate the importance of serum proteins in an intact embryo and to study how serum proteins may regulate the intact embryo. Thus, although the isolated brain has not been in line with our primary objective we started work with this system before the intact rat or chick embryo without yolk-sac. Furthermore, we have maintained an interest in the brain because our studies with protein starvation as well as those on regional patterns of protein synthesis with nutrition showed that the brain was the most responsive of the various embryo regions.

We have tried a variety of techniques to culture isolated brains and have found that placing the brains on Millipore filters which were in turn placed on semi-solid medium to be reasonably adequate in maintaining brain morphology, protein content and protein synthetic activity. For example, placing brains directly on the semi-solid medium without a Millipore filter resulted in a rapid loss of brain morphology.

A major question, regardless of which system, isolated brains or intact embryos, has been just what should be measured to evaluate the response to serum proteins. In the past with various studies we have done such work as morphology; accumulations of DNA, RNA, and protein; work with radioactive precursors for the synthesis and breakdown of DNA, RNA, and protein; isolated polyribosome activities; evaluations of some specific proteins and acrylamide gel analysis of general proteins synthesized. With the isolated brains we have been trying to perfect acrylamide gel analysis of general proeins synthesized.

Our experimental design has been to culture chick embryos (with yolk-sacs) on

whole egg growth medium following our standard procedures. After 24 hours of culture, brains were removed and cultured for a second 24 hours on Millipore filters which were in turn placed on whole egg medium. After the total of 48 hours they were removed from the filters and exposed to ^{3}H-valine for 3 hours in buffered chick Ringer's. These brains were then compared to brains which had spent the entire 48 hours with the embryo. These were removed from the embryo after 48 hours of culture and exposed to ^{14}C-valine for 3 hours. Extracts from both brains were then compared by electrophoresis on single acrylamide gels. We have compared various forms of acrylamide gels, sample sizes, etc. and have worked out a procedure with sodium dodecyl acrylamide gels which we feel have met our requirements (i.e., resolution and reproducibility). The comparison between brains cultured with and without their embryos showed a "total" difference that was double that observed for control gels. (The "total" difference was obtained by first expressing the amounts of ^{3}H and ^{14}C in each slice as a percentage of the total redioactivity on the gel. Then, the difference between ^{3}H and ^{14}C for each slice were summated.) The control gels were obtained from brains removed from embryos after 48 hours of culture and divided into ^{14}C-valine and ^{3}H-valine groups. In addition to the overall gel differences certain major peaks of radioactivity observed with the isolated brains were not present in the brains taken from the cultured embryos. (These patterns are presented in the Progress Report for 1976-1977 included with this report).

Another comparison of some interest was that between brains from embryos cultured for 48 hours on whole egg medium (starting with 11 to 13 pairs of somites) with brains from embryos allowed to remain within the egg for a comparable period of time. Again, after the brains were removed from the embryos they were either exposed to ^{3}H or ^{14}C-valine and their proteins were compared on single acrylamide gels. The "total" gel difference between brains from cultured and *in ovo* embryos was as small as that achieved with a control gel.

In the very near future we hope to culture isolated brains on chick embryo

serum proteins. We would naturally hope to find that the difference between isolated brains and those left with the embryo would be reduced when the isolated brains were cultured on embryo serum proteins.

L. Protein starvation.

Our previous studies on the protein starved embryo have been reviewed in the Background Section of this report. These previous studies showed that the brain was particularly sensitive to starvation in that breakdown of protein and RNA was greater in the brain than other regions (18) and that a large percentage of the RNA and protein lost was derived from the ribosome fraction (19). Studies with isolated polysomes showed that protein synthesis was reduced by starvation but both the brain and heart polysomes showed comparable reductions (21). During the last three years some additional experiments were done on the question of reduced polysome activity in the starved embryo as well as on some related problems.

Our studies with patterns of soluble proteins synthesized in embryo regions showed that a particular peak of radioactivity was greatly reduced in the starved relative to the growing embryos. A fractionation study showed that this peak (or material that co-migrated) was particularly concentrated in a high salt (1M KCl in ribosome suspension buffer) wash of ribosomes. Working with isolated polysomes, we could relate the amount of this particular protein directly to the protein synthetic activity of the polysomes. Some preliminary attempts to isolate the protein were made but frustrations over the relatively small amounts of tissue we have to work with and our desire to spend more time on the serum protein problem has led to a suspension of work on this problem for the present time.

Although the use of acrylamide gels to study the general patterns of proteins synthesized were of value we have been interested in studying the effects of starvation on a specific protein. The brain, at the stage we use, does not as yet contain brain specific markers. However, the brain of the chick embryo was reported to contain relatively large amounts of the microtubule protein, tubulin (46). We have looked at

tubulin in brains of cultured chick embryos using both the radioactive colchicine binding assay and the vinblastine precipitation-acrylamide gel separation assay. Both techniques gave comparable results (47). Starvation appeared to selectively inhibit the accumulation of tubulin by the brain. For example, with vinblastine precipitation, after 48 hours of culture on whole egg growth medium the brains contained 10.8 ug of tubulin per 100 ug of supernatant protein while this figure for 48 hours protein starved was 5.8 μg per 100 μg of supernatant protein. Similar to the "ribosome" protein we have not continued work on tubulin. However, we could well return to tubulin and to the "ribosome" protein, particulary in conjunction with our studies on the effects of serum proteins on embryos.

One final study on protein starvation that was completed during the past three years may be of interest. During protein starvation radioactivity was lost from the proteins of the brain but not the heart. We attempted to determine if this difference in response was the result of the relationship of these organs to the entire embryo or to the metabolism of the cells contained in the organs. Following precisely the experimental design used with intact embryos, chick embryos of 11 to 13 somites were exposed to ^{14}C-valine for 3 hours and then cultured for 6 hours to reduce the free ^{14}C-valine pool. The brains and hearts were then removed from the embryo, placed on Millipore filters and then onto protein starvation medium. After an additional 36 hours of culture both the brains and hearts on starvation medium maintained approximately 95% of their zero-time protein content. However, the hearts lost 51% of their protein radioactivity and the brains 31%. Thus, under these conditions the heart appeared at least as sensitive (or insensitive) to starvation as the brain (48). In this regard some data have been published suggesting that the resistance of the young rat brain to a low protein diet was in part due to the ability of the brain to utilize amino acids derived from breakdown of proteins in other parts of the body (49).

M. The two protein requirement.

As previously discussed in the Background section, we have found that the early chick embryo required at least two proteins in the nutrient medium for growth and development. It was found that one of the required proteins was a transferrin while the second could be either ovalbumin or lipovitellin (9). For some time we have been attempting to find the basis for this requirement. In a recent series of experiments we have been examining the effects of conalbumin (egg white transferrin) and ovalbumin on intracellular breakdown of yolk-sac proteins and the ability of these two proteins to serve as substrates for preparations of yolk-sac proteases. Our major findings have been that conalbumin inhibited intracellular protein breakdown to a greater extent then ovalbumin and that transferrin was preferred over ovalbumin as a substrate for yolk-sac proteases (50). Details of these experiments are presented in a preliminary draft of a manuscript included with this proposal. However, it should be noted that for the intracellular breakdown studies proteins with long half-lives were selectively followed and that cycloheximide was used to block amino acid reutilization. These studies, we feel, suggest that transferrin (conalbumin) may regulate intracellular protein "turnover" by acting on yolk-sac proteases.

PROPOSED STUDIES

I. Introduction.

At times in the past it may have been difficult for a reviewer to precisely relate our proposed studies to our objectives. With the progress we have made this should no longer be a problem. We propose to determine the mechanisms regulating serum protein synthesis by the yolk-sac of the chick embryo and the mechanisms by which serum proteins regulate the growth and development of embryos. The term "regulate" is used here in the broadest sense. For example, serum proteins may "regulate""modulate" or even provide "signals" to the early embryo.

The research proposed will be restricted to two major areas. The reviewer, however, should note that as in the past we will continue to seek possible applications of our research to such areas as embryo toxicology-teratology and the use of the systems that we develop to test the actions of drugs and environmental pollutants.

II. The regulation of serum protein synthesis.

A. Yolk-sac cell cultures

As discussed under Research Accomplishments we have been attempting to set up cell cultures derived from the yolk-sac of the chick embryo that would synthesize and secrete serum proteins. We have used published procedure for mixed yolk-sac cell cultures (34). In addition, we have tried a variety of procedures to measure serum protein synthesis including the elegant crossed immunoelectrophoresis technique that has been used to measure serum protein synthesis by chick embryo liver cells in culture (35). Although we have had some indication that serum proteins were being synthesized by these mixed cultures of yolk-sac cells, we have decided to first separate the cell types before returning to the problem of serum protein synthesis.

The yolk-sac in the area opaca (or area vasculosa) consists of the three germ layers, ectoderm, mesoderm and endoderm. Although our studies clearly show that the yolk-sac is the site of serum protein synthesis in an early embryo, the cell type or types involved are not known. One might assume that it is the endoderm as the liver eventually synthesizes serum proteins and it is an endodermal derivative. Nevertheless, this has not been demonstrated for the yolk-sac to this authors knowledge. It should be noted that in our previous cultures of mixed yolk-sac cells we did not determine the frequencies of the various cell types. Indeed, the cell types that synthesize the serum proteins may have been in the minority.

Recently, we started to work on the problem of isolating the various cell types of the yolk-sac. Because the endoderm appeared to contain large amounts of yolk, we initially considered a separation based on density. However, the literature suggested a more direct approach and we have found it to work (51). Quite simply, yolk-sacs were rinsed in several changes of calcium - and magnesium - free Tyrode's over a period of approximately one hour and the layers of the yolk-sac could be gently teased apart with forceps and a pipette stream. Working with 3 and 4 day yolk-sacs the ectoderm (probably somatopleure, that is ectoderm plus a small layer of mesoderm) could be easily separated while separating splanchnopleure into endoderm and mesoderm required some patience. This latter mesoderm was isolated as a single sheet with blood vessels while the endoderm came off more as large chunks of tissue. (The reviewer is asked to please examine the Progress Report 1976-1977 submitted with this report for photographs of these isolated cell sheets.)

We propose, as a first experiment, to determine which cell type or types synthesize serum proteins. The sheets of ectoderm, mesoderm and endoderm will be separated in calcium - and magnesium - free Tyrode's. Histological examinations of the three sheets will be used to verify the cell types in each

layer. An enzyme, cysteine lyase is apparently endoderm specific (52) and could be used to aid identification. Following the procedures that we have worked out for serum protein synthesis with the intact yolk-sac (22), each of the three sheets of cells will be incubated for 3 hours in buffered chick Ringer's containing ^{14}C-valine. The sheets of cells will be removed and the Ringer's will be analyzed on acrylamide gels for radioactive serum proteins. We will look for the synthesis of transferrin, alpha globulin-a and b, albumin and prealbumin. The disc acrylamide gels should be sufficiently sensitive as we can obtain relatively large amounts of this tissue. However, we have already prepared rabbit antiserum to the total embryo serum proteins (8 day) as well as to purified embryo serum transferrin and the embryo specific prealbumin. Thus, if greater sensitivity is required we could use the quantitative immunoassay of Laurell as used in the study of serum albumin synthesis by mouse hepatoma cells (53) or the crossed immunoelectrophoresis technique used with serum proteins synthesized by chick liver cells in culture (35). These experiments should clearly define which cell type is synthesizing which serum protein. In addition, by comparing the relative synthesis of serum proteins with the separate tissue sheets to that obtained with the intact yolk-sac, we may gain some insights into the importance of tissue organization (or tissue interaction) in the regulation of serum protein synthesis.

After we determine which cell types synthesize the serum proteins, that tissue sheet or those sheets will be dissociated with trypsin so that the cells can be cultured. We feel rather confident that separation of the cell types by tissue sheets before dissociation should provide cultures of a limited number of cell types. After dissociation the cells will be cultured with radioactive valine in a medium that has been used for mixed yolk-sac cells (34) or chick embryo liver cells (35) (Ham's F-12 with 10% fetal bovine serum) and once again the ability of these dissociated cells to synthesize radioactive

serum proteins will be tested. Again, depending on the sensitivity required, disc electrophoresis, quantitative immunoassay or crossed immunolectrophoresis will be used to determine if serum proteins are being synthesized.

Once we have demonstrated which tissue sheet synthesizes which serum protein and have demonstrated the same for dissociated cells derived from the sheet or sheets the number of experiments that can be envisioned is difficult to restrict. Following the establishment of such basic parameters as the kinetics of serum protein synthesis and the ability of cells to divide and be sub-cultured, we will address ourselves to the question of regulation of serum protein synthesis. Thus, we have clearly demonstrated that the relative synthesis of the serum proteins by the intact yolk-sac changes with the protein nutrition of the embryo and with development (22). Therefore, our first experiments will be designed to determine if the cells in culture will respond to changes of nutrition and developmental stage like the intact yolk-sac. For example, we will determine the effects of adding ovalbumin or conalbumin to the culture medium on the relative synthesis of serum proteins. In the case of development, we will isolate cells from yolk-sacs of various developmental stages and determine if the cells in culture exhibit the same relative synthesis of serum proteins as the intact yolk-sac. In addition, we will determine if the relative synthesis changes with length of time of culture or remains constant. That is, do the cells that synthesize serum proteins have a built in "clock" or do "environmental factors" regulate the developmental changes?

Allowing assumption to follow assumption, we will next turn to questions of transcriptional or translational control as outlined below in our studies with polysomes isolated from the intact yolk-sac. I do not wish to appear ludicrous but to indicate the ultimate direction one might follow there is the work of Heywood on translational control factors (54) or the work of

O'Malley on transcriptional control (55).

I would like to mention two other areas of research in which I feel that cell cultures would be particularly useful. First, comparative studies of yolk-sac and liver cell cultures would be of interest because the yolk-sac but not the liver synthesizes alpha fetoprotein. This serum protein is of considerable interest because it is synthesized by certain cancers and is present in high concentrations under certain teratological conditions. It has been demonstrated that the synthesis of serum proteins by chick embryo liver cells can be altered by insulin (35). Would the yolk-sac cells also respond to insulin and how might insulin influence the synthesis of alpha fetoprotein? In addition, we have argued that the protein nutrition of the early embryo is important in regulation because regulation is apparent before such homeostatic systems as the endocrine glands function. For example, the pancreas does not appear to synthesize insulin until day 5 (56). We would like to know if yolk-sac cells would respond to insulin if isolated from yolk-sacs prior to day 5. Alternatively, we would like to determine if the response of yolk-sac cells to ovalbumin and conalbumin would change at day 5.

A second area of some interest is that of cell interaction. An interaction between cell layers of the yolk-sac has been implicated in blood island formation (51) and in the induction of the primitive streak (57). It would be of considerable interest to determine if cells interact in the regulation of serum protein synthesis. With separate cell cultures of the three cell types that constitute the yolk-sac, we propose to study serum protein synthesis in mixed cell cultures containing precise numbers of the three cell types.

B. Cell-free synthesis of serum proteins

Our progress in developing a cell-free serum protein synthesizing system to study the transcription-translation question in regulating the

synthesis of these proteins has been discussed under Research Accomplishments of the Last Three Years under section "G". The reviewer is also directed to the Progress Report 1976-1977 for an acrylamide gel pattern of serum proteins synthesized by isolated polysomes, kinetics of the cell-free synthesis of protein and a polysome profile. To work out the conditions for cell-free serum protein synthesis we have used yolk-sacs from 4 day embryos. The large amount of yolk fat associated with the yolk-sac has caused some unique problems in obtaining good polysome profiles as well as recovery of RNA for the protein synthesis reaction mixtures. By optimizing deoxycholate concentration and volume in relationship to the number of yolk-sacs we have now been able to produce excellent polysome profiles. This same treatment in conjunction with the reextraction of a low speed pellet and a floating fat fraction has increased the recovery of yolk-sac RNA for the reaction mixture from 35% to greater than 90%. (This recovery was based on quantitative RNA estimations with orcinol.) By using a large number of yolk-sacs we could obtain sufficient RNA for the reaction mixture at 35% recovery. Furthermore, this material synthesized transferrin, the alpha globulins, prealbumin and globin. However, the low recovery was of some concern because of the possibility that it was not representative of the total yolk-sac polysome population.

The following procedure is presently being used to isolate yolk-sac polysomes. Yolk-sacs from 4 day embryos (one yolk-sac per ml) are homogenized in a loose fitting Dounce homogenizer with a buffer containing 0.5% deoxycholate. The homogenate is centrifuged at 500 x g which gives three layers. The top floating fat fraction contains approximately 7% of the total RNA, a supernatant with 73.5% of the RNA and a pellet with 19.5% of the RNA. The pellet and floating fractions are extracted again with 0.5% deoxycholate and these supernatants are combined with the first supernatant. The supernatants are then layered onto 1.46 M sucrose and centrifuged at 55,000 x g for 1.5 hours to pellet

the polysomes. The polysomes are resuspended, their concentration determined spectrophotometrically and they are added to a protein synthesizing reaction mixture containing radioactive valine and other components previously described (see preprint submitted with this proposal (21)). After an incubation period of 90 minutes (the reaction starts to plateau after 60 minutes) the reaction mixture is dialyzed against a buffer solution containing sucrose, urea, sodium dodecylsulfate and mercoptoethanol and finally the reaction mixture is analyzed by sodium dodecylsulfate gel electrophoresis (gels are stained, destained, cut at 1 mm, and each slice is analyzed for radioactivity).

Employing this procedure we propose to determine the relative synthesis of serum proteins by polysomes isolated from yolk-sacs. We will consider changes with nutrition and development. For example, when chick embryos are cultured on a medium containing conalbumin and ovalbumin the relative synthesis of serum transferrin is high relative to other media and using whole egg medium the synthesis of alpha globulin-a is quite high. We will determine if these changes in synthesis with nutrition observed with the intact yolk-sac are reflected by the isolated polysomes. With regard to development, using the intact yolk-sac the relative synthesis of serum albumin increases from 4.2% on day 5 to 41.7% on day 17 while prealbumin declines during this same period from 26% to 14%. Again, we will determine if these changes are reflected in the relative synthesis of serum proteins by isolated polysomes.

Through the above studies we will indirectly determine if serum protein synthesis is regulated through "functional" messenger RNA availability. An alternative more direct approach to the problem that we also propose to use involves the isolation of messenger RNA molecules and their subsequent identification through their protein products (in this case serum proteins). That is to say, in the approach that we have been using messenger RNA molecules and polysomes are treated as a single unit. In the alternative approach the

messenger RNA molecules are isolated and added to a cell-free synthesizing system. There are several advantages to this alternative approach. First, we can examine total messenger RNA rather than just those molecules associated with ribosomes. Second, protein synthesizing systems such as the one derived from wheat germ are highly efficient and should provide greater resolution of the messenger RNA molecules than the polysome system that we are presently using. Finally, we hope the use of an alternative procedure will confirm and support the conclusions derived from our original approach.

Two experimental designs will be used in the messenger RNA isolation approach. We propose to examine total messenger RNA as well as messenger RNA attached to polysomes. The polysome messenger RNA will provide a direct comparison to our original studies with intact polysomes. The total cellular messenger RNA can be compared with polysomal messenger RNA in considering possible involvement of such elements as messenger ribonucleoprotein particles (a possible "storage" form of inactive messenger RNA molecules (58)).

For total RNA, following homogenization the RNA will be extracted by the cold phenol method. For polysomal RNA, the polysomes will be pelleted through 1.46 M sucrose and the polysome pellet will be extracted with cold phenol. Subsequently both RNA preparations will be handled in an identical manner. The initial homogenization step is critical for yolk-sacs and as previously noted we have already worked out a procedure which provided RNA recoveries of greater than 90%. Following phenol extraction the RNA will be precipitated with ethanol and resuspended in buffer. At this point we plan to treat the RNA with proteinase K (59) which removes protein from RNA and destroys any remaining RNase. Next, the RNA preparation will be chromatographed on oligo dT cellulose which allows the selective separation of polyadenylated messenger RNA from the rest of the RNA (60). The polyadenylated messenger RNA molecules will then be added to the wheat germ protein synthesizing assay system con-

taining radioactive valine (61). Finally, the reaction mixture will be run on sodium dodecyl sulfate acrylamide gels for the determination of relative serum protein synthesis.

It should be noted, similar to our work with polysomes, we will examine isolated messenger RNA molecules taken from yolk-sacs cultured under various conditions of nutrition as well as at various stages of development. It should also be noted that although we have been using intact yolk-sacs for these studies, our anticipated success with yolk-sac cell cultures will allow us to work with a particular cell type involved in the synthesis of a particular serum protein.

Similar to the polysome work, the above studies should ultimately direct us to the mechanisms regulating serum protein synthesis be it transcription, translation or post-translational modifications.

Finally, our laboratory has a rather close working relationship with the laboratories of Dr. S. Heywood and Dr. J. Lucas-Lennard. Both of these laboratories are involved in protein synthesis and are currently making use of the procedures outlined above. Both have assured me of their continued expert advice.

III. The regulation of growth and development by serum proteins.

The above title is a bit pretentious because at this time we are attempting to demonstrate that serum protein do indeed act on developing system. As discussed under Research Accomplishments we have amassed considerable evidence which indirectly points to the importance of serum proteins. Furthermore, we have perfected several test systems to evaluate the biological action of serum proteins (cultures of rat embryos, chick embryos without yolk-sacs and isolated brains). We are, therefore, in position to isolate individual serum proteins and to demonstrate that they effect developmental processes (growth and differentiation).

I will divide the next section by test system because of some minor differences in experimental approach.

A. The rat embryo.

Our recent studies with the rat embryo have been discussed under Research Accomplishments in Section I and the preprint of a paper submitted to Nature is included with this report (43). Needless to say, we are quite excited about our rat embryo work. Briefly, we discovered that a protein band of approximately 125,000 molecular weight was selectively depleted from the serum medium used to culture rat embryos. Furthermore, regular serum (formed after clotting) which does not support embryo growth or allow normal heart development has a reduced level of this same protein band relative to the concentration in immediately centrifuged (true) serum. Immediately centrifuged serum allows normal heart development and superior embryo growth. Clearly, the next step must be the isolation of this protein (assuming it is a single protein) followed by tests for biological activity and characterization of the protein.

Several approaches can be used to isolate a protein and I know of no material (with the possible exception of chicken egg white proteins) that has received more consideration in regard to isolation than the proteins of serum. For example, Dietrich describes a method for simultaneous gel filtration and electrophoresis (62), Freeman describes salting out techniques, gel filtration, absorption chromatography and preparative electrophoresis (63) while Putnam reviews the whole subject of serum protein isolation and identification (64). Possibly the most direct approach for us would be to cut the protein band out of an acrylamide gel and to elude it from the acrylamide. Although this approach should provide excellent purity, yield and the maintenance of biological activity could be a problem. Thus, after attempting the preparative acrylamide approach we will turn to column chromatography. Once again we are faced with a variety of materials that can be used. Working with serum from 8 day embryos we have had experience and success using first carboxymethycellulose

column chromatography (65) with a pH gradient followed by placing these individual fractions on QAE-50 Sephadex exchange with an NaCl gradient (described by Pharmacia in "Sephadex Ion Exchangers, A Guide to Ion Exchange Chhromatography"). Certainly, we are prepared to try a variety of protein isolation procedures. Indeed, with a protein of M.W. 125,000 we may attempt direct use of Sephadex which separates mainly by molecular weight. Regardless of the isolation procedure used, we will monitor purity by acrylamide gels.

We will use headfold rat embryos (10 days gestation) to test the isolated protein for growth promoting properties and to monitor progress in purification. For example, we will compare the ability of various serum protein fractions to overcome the inadequacy of embryos depleted serum to support embryo growth. Alternatively, we could add various serum protein fractions to regular serum (delayed centrifuged) and determine if growth can be improved. Embryo growth in these studies will be measured by embryo DNA, RNA and protein content (quantitative colorimetric assay; diphenylamine, orcinol, and Lowry that we have used extensively with chick embryos.) The effect of regular serum as opposed to immediately centrifuged serum on heart development is the formation of a double heart (42). Thus, in addition to growth we will evaluate the effect of the various serum protein fractions on heart development. For this purpose we will culture embryos of 8 1/2 to 9 days gestation and although the double heart can be seen by gross examination we will probably do histological examinations.

In regard to protein characterization, it would be difficult for us to go much beyond molecular weight determinations and amino acid analysis. We have already determined molecular weight. Dr. E. Kharrallah at the University of Connecticut operates an amino acid analyzer and he has assured me that he will do an amino acid analysis for us. We have had experience working with anti-serum and an immunoassay would be no problem for us should the need arise. I should add, there are several protein biophysicists at the University of Connecticut who could be of some help or possibly they would become interested in the problem.

Rather than work on protein characterization, it would be more appropriate for us to follow the basis for biological activity. Although presumptious, it may be of some value to the reviewer to have some idea of the directions we would follow with a biologically active purified protein in hand. First, assuming the 125,000 molecular weight protein is biologically active, we would determine if it selectively binds to DNA. As previously noted, two human serum proteins have a high binding affinity for DNA (44). One of these proteins has a molecular weight of 126,000. Such binding affinity may indicate a regulatory function for the protein at the level of DNA. Second, following the elegant work of Dr. S. Cohen on the epidermal growth factor (66), we would iodinate the protein with ^{125}I and follow the distribution of the protein. For example, does the protein act on the yolk-sac of the rat embryo or does it reach the embryo proper? At the cellular level, does the protein remain on the cell surface or is it internalized? Is it readily degraded? Finally, if the protein did enhance embryo growth, the question of transcription versus translation regulation would be approached in the manner that has been described for studies of the regulation of serum protein synthesis.

Without going into great detail, there are two additional areas in which we plan to use rat embryo cultures. First, we plan to continue our work on the response of rat embryos to embryo serum proteins. In this case individual serum proteins are isolated from the serum of 8-day chick embryos and are added to the rat culture medium. It may appear a bit strange to add chick embyo serum proteins to rat embryo culture media but by working with chick embryos we can obtain relatively large quantities of embryo serum. Furthermore, we feel that certain serum proteins that are specific for embryos may have unique properties. For example, in addition to the embryo, certain cancers synthesize embryo-specific serum proteins. Our studies suggest that the relative synthesis of alpha fetoprotein may be related to embryo growth and the precise function of these so called "oncodevelopmental" proteins is unknown. In general terms, the individual chick embryo serum proteins will be added as supplements to serum medium or to a more defined medium for the culture of 10 day rat embryos.

We will measure growth after 48 hours of culture and examine the embryos for morphological development. Depending on these results we will follow the directions with chick embryos serum proteins as outlined above for the rat serum protein.

The second area, concerns the possible use of rat embryo cultures for sereening various sera from animals as well as man. The rationale being that the cultured rat embryo can distinguish relatively small changes in serum. The differences, for example, between immediate centrifuged and delayed centrifuged serum are quantitatively and quantitavely small. However, this difference brings about a rather dramatic response in terms of the growth and development of the embryo. Thus, might the embryos respond differently to serum from individuals or animals with a pathological condition such as cancer or might they respond in a unique manner to serum from individuals or animals exposed to some environmental pollutant? Although rat embryos in culture are being used in several laboratories for the study of the direct action of teratogens on embryos, I am not aware of studies being done comparable to what I have suggested here.

B. <u>Chick embryos without yolk-sacs</u>.

Chick embryos with an intact yolk-sac cannot be cultured on serum. In an attempt to study the effect of serum proteins on the chick embryo we have by-passed the yolk-sac by simply removing it. After various trials we have tentatively settled on culturing these embyos in the same roller tubes as the rat embryos. The morpho-olgical development of the embryo under these conditions is quite poor relative to culture on semi-solid whole egg medium with an intact yolk-sac. Furthermore, growth is also restricted (see Progress Report 1976-77). Nevertheless, these embryos do respond favorably to chick embryo serum. Indeed, the most favorable growth and development, to date, has been achieved with chick embryo serum.

We propose to use this system in conjunction with rat embryo cultures and cultures of isolated brains to study the effects of individual serum proteins on developmental processes. Chick embryos containing 8 to 10 pairs of somites are

cut free of yolk-sacs and placed into glass culture vessels as described for rat embryos (43). The vessels are gassed with 100% O_2 and rolled at 60 rpm. At the end of 24 hours we will evaluate embryo growth and development using quantitative determinations for DNA, RNA and protein content as well as histological examination. Depending on these results we could do a variety of other determinations to evaluate the response. For example, based on our other studies we could determine patterns of protein synthesis or polysome protein synthetic activity or even consider the problem of transcription and translation.

We have started to isolate and purify individual serum proteins from 8 day chick embryos in sufficient quantities for this type of study. Serum from 8 day chick embryos is obtained by plucking embryos from eggs without their extraembryonic membranes, rinsing them quickly in chick Ringer's and placing them in large (1000 ml) flasks. Embryos are added to the flask until they cover the bottom and the flask is then rocked (wrist action) for one hour at 37.5°C. The embryos are separated from the blood by passage through cheese cloth and the blood is centrifuged to pellet the blood cells. The serum consists of transferrin, alpha globulin a and b, albumin and prealbumin as judged by acrylamide gel analysis. Non-serum proteins are essentially absent from blood prepared by this procedure.

As has been noted, we have developed a fractionation procedure involving carboxymethylcellulose and QAE-50 Sephadex. The carboxymethcellulose is discharged with NaOH and NaCl and charged with HCl. After rinsing with water and starting buffer (acetic acid-ammonium hydroxide) the material is packed into a column and equilibrated with starting buffer. Starting at pH 4.3, the serum proteins are eluted at pH 4.8 (prealbumin), pH 5.2 (albumin), pH 5.7 (alpha and beta globulin) and 6.6 (transferrin). Acrylamide gel analysis indicated that prealbumin was quite pure but all fractions were contaminated with other serum proteins. Each fraction obtained from carboxymethylcellulose is now placed on QAE-50 Sephadex and the serum fractions are eluted at the following concentrations of NaCl: transferrin, 0.15M; alpha and beta globulin, 0.2M; albumin, 0.22M; and prealbumin, 0.26M. These fractions

were found to be of greater purity than the carboxymethcellulose alone. The individual fractions are dialyzed and finally lyophilized to dryness. The experimental design is to simply add the various serum proteins individually or in combinations to the culture medium (a basal medium) and after 24 hours to evaluate the response of these embryos without their yolk-sacs. We hope by this study to show that the serum proteins of an embryo have unique properties in regulating growth and development.

Naturally, when we isolate the 125,000 molecular weight rat serum protein and find it to be active with rat embryos we will also study its action on this chick embryo system for comparative purposes and to demonstrate general developmental significances.

D. <u>Isolated brains</u>

The pattern of proteins synthesized by isolated brains is distinct from the pattern obtained with brains left intact with the embryo. This is observed under conditions in which both the intact embryo and isolated brain are cultured on whole egg medium (see Research Accomplishments of the Last Three Years section K for additional details). We believe that this difference may be due to differences in the cellular environments. Thus, with the intact embryo the brain is exposed to "signals" from the serum proteins synthesized in the yolk-sac and passed into the circulatory system. The isolated brain, on the other hand, is exposed directly to the proteins of egg white and egg yolk. As already discussed, we have worked out the procedures and the control experiments and are presently prepared to run the "crucial" experiment.

Chick embryos of 11 to 13 pairs of somites will be cultured for 24 hours and then their brains will be removed and cultured for an additional 24 hours on chick embryo serum. A second group of 11 to 13 somite embryos will be cultured intact for 48 hours and then their brains will be removed. One group of brains will be

exposed to ^{14}C-valine and the second to ^{3}H-valine by incubation for 3 hours in buffered chick Ringer's with radioactive valine. Next, the two groups of brains will be homogenized and centrifuged. The soluble proteins from the two groups will be mixed and run on single sodium dodecyl sulfate acrylamide gels. The gel is next sliced and the amount of ^{14}C and ^{3}H in each slice is determined, plotted and analyzed by computer program. We have had considerable experience with all the many steps involved in this type of analysis (37). We hope to show by this study that brains cultured on chick embryo serum synthesize a pattern of proteins that resembles more closely the brain of the intact embryo than isolated brains cultured on whole egg medium.

The experiment described above will be run in the immediate future. It is presented as an example of the type of approach we can use with isolated brains. I do not wish to become repetitious but like the rat embryo cultures and the cultures of chick embryos without yolk-sacs the isolate brains will be used to study the 125,000 molecular weight rat serum protein as well as the individual serum proteins from chick embryo serum. I don't wish to suggest that we will routinely repeat the same experiment in the three different systems. Rather, I would like to indicate that if we do propose to demonstrate that serum proteins are generally important in the regulation of developmental processes that we have more than just a single developmental system to study.

SIGNIFICANCE

During early stages of development embryos are particularly vulnerable to environmental insults. We are interested in providing insights into the mechanisms by which growth and development are regulated during this early stage of development. Our initial studies were concerned with the nutrition of the early embryo because we felt that prior to the establishment of such regulating systems as the endocrines, nutrition itself might serve a regulatory function. Recently, our research directed us to the serum proteins as possible regulatory agents and as mediators in the response of embryos to nutritional changes. Serum proteins and serum factors have played a central position throughout the history of cell culture. Serum proteins and other serum components have been analyzed for countless years in the diagnosis of pathological conditions. Finally, serum proteins have received attention in developing systems but possibly not with the emphasis of this proposal. I would like to briefly cover these three areas of serum proteins, concentrating on recent studies which I feel are relevant to this proposal.

A great deal of effort has been made to replace serum in the culture of cells. Yet, in the opening sentence of a recent review of growth factors in cell culture it was stated that "nearly all animal cells in culture require serum for proliferation" (67). Fractionation of serum has led to a variety of proteins which stimulate the multiplication of cells in culture (67). The relevant question is, however, to what extent is serum actually required? The answer is not available but I would like to point out two recent contributions. A paper by Hayashi and Sato (68) suggests that serum can be replaced by a mixture of hormones. They worked mainly with a line of cells derived from the rat pituitary gland. Although they claim replacement of serum, I would like to point out that their medium contained transferrin, a serum protein. Second, they compared their

hormone mixture to 8% foetal calf serum which may be sub-optimal. In another paper (69), cells were treated with the inhibitor cycloheximide and then attempts were made to stimulate the cells to synthesize DNA with hormones or serum . The cells responded to serum but not to hormones, suggesting to the authors that there was more to serum than just hormones. However, cycloheximide may create a unique situation. The requirement for serum and serum factors remains unresolved. In their review (67) Gospodarowicz and Moran state, "the definitive test that allows one to conclude that any growth factor is truly a mitogen of physiological significance is to demonstrate that the growth factor is active _in vivo_". They go on to point out that this approach is difficult because more than one organ may make a particular factor and it would be virtually impossible to deplete an animal. I would like to suggest that the answer to this dilemma lies in the use of intact embryo model systems as presented in this proposal.

Although numerous changes in serum have been reported with pathological conditions, like many others, we have become interested in the so called oncodevelopmental proteins. We are particularly interested in alpha fetoprotein because we feel that we can make important contributions to an understanding of the mechanisms regulating the synthesis of this protein as well as to the mechanisms by which it acts. High levels of alpha fetoprotein have been observed in the amniotic fluid of developmental central nervous system abnormalities and it is being suggested that when amniotic fluid is taken it should be analyzed for this protein (70, 71). High levels are also found with liver and intestinal cancers (see 72 and 73) as well as in liver injury (74 for a rat study). Alpha fetoprotein has immunosuppressive properties (75, 76) and has a high affinity for estrogen (77). In the fetal mouse liver, alpha fetoprotein was syntehsized mainly on membrane bound polysomes (78) and the polysomes _in vitro_ synthesized relative amounts of this protein comparable to that observed _in vivo_. We have proposed two unique

approaches for the study of this interesting and important protein as well as serum proteins in general. First, we propose to study the regulation of serum protein synthesis as modulated through nutrition and development. For this we will use yolk-sac cell cultures and a cell-free protein synthesizing system derived from isolated yolk-sac polysomes and messenger RNA. Second, we propose to study the action of this protein and other serum proteins directly on three different developing systems. In this regard, it should be noted that in a recent report antisera prepared against alpha fetoprotein was found not to be toxic when injected into the pregnant rat (79). Such negative results, however, do not negate the possibility that this protein is biologically active in an embryo.

Although changes in serum proteins with development has received a great deal of consideration, their possible role as regulators has not received much attention. Although serum will not support the growth of cultured chick embryos, it is the most widely used medium for rat (38) and mouse embryos (80). We believe that this difference between the chick and mammalian embryos is due to the more extensive development of the yolk-sac in the chick. Indeed, our work with the chick embryo without yolk-sac would support this contention. It appears possible that the use of serum for cultured embryos may reflect a certain artifact of the culture condition. In this regard, an analysis of human uterine fluid indicated that the proteins were mainly serum proteins (81). But, do these proteins actually influence developmental processes? We have identified a protein band on gel electrophoresis of rat serum that may play a role in the growth and heart development of the rat embryo (43). Blastokinin, a protein isolated from the rabbit uterus, was found to promote blastulation and blastocyst expansion (82). A major portion of our proposal is concerned with an examination of the role that serum proteins play in the regulation of development. In a recent review Sherman states, "it seemed conceivable that the uterine milieu might not only play a protective and nutritive role, but might also provide signals which trigger various events involved

in differentiation of the embryo" (83).

Finally, it should be noted that we are not only interested in what serum proteins may do to a developing system but ultimately how might they do it. In their review, Gospodarowicz and Moran (67) thoroughly cover the responses of cells in culture to serum and serum factors. But, no mention is made of possible mechanisms of action. I would simply like to mention here that there are indications for both transcriptional and translational controls exerted by serum proteins. I would like to suggest, although not at all convincing, that the binding of a serum protein to DNA may indicate transcriptional control. Thus, DNA binding proteins from serum have been identified (44) and a serum protein with DNA affinity has been identified in association with various neoplasms (84, 85). In addition, a serum protein was identified with isolated chromatin from cultured fibroblasts (86). For translational control, a study with Vero cells showed that upon serum removal there was an immediate decline in peptide chain elongation (87). Assay for elongation factor I indicated a reduction in the activity of this translation factor. We propose to study the question of transcription versus translation not only in the regulation of serum protein synthesis but ultimately in the action of serum proteins on embryos as well.

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Graduate Students

Ph.D. Degrees

Dr. Judith Piper-Airhardt, Ph.D., Marquette University, 1969. Research Associate, Dept. Physiology and Biophysics, College of Medicine, University of Vermont.

Dr. James D. Yager, Jr., Ph.D., University of Connecticut, 1971. Assistant Professor, Dept. Pathology, School of Medicine, Dartmouth College.

Dr. John Hassell, Ph.D., University of Connecticut, 1972. Senior Staff Fellow, National Institute of Dental Research, National Institute of Health.

Dr. Darrell H. Carney, Ph.D., University of Connecticut, 1975. Post-Doctoral Trainee, Dept. Medical Microbiology, California College of Medicine, University of California, Irvine.

Dr. David Kram, Ph.D., University of Connecticut, 1976. Staff Fellow, Laboratory of Cellular and Comparative Physiology, Gerontology Research Center, National Institute on Aging.

Ph.D. Candidates

Phillip P. Minghetti has completed his third year, should receive degree in 1978.

Franklin Longo, has completed his third year, should receive degree in 1978.

Marian F. Young, has completed her first year.

Clare Oesterle-Chatot, will join the laboratory August 1, 1977. She has just completed her Master's Degree, Villanova College.

Master's Degree

Eileen D. Hickey-Weber, Master's; Marquette University, 1967. Received Ph.D., University of Bern, 1970. Research Associate, State University of New York, Albany.

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Audrey G. Sawyer-Lynch, Master's, University of Connecticut, 1972. Research Associate, Edmonton, Canada.

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Post Doctorals

Dr. Robert C. Fitzsimmons, Post-Doctoral 1965 to 1967. Associate Professor (I think), University of British Columbia.

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Dr. Judith A. Piper, 1969
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Dr. James D. Yager, 1971
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Dr. John R. Hassell, 1972.
An Analysis of Nutrient Protein Utilization by the Cultured Chick Embryo and Its Relationship to Growth Regulation During Development.

Dr. Darrell H. Carney, 1975
Developmental and Nutritional Effects on the Synthesis of Specific Proteins in Regions of the Cultured Chick Embryo.

Dr. David Kram, 1976
Serum Protein Synthesis in the Early Chick Embryo.

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