PROTEINS IN GROWTH REGULATION
DURING EARLY DEVELOPMENT

Progress Report
for Period 1976 to 1977

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INTRODUCTION

The present report covers the period May 1976 to July 1977. In addition to this report the reader is referred to a paper that appeared last year on serum protein synthesis, a paper submitted for publication on polyribosome activity, another paper submitted concerned with serum protein utilization by the rat embryo, a manuscript being prepared on nutrition and patterns of proteins synthesized and a manuscript being prepared on the role of transferrin (conalbumin) in the regulation of intracellular protein degradation. In addition, the reader should note that a Comprehensive Three Year Report was prepared at this time.

My approach in preparing this progress report is to provide examples of data from those studies not covered in some other form (i.e. manuscript).

Our previous work has directed us to the yolk-sac as a central organ in mediating the response of embryos to environmental factors and particularly to nutrition. Furthermore, because the yolk-sac is the exclusive site for the synthesis of serum proteins we have been considering the possibility that serum proteins provide signals of developmental significance to the developing embryo. Thus, on the one hand we have been interested in the mechanisms regulating the synthesis of serum proteins by the yolk-sac while on the other hand we have been interested in determining if indeed serum proteins are developmental signals.

I. Effects on serum protein synthesis.

We have published a paper demonstrating that in an early chick embryo the yolk-sac is the exclusive site of serum protein synthesis and that serum proteins are synthesized as early as the primitive streak stage or prior to the establishment of a functional circulatory system. In addition, in this same paper, we

showed that when embryos of 11-13 somites are cultured on various media for 48 hours the relative synthesis of serum proteins was altered. It was particularly interesting to find that the relative synthesis of one serum protein, alpha globulin-a (alpha fetoprotein) was directly related to the ability of a nutrient medium to support embryo growth.

More recently, we have completed a developmental study on the relative synthesis of serum proteins by the yolk-sac and liver, and have examined the effects of several drugs and a mutation on serum protein synthesis.

A. Development

At various stages of development, yolk-sacs and livers were incubated with radioactive valine and the secreted serum proteins were analyzed by acrylamide gel electrophoresis. The amount of radioactivity in each serum protein was corrected for the valine content of that protein. In this way, the relative synthesis of each serum protein could be determined. In addition, samples of embryo blood were analyzed so that the circulating levels of each serum protein could be determined. In this case, radioactivity was not used but gels were spectrophotometrically scanned and the areas under the peaks determined.

The results are summarized on Tables 1-3. Several interesting points should be noted. Both yolk-sac and liver synthesized transferrin, alpha globulin-b, albumin and prealbumin. The synthesis of alpha globulin-a, however, was restricted to the yolk-sac. A peak in the synthesis of this protein was noted on day 7 while circulating levels peaked on day 12. The synthesis of alpha globulin-b stopped in both yolk-sac and liver on day 11. The electrophoretic mobility (not shown) of transferrin synthesized by the yolk-sac was different from that of the liver. The circulating level of transferrin remained constant while synthesis in the liver and yolk-sac declined. Dramatic changes were noted in the synthesis of albumin and prealbumin. During de-

velopment the synthesis of albumin increased for both liver and yolk-sac while the synthesis of prealbumin decreased in both. These changes in synthesis of albumin and prealbumin were reflected in the circulating levels of these proteins.

Table 1

Relative synthesis of serum proteins
by the chick embryo yolk-sac

day of incubation	trans. %	α glob-a. %	α glob-b. %	alb. %	pre-alb. %
5	18	44	7	4	26
7	13	55	4	7	22
9	14	53	3	8	23
11	12	50	3	16	21
13	8	47	0	27	18
15	7	40	0	32	21
17	7	37	0	42	14

Table 2

Relative synthesis of serum proteins
by the chick embryo liver

day of incubation	trans. %	α glob-b %	alb. %	pre-alb. %
5	51	11	15	24
7	42	14	14	30
9	32	7	47	14
11	23	5	65	8
13	19	0	76	6
15	20	0	76	4
17	18	0	77	5
19	26	0	68	6

Table 3

Circulating levels of serum
proteins in the chick embryo
(Note, the amount of gamma globulin and
another protein, probably globin, have been omitted)

day of incubation	trans. %	α glob-a %	α glob-b %	alb. %	pre-alb. %
6	18	29	21	6	14
8	20	32	17	7	12
10	20	34	13	9	12
12	19	36	0	19	10
14	14	26	0	19	6
16	17	22	0	19	6
18	16	13	0	19	5
20	18	11	0	17	2

B. Teratogenic agents.

In considering the effects of various environmental factors on embryos, it was natural for us to consider the possibility that they could act on the embryo through alterations in serum protein synthesis. This seemed particularly possible because substances that reach the embryo must first contact the yolk-sac. In considering various drugs to study, we turned to the National Cancer Institute because of a possible relationship between the synthesis of serum proteins by the yolk-sac and the synthesis of embryo specific proteins by certain forms of cancer.

Two approaches have been used. First, embryos of 11 to 13 pairs of somites were cultured on a drug for 48 hours. Their yolk-sacs were then removed and incubated for 3 hours in buffered chick Ringer's in the presence of ^{14}C -valine. The secreted serum proteins were then analyzed by acrylamide gels (sliced and radioactivity in each slice determined). In the second approach, yolk-sacs were isolated from 11 to 13 somite embryos and incubated in buffered chick Ringer's containing not only radioactive valine but also a drug. After three hours, the secreted serum proteins were analyzed by acrylamide gels. It should

be noted that for each drug a concentration response curve was determined. For example, with cultured embryos a concentration was selected that would produce abnormal embryos and still allow survival of at least 50% of the embryos.

The results of these studies are presented in tables 4 and 5. Differences between the various drugs in relative serum protein synthesis can be noted and these differences were reasonably reproducible. However, with few exceptions, we have been more impressed by the lack of response to the drugs than by the rather small differences. It should be noted that three compounds, adriamycin, daunomycin and actinomycin D, cause the similar abnormality of rumplessness or degeneration of cells in the posterior trunk. In this regard, adriamycin and daunomycin gave similar reductions in transferrin and increases in prealbumin when provided in the 48 hour culture medium. Probably the largest serum protein response came with cyclophosphamide. This was true if cyclophosphamide was added to the culture medium for 48 hours (Table 4) or to the 3 hour incubation medium. In one situation, however prealbumin was high and globulins low while in the other situations globulins were high and prealbumin low. Clearly, the number of compounds tested to date has been small. In the literature there are reports that insulin and cycloheximide influence serum protein synthesis. The systems used in these published reports were different from the one used here but it still may be of some value to test these compounds. We do plan additional work on a possible relationship between drugs and other environmental factors, serum protein synthesis and developmental abnormalities.

Table 4

Effects of various drugs on
yolk-sac serum protein synthesis
(Embryos cultured for 48 hours with drug)

drug	trans. %	* glob a+b %	alb. %	prealb. %
control (4)	23	43	5	29
adriamycin (3)	18	42	5	35
daunomycin (1)	19	39	5	37
cyclophosphamide (5)	21	34	6	39
dibromomannitol (2)	22	41	9	27

Table 5

Effects of various drugs on
serum protein synthesis by yolk-sacs
from 11-13 somite embryos.
(drug added to isolated yolk-sacs
during three hour period)

drug	trans. %	* glob a+b %	prealb. %
control (2)	21	30	50
adriamycin (1)	17	34	49
cyclophosphamide (3)	24	38	39
actinomycin D (1)	19	29	53

C. Genetic mutation

The Department of Animal Genetics of the University of Connecticut maintains flocks of birds with various mutations. One of the most studied of these mutations has been the Creeper gene which is autosomal and dominant. An interesting and pertinent effect of this mutation is poor development of the yolk-sac. Although the yolk-sac forms blood islands, a functional circulatory does not develop. For this reason it was of interest to examine serum protein synthesis in the Creeper. It should be noted that in a recent publication a deficiency in plasma protein synthesis was found in association with the X-ray

induced lethal mutation at the albino locus in the mouse.

Yolk-sacs from Creeper embryos were isolated after 3 and 7 days of incubation. The yolk-sacs were incubated in buffered chick Ringer's containing ^{14}C -valine. The Ringer's was then analyzed for serum proteins by acrylamide gels (by our standard procedure).

Like the drug study previously discussed, the difference between Creeper and control were relatively small (Table 6). At 3 days, however, there was a 9% reduction in alpha globulin-a synthesis in the Creeper relative to control while the Creeper synthesized approximately 10% more prealbumin. At 7 days Creeper and control were quite similar. It should be noted that homozygous Creepers were used in both studies.

Table 6

Effects of Creeper mutation on
relative synthesis of serum proteins

3 days		trans.	glob-a	glob-b	alb.	prealb.
		%	%	%	%	%
Control		18	50	6	2	26
Creeper		16	41	5	2	36
7 days						
control		10	42	3	4	22
Creeper		13	42	3	5	18

In addition to the study of relative synthesis, we have been attempting to measure the kinetics of serum protein synthesis. Because of the difficulty in defining precisely the non-radioactive free amino acid pool, it is quite a problem to measure the kinetics of protein synthesis in an intact tissue. For example, it is extremely difficult to identify the pool of amino acids which dilutes the radioactive amino acid tracer. Nevertheless, we have made an attempt. Sure enough, we found that the Creeper yolk-sacs secreted radioactive protein

at a much greater rate than control. We have tried increasing the amount of radioactive amino acid and this did produce a small increase in the control. We are now in the process of examining free amino acid pools. We suspect that the difference in the incorporation kinetics may be due simply to a greater release of non-radioactive amino acids into the incubation medium by control relative to Creeper. Although this study has lost some significance to the problem of serum proteins, we are interested in yolk-sac proteases with regard to protein utilization. For this reason we have planned some additional work with the Creeper gene.

II The regulation of serum protein synthesis

We are using two approaches to study the mechanisms regulating serum protein synthesis. In one approach we are attempting to set up cell cultures of specific cell types derived from the yolk-sac that will synthesize and secrete serum proteins. In the second approach we are working with cell-free protein synthesizing systems derived from the yolk-sac. To date, we have used isolated polysomes but soon we will start to work on isolated messenger RNA.

A. Cell cultures.

In the past we have worked with cell cultures derived from the total yolk-sac and have made several attempts to determine if these cells synthesized serum proteins. First, we simply incubated cells with radioactive amino-acids and analyzed the medium for radioactive serum proteins. Although radioactivity was detected in secreted proteins, the acrylamide gels did not show appreciable radioactivity in serum proteins. We felt that this was due to the possibility that the amount of synthesized serum protein was too small relative to the total protein placed on the gel. Therefore, we next made rabbit anti-serum to chick embryo serum proteins. Repeating the previous experiment, we now used antibody to precipitate serum proteins and analyzed the precipitate by acrylamide gels. Although we detected radioactivity in the precipitate, the gels were again

negative for serum proteins. Once again we felt that the total protein placed on the gel was too great relative to the amount of radioactive serum proteins. So, with our antiserum already prepared we started to use the sensitive and elegant crossed immunoelectrophoresis technique. We ran the same experiment again, and once again we detected serum proteins on the electrophoresis gel but when exposed for autoradiography we only detected a small peak. We have not repeated this last experiment. It is quite possible that we did not expose the autoradiograph for a sufficient period of time. But, rather than repeat this experiment we have been having excellent success at separating the cell layers of the yolk-sac. Thus, we plan to first determine which cell layer synthesizes which serum protein. Then, we will dissociate the appropriate cell layer or layers for cell culture.

At this point, I would simply like to describe the cell layer separation and show you a few pictures of how these separated cell layers appear. To separate the cell layers, they were simply teased apart after being rinsed for about one hour in several changes of calcium—and magnesium—free Tyrodes solution. The pictures presented (Figs. 1-3) were taken after separating the cell layers from a single piece of 3-day yolk-sac. The distinct morphology of each cell layer should be noted. As far as we can tell, the separation is quite clean in that one layer does not appear to contaminate another layer. Histology will be used to verify this point. Although the endoderm and mesoderm appear quite distinct we suspect that the ectoderm may actually be somatopleure. Thus, a small mesodermal layer may be associated with the ectoderm.

B. Cell-free serum protein synthesis.

We have observed two conditions which lead to rather dramatic changes in the relative synthesis of the serum proteins; the protein composition of the nutrient medium and developmental age. We would like to determine if these serum protein changes are regulated at the transcription and/or translation

ISOLATED CELL LAYERS FROM 3-DAY CHICK EMBRO YOLK SACS

FIG. 1 ECTODERM

FIG. 2 MESODERM

FIG. 3 ENDODERM

level of protein synthesis or possibly at the post-translation level. Our first approach has involved a comparison between the synthesis of serum proteins by isolated polysomes and the intact yolk-sac. This study will allow us to determine if the levels of "functional" messenger RNA are related to the relative amounts of serum proteins synthesized and secreted by intact yolk-sacs.

In working out the details of isolating active polysomes from the yolk-sac we ran into some difficulties with RNA yield. This problem has now been solved as we found that the large amount of fat associated with the yolk-sacs interfered with the liberation of the polysomes by deoxycholate. We had been working with only 35% of the yolk-sac RNA but now with improved conditions we obtained a yield of greater than 90%. Before the improved yield we were able to obtain excellent polysome profiles, work out reaction kinetics and even obtain serum protein synthesis. However, the low RNA yield was of some concern because of the possibility that the polysomes used in the reaction were not representative of the entire yolk-sac polysome population. The following results were obtained with the low yield RNA.

The polysome profile that we now obtain from 4-day yolk-sac polysomes has a relatively large proportion of heavier (greater than dimers) polysomes (Fig. 4). Titration of these polysomes in our reactions mixture shows an increase in incorporation of radioactive amino acids into protein up to 18 mg of ribosomal RNA per reaction (Fig. 5). Incorporation kinetics of 18 mg ribosomal RNA shows linearity up to approximately 45 minutes (Fig. 6). Finally, the analysis by acrylamide gels shows that transferrin, alpha globulin, prealbumin and globin were synthesized (Fig. 7). It should be noted again that these studies were run with the low yield RNA. We feel certain that by working with the high yield RNA the synthesis of serum proteins will be greatly improved.

FIG. 4

POLYSOME PROFILE FROM 4-DAY CHICK EMBRO YOLK SACS

(10% to 50% SUCROSE GRADIENT, 90 MIN., 35,000 X G)

FIG. 5 PROTEIN SYNTHESIS BY ISOLATED 4-DAY CHICK EMBRYO YOLK SAC POLYSOMES. VARIOUS QUANTITIES OF RIBOSOMAL RNA WERE ADDED TO THE REACTION MIXTURE.

FIG. 6 PROTEIN SYNTHESIS BY ISOLATED 4-DAY CHICK EMBRYO YOLK SAC POLYSOMES. SAMPLES WERE TAKEN AT VARIOUS TIME POINTS. RIBOSOMAL RNA WAS AT 18MG./ REACTION MIXTURE.

FIG. 7

PROTEIN SYNTHESIS BY ISOLATED 4-DAY CHICK EMBRYO YOLK-SAC
POLYSOMES. THE REACTION MIXTURE CONTAINED 18MG OF POLYSOMAL
RNA AND WAS RUN FOR 45 MIN. THE REACTION MIXTURE WAS ANALYZED
ON SDS POLYACRYLAMIDE GELS.

III Effects of serum proteins on embryos

A. Rat embryo.

The manuscript included with this report covers our major findings on the depletion of a serum protein by cultured rat embryos. This protein band of 125,000 molecular weight was depleted from rat serum medium by cultured head fold stage rat embryos (10-day). The concentration of this same band was reduced in so called "delayed centrifuged" rat serum relative to immediately centrifuged (true) rat serum. Immediately centrifuged serum in comparison to delayed centrifuged supports higher levels of embryo growth and allows normal heart development.

In addition to the above study, we have added embryo specific serum proteins to the rat serum used as a culture medium for rat embryos. The embryo proteins were isolated from the serum of 8-day chick embryos. Alpha globulin-a (alpha fetoprotein) and prealbumin were added at concentrations of 1 mg per ml and 0.27 mg per ml, respectively. With both proteins we noted an enhancement of embryo growth relative to controls (not supplemented serum). Naturally, these experiments must be repeated and various other proteins must be studied before we can suggest a specificity in the response of embryos to embryo serum proteins.

B. Chick embryos without yolk-sacs.

Chick embryos unlike rat embryos cannot be cultured on serum. We believe that this is due in part to the more extensive development of the yolk-sac in the chick relative to the rat and in part to the unique nutrient requirements of the chick yolk-sac. In any case, in order to study the effects of serum proteins on the chick embryo we have been attempting to culture these embryos without yolk-sacs. We have tried numerous approaches. However, when compared to our cultures of embryos with yolk-sacs, the yolk-sac free embryos show limited growth and poor morphological development. At this time, we are using the same apparatus we built for rat embryos for these chick embryos with-

out yolk-sacs. The embryos are turned in glass culture bottles at 60 rpm with a gaseous environment of 100% oxygen.

In spite of the limited growth and poor development, we have found to date that chick embryo serum supports growth and development to a greater extent than the various other media we have used.

In this regard, I would like to describe one experiment. Embryos of 8 to 10 somites were cultured for 24 hours without their yolk-sacs on a basal medium (salts, glucose and vitamins) supplemented with each of the following; amino acids, ovalbumin (20 mg/ml), conalbumin (20 mg/ml), ovalbumin plus conalbumin at an equal ratio by weight (20 mg/ml total) and 8-day chick embryo serum (approximately 16 mg of protein/ml). Chick embryo serum supported embryo growth to a greater extent than the other media (Fig. 8). It should be clearly noted that one of the media, ovalbumin plus conalbumin supported the growth and development of chick embryos cultured with intact yolk-sacs. This study, therefore, followed our prediction that the embryo proper depends on serum proteins synthesized by the yolk sac.

C. Isolated brains

In our previous studies on protein starvation as well as those on regional patterns of embryo protein synthesis, the brain was found to be more sensitive than the other regions of the embryo. We initially started work with isolated brain cultures in order to determine if the sensitivity of brains to starvation was a cellular or an organismal response. More recently, we have been working on various preliminary experiments so that we can use the isolated brain in testing the biological activity of serum proteins.

Our background studies have involved comparisons of the patterns of proteins synthesized by isolated brains with the patterns from brains left to reside for a comparable period of time with their embryos. In this latter group we have

CULTURES OF CHICK EMBRYOS WITHOUT YOLK-SACS ON VARIOUS MEDIA. EMBRYOS OF 8 TO 10 PAIRS OF SOMITES WERE USED. GAS WAS 100% OXYGEN AND CULTURE PERIOD WAS FOR 24 HOURS. THE MEDIUM WAS A MIXTURE OF SALTS, GLUCOSE AND VITAMINS SUPPLEMENTED WITH EACH OF THE FOLLOWING: (A), AMINO ACIDS; (B), OVALBUMIN; (C), CONALBUMIN; (D), OVALBUMIN PLUS CONALBUMIN; and (E), 8-DAY CHICK EMBRYO SERUM.

used both brains from embryos cultured for 48 hours on whole egg medium and brains from embryos allowed to remain in the egg for a comparable period. The experiments were designed as follows. Embryos of 11 to 13 somites were cultured on whole egg medium for 24 hours and their brains were removed. These brains were then cultured for an additional 24 hours on Millipore filters placed on whole egg medium. A second group of brains were taken from embryos at the end of 48 hours of culture on whole egg medium. A third group of brains were taken from embryos after a comparable period of time left within their eggs. To summarize, the groups were cultured isolated brains, brains from cultured embryos and brains from in ovo embryos. All three groups of brains were exposed to either ^{14}C or ^3H -valine for 3 hours, homogenized and analyzed by sodium dodecyl sulfate acrylamide gels. By using both ^{14}C and ^3H comparisons could be run on a single gel. Gels were sliced and the amounts of ^{14}C and ^3H in each slice was determined. The results were plotted (Figs 9-11) and a computer program was used to calculate the "total percent differences".

A control gel was run with ^{14}C and ^3H labeled brains taken from embryos cultured for 48 hours on whole egg (Fig. 9). Small differences were noted in the plot of the data and a value of approximately 7 was obtained for the total percent difference. In comparing 48 hours of culture on whole egg with a comparable period in ovo the total percent difference was only 5 (Fig. 10). This suggested that our whole egg culture technique with regard to this parameter was comparable to the in ovo condition. Finally, the comparison between isolated brains and brains from 48 hour whole egg cultured embryos showed major differences (Fig. 11). It was particularly interesting that the isolated brain showed two large peaks absent from the brains allowed to reside with their embryos. The total percent difference was 15 or more than double the control values. Our next experiment will involve a comparison in which the isolated brains are cultured on chick embryo serum.