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Synthesis of Serum Proteins by Cultured Aggregates of Endodermal Cells from the *Area Opaca* of the Early Chick Embryo

by

E. ANNE DARRAGH

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

CELL BIOLOGY

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA

SPRING 1987

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Supervisor

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Date Nov/13/86..............

DEDICATION

This thesis is dedicated to my parents, Alfred and Marina, in appreciation for their continued efforts to inspire curiosity in their children.

ABSTRACT

The pattern of serum protein synthesis and secretion in aggregates of extraembryonic endoderm cells (EEC) from the *area opaca* of primitive streak chick embryos was studied. EEC aggregates were cultured for various time intervals and serum proteins which were synthesized and secreted by aggregates were detected using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography. Serum proteins were identified in EEC extracts and culture medium, based on their comigration with reference proteins from 4 day chick embryo serum and with reference proteins from egg white albumen and chicken serum which were obtained from commercial sources. A number of serum proteins were detected in EEC aggregates including: two isomorphs of immunoglobulin (IgG), four isomorphs of transferrin, a protein fraction with a molecular weight of 66,500 which may correspond to α globulins, as well as prealbumin and a protein with a molecular weight of 38,600 (serum protein 11) which remains unidentified. These proteins were also detected in the culture medium. The banding profiles of EEC extracts and culture medium were compared over various time intervals of culture (6h, 18h and 30h). It was found that the IgGs, transferrins and serum protein 11 decreased in concentration in EEC extracts over the culture interval. These proteins were detected in the

culture medium and one transferrin isomorph and prealbumin increased in concentration over the same time interval. Incorporation of amino acids into protein was detected by adding radiolabelled amino acids to the medium. A number of proteins were synthesized by EEC, but all of the labelled serum proteins were detected in the culture medium, not in EEC extracts. These results suggest that serum proteins are synthesized by EEC then rapidly released into the medium. Labelled serum proteins detected in the culture medium include prealbumin and an unidentified serum protein (serum protein 14) with a molecular weight of 15,400, both synthesized early in culture (6h), and transferrin which was synthesized later (18h). Other synthesized proteins were detected in EEC extracts, but none of these were serum proteins and they are unidentified at this time. When aggregates were prepared for autoradiography, silver grains were detected in nascent cavities within the aggregates and at the peripheral border of the aggregates.

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. Sara Zalik for her professional guidance and encouragement throughout the course of this project. I would also like to thank Irene Ledsham and Eva Dimitrov for their expert technical advice and cheerful assistance with the dissection of numerous chick embryos. And to all of the people who helped to create an atmosphere of comraderie and friendship, I am very grateful. Thank you Dr. Sara Zalik, Geraldine Mbamalu, Irene Ledsham, Eva Dimitrov, Karen Elgert, Dr. Harriet Harris, Dr. Bill Kulyk, Dr. Nadine Milos, Dr. R.F. Ruth, Dr. Rueben Kaufman, Milan Makale, Paul Lindsay, Liz Stinson and Kerry Irons. And to my brother John, a special thank you for his critical review of this manuscript and his many helpful suggestions. This project was supported by an Alberta Heritage Foundation for Medical Research Studentship and, in part, by the National Science and Engineering Research Council and Medical Research Council of Canada. This support is gratefully acknowledged.

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I. INTRODUCTION

A. Serum Proteins as Regulators of Growth and Development

The development of an embryo from a fertilized egg involves a great deal of cell proliferation, cell rearrangement and differentiation into specific tissues and organs. These developmental events take place according to a very specific timetable but the regulatory mechanisms involved are not clearly understood. Embryogenesis is ultimately controlled by gene expression and changes in gene expression are often reflected by changes in protein synthesis patterns. By studying how specific proteins affect cell proliferation, cell adhesion and differentiation in embryos, some of the molecules which regulate these processes can be identified.

Early attempts to grow vascular tissue excised from chick embryos under culture conditions demonstrated that cell proliferation required fresh plasma or serum (Carrell, 1912). Serum proteins were eventually recognized as a nutritional requirement for cells cultured *in vitro* (Eagle, 1955). Cultured cells normally undergo mitosis until growth is inhibited by cell to cell contact but cells at this stage can be induced to divide again if fresh serum is added to the culture medium (Todaro *et al*, 1965; Yoshikura and Hirokawa, 1968). One of the factors involved in regulating mitogenic activity is a substance called platelet-derived growth factor which is released from blood platelets during

serum preparation (reviewed by Ross and Vogel, 1978). *In vivo*, this substance is released when tissue injury occurs and likely plays a role in cell proliferation during tissue repair. Many other growth factors have been reported which are present in serum. When cells are cultured in serum-free medium, they require serum protein supplements which are specific for each cell type (reviewed by Barnes and Sato, 1980). *In vitro*, these molecules play a role in cell adhesion and cell proliferation and both of these processes are important *in vivo* during embryogenesis.

Cell proliferation also occurs *in vivo* during neoplastic transformation. Cells infected with tumorgenic viruses (eg. SV40, polyoma and Rous sarcoma) as well as neoplastic cell lines have a reduced requirement for serum (Birch and Pirt, 1969; Takaoka and Katsuta, 1971). This may mean that they have somehow overcome their requirement for serum or that they are synthesizing the proteins required for growth *in vitro*. Adults with tumors of the liver or structures derived from the yolk sac have elevated levels of a serum protein normally present only during fetal development (reviewed by Hirai, 1982). This protein, called α fetoprotein, is normally produced by the liver and yolk sac cells of the developing mammalian embryo. Its concentration in serum diminishes prior to birth and it is detectable only in nanogram quantities in adults (Seppala and Ruoslahti, 1973; Massayeff *et al*, 1975). When tumor formation is induced in mice, there is a transient elevation

of α fetoprotein in serum prior to the appearance of a tumor. After tumor formation, α fetoprotein levels are once again highly elevated (Watabe, 1971; Hirai *et al*, 1973). The gene or genes regulating α fetoprotein synthesis are apparently active in the embryo, shut down in the adult and reactivated in association with certain cancers. This process is referred to as "oncodevelopmental gene expression". The re-expression of genes which code for serum proteins specific to the embryo may account for the reduced serum requirement of neoplastic cells. These embryo-specific serum proteins may play a role in cell proliferation during embryogenesis.

B. Sources of Serum Proteins for the Developing Chick Embryo

All those nutrients required for normal growth and development of the chick embryo, aside from respiratory gases, must be contained in the albumen and yolk of the egg. In the laying hen, albumen proteins are produced in the oviduct in response to steroid hormones (Gilbert, 1971). Prior to this, oocyte differentiation involves accumulation of yolk and it is at this time that a carefully balanced nutrient pool is deposited for utilization during embryo development (Gilbert, 1972). If serum proteins are necessary for normal development of the chick embryo then these proteins must either be provided by the albumen and/or yolk or they must be produced by the developing embryo or its associated extraembryonic structures.

Weller (1966a) compared serum from 12 day old chick embryos with albumen and with the water soluble fraction of yolk, referred to as livetin. Using immunoelectrophoresis, he observed that transferrin was present in both albumen (the transferrin in albumen is referred to as conalbumin or ovotransferrin) and 12 day embryo serum. When livetin and serum were compared, he noted that several proteins were common to both, including prealbumin, α 3-globulin and transferrin. By comparing adult hen serum to 12 day embryo serum, Weller showed that two serum proteins were unique to the embryo serum. These proteins, referred to by Weller as embryo-specific α 3-globulin and α 4-globulin, were not detected in either albumen or livetin. He concluded that these proteins were specific to the embryo.

It was suggested by Schechtman (1952, 1956) that serum proteins contained in egg yolk and egg white may be transferred into embryonic circulation. While Weller (1966a) acknowledges that this may be true, his detection of embryo-specific proteins in embryo serum led him to propose that at least some serum proteins are synthesized by the embryo.

C. The Role of the Yolk Sac as a Mediator of Nutrient Uptake

The yolk sac of the chick embryo mediates the transfer of material from the yolk into the extraembryonic circulation. Once it has entered the circulatory system, material can be transferred to the embryo proper. Because

the yolk sac acts as a "nutritional sieve", it plays an important role in regulating which serum proteins are transferred directly from yolk into the embryonic circulation. It also mediates the uptake of yolk protein which can be degraded for use as precursors in serum protein synthesis.

Development of the Yolk Sac

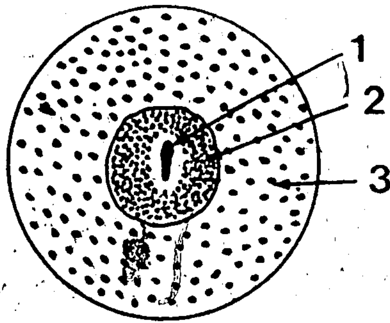
During the first day of chick embryo development the blastodisc differentiates into the *area pellucida*, which forms the embryo proper, and the *area opaca*, which forms the yolk sac and other extraembryonic membranes (Figure 1a). At the primitive streak stage of development, the *area opaca* consists of an epithelium of ectoderm cells with an underlying layer of endoderm cells. Mesoderm is only present in the posterior region of the *area opaca*. By the one somite stage, the mesoderm expands into the anterior region of the *area opaca* along both lateral margins of the *area pellucida* and by the 14 somite stage, it fuses at the anterior midline. Concomitant with this process, a horizontal division arises in the mesoderm of the *area pellucida* (1 somite stage), dividing this tissue into somatic and splanchnic mesoderm. By the 7 somite stage, this separation extends into the *area opaca* to the distal region of the mesoderm (Romanoff, 1960; Balinsky, 1975).

Mesoderm invades the *area opaca* at early somite stages and the splanchnic mesoderm differentiates into both

Figure 1. Diagramatic representation of growth of the chick embryo yolk sac during the first 3 days of development (redrawn from Romanoff, 1960). a and a', dorsal and lateral views of the chick embryo after 1 day of development; b and b', after 2 days; c and c', after 3 days. Structures are indicated with arrows as follows: 1, primitive streak in the *area pellucida*; 2, *area opaca*; 3, yolk; 4, *area vasculosa*; 5, *area vitellina*; 6, *sinus terminalis*.

FIGURE 1.

a



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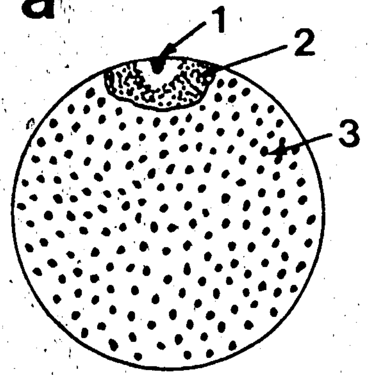

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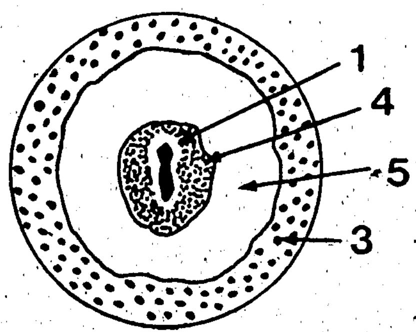

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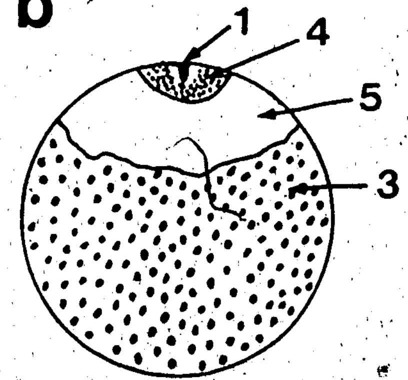
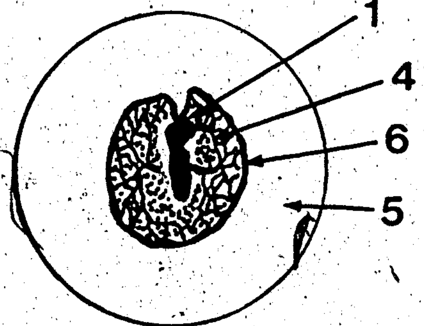


c



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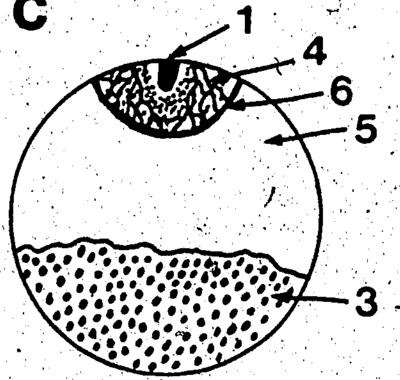


mesenchymal tissue and vascular tissue which consists of densely packed regions of mesoderm called blood islands. This vascular region of the *area opaca* is now referred to as the *area vasculosa* and the region peripheral to it is the *area vitellina*. Blood islands differentiate into endothelium and blood cells, which form an irregular network of capillaries by the 5-7 somite stage. Blood begins to flow at about the 16 somite stage. At the same time as circulation is being established, a large vessel called the *sinus terminalis* differentiates at the distal edge of the *area vasculosa* (Figure 1b). By the end of 2 days incubation, the *area vasculosa* is well defined. After a further 12-14h of incubation, the vitelline arteries differentiate and vitelline veins develop by the third day (Figure 1c). The network of extraembryonic vessels drain into the vitelline veins which are continuous with the ductus venosus of the embryo. The vitelline arteries complete the circulatory route as they connect the dorsal aorta of the embryo to the network of vessels in the *area vasculosa* (Romanoff, 1960; Balinsky, 1975).

The Yolk Sac Endoderm

The yolk sac consists of a layer of endoderm cells which lie directly over the yolk and a layer of splanchnic mesoderm which differentiates into blood vessels, blood cells and mesenchymal cells as described above. The developing yolk sac spreads over the surface of the yolk and

the *area vitellina* passes the "equator" of the yolk during day 3 of incubation (Figure 1c). By 5 days, the yolk is completely surrounded by yolk sac endoderm. The endoderm cells of the yolk sac mediate the transfer of material from the yolk to the circulatory system in the *area vasculosa* and therefore play a crucial role in providing nutrients to the developing embryo.

Yolk sac endoderm cells, herein referred to as extraembryonic endoderm cells or EEC, take up yolk by phagocytosis during the first week of development (Bellairs, 1963). Detailed electron microscope studies of the EEC showed that these cells form a simple columnar epithelium in the *area vasculosa* and contain large intracellular yolk droplets (Lambson, 1970; Mobbs and McMillan, 1979). The apical surface of the EEC is in direct contact with yolk and has numerous microvilli associated with it. Mobbs and McMillan (1979) observed numerous bristle coated regions in the apical membrane and some of these regions formed bristle coated vesicles by invagination. Vacuoles were present between the apical membrane and intracellular yolk droplets, and the cytoplasm in this region contained smooth canaliculi associated with apical and lateral plasma membranes. Intercellular tight junctions were observed at the apical pole of EEC. In addition to large yolk droplets, the basolateral cytoplasm contained the nucleus, rough endoplasmic reticulum, ribosomes, mitochondria and occasionally, small Golgi complexes. Extracellular yolk

granules were sometimes observed in association with regions of bristle coated membrane and similar material was seen in apical vacuoles. These workers suggested that tight junctions seal the extracellular yolk compartment from the vitelline circulation and that uptake of yolk is mediated by the EEC and involves bristle coated vesicles and apical vacuoles. By introducing horseradish peroxidase (HRP), ferritin and latex spheres to the extracellular yolk of embryos incubated both *in ovo* and *in vitro*, Mobbs and McMillan (1981) examined the process of protein transport through the cells of the extraembryonic endoderm. When embryos were fixed within 1 min of exposure to HRP, this protein was detected along apical bristle coated membrane, in bristle coated pits and vesicles and in smooth canaliculi. After 15 min of exposure to the tracer, HRP was observed in apical vacuoles and by 2-6h, this protein was detected in intracellular yolk droplets and on the extracellular side of the ~~basal membrane~~ of ~~cells~~. Ferritin uptake followed a similar pathway but was also observed in the basal half of intercellular spaces. Latex spheres which were similar in size to extracellular yolk granules were observed in bristle coated vesicles, apical vacuoles and intracellular yolk droplets but never in intercellular spaces or on the extracellular side of the basal membrane.

Because all tracer substances were observed in bristle coated vesicles and apical vacuoles but only ferritin was observed in intercellular spaces between cells, these

workers propose two alternate pathways for protein transport in EEC. In one pathway, proteins are transported to the intracellular yolk droplet for lytic digestion. Continuous endocytosis of yolk granules would replenish the intracellular yolk droplet as nutrient protein is degraded. Rough endoplasmic reticulum and Golgi were observed in the basal cytoplasm of EEC suggesting that new proteins are synthesized from raw materials provided by the breakdown of nutrient protein. Vesicles and canaliculi were observed in association with the lateral membrane but not at the basal surface. It may be that products of intracellular digestion and/or protein synthesis cross the basal membrane by diffusion or transport. Hassel and Klein (1971) observed that chick embryos with intact yolk sacs cultured on medium containing radiolabelled ovalbumin accumulated ovalbumin in the *area opaca* then degraded it. These workers detected label in newly synthesized protein throughout the embryo. Ovalbumin is apparently used as raw material for protein synthesis and may be routed by some mechanism into the lytic digestion pathway in EEC.

In the second pathway, certain proteins may bypass the intracellular yolk droplet and be transported directly to the lateral membrane of EEC. Vesicles and canaliculi were observed in association with this membrane and they could, in principle, release intact protein by exocytosis into intercellular spaces for diffusion to the vitelline circulation. Linden and Roth (1978) showed that the chick

yolk sac has specific binding sites for immunoglogulin G (IgG). This molecule localizes along coated regions of the apical membrane of EEC and is then internalized. They propose that this process is mediated by receptor molecules. IgG is transferred intact from yolk into the vitelline circulation.

In the guinea pig yolk sac, exogenous IgG localizes at the apical surface of EEC, enters endocytic vesicles and is then exocytosed at the lateral plasma membrane into intercellular spaces (King, 1977). Similar observations were made in rabbit yolk sac (Moxon and Wild, 1976). These workers proposed that protein specific receptor molecules in coated regions of the apical plasma membrane mediate the segregation of yolk protein. This could be accomplished by partitioning proteins into separate vesicles depending on whether they are to be transported to the yolk droplet or to the lateral membrane for exocytosis.

If serum proteins are transported intact from yolk to the vitelline circulation of the chick embryo then changes in the types or amounts of serum proteins as development proceeds could be mediated by changes in selective uptake and/or transport processes in EEC. If serum proteins are synthesized during development then degradation of nutrient protein in the EEC may be providing raw materials for this process.

Yolk Protein as a Nutrient for the Developing Chick Embryo

Yolk is a heterogeneous mixture of protein and lipid and is particularly suited to provide nutrients to the developing chick embryo (Cook, 1968; Shenstone, 1968). Bellairs (1961) examined yolk from unincubated eggs by electron microscopy and observed both high density granules and low density globules; both of these were suspended in dissolved material. When yolk is centrifuged at high speeds, it separates into 3 fractions. Cook (1968) described a low density fraction (LDF) which contains lipovitellenin (a lipoprotein) and a granular fraction which contains phosvitin (a phosphoprotein) and α and β lipovitellin (lipoproteins). The third fraction is water soluble and is referred to as livetin. This fraction can be separated by electrophoresis into 3 components, α, β and γ livetin (Martin *et al*, 1957). These livetin proteins were later identified as the serum proteins serum albumin, transferrin and γ globulin respectively (Williams, 1962a).

In order to determine how chick embryos utilize protein as a nutrient, Walter and Mahler (1958) injected radiolabelled proteins, peptides and amino acids into the yolk and albumen of unincubated eggs. After 5-9 days incubation, embryos were harvested and the labelled protein in embryos was recovered by precipitation with trichloroacetic acid. Very little label was recovered from embryos when precursor proteins were injected into albumen and they concluded that proteins in albumen are not used as

precursors for embryonic proteins. In contrast, proteins, peptides and amino acids injected into yolk were all utilized with similar efficiency. Because yolk contains a large amount of protein but very few free amino acids or peptides (Romanoff and Romanoff, 1949), these workers concluded that the exogenous proteins were diluted to a great extent by endogenous yolk proteins whereas peptides and amino acids were not. Based on this assumption, they concluded that yolk proteins are the preferred precursors for protein synthesis in the embryo. Their conclusion was corroborated by Grau *et al* (1962) who replaced yolk with saline in eggs incubated for 3 days, allowed one day for recovery then replaced the saline with various nutrient media. The percent survival of embryos over 120 hours of incubation was compared for media containing free amino acids and media containing the milk protein, casein. Their results showed that embryo survival depended on the presence of protein in the medium, not amino acids.

Under culture conditions, proteins from yolk support the growth of chick embryos to a greater extent than media prepared from non-yolk proteins (Klein, 1968). Yager and Klein (1974) compared the ability of individual yolk proteins to support chick embryo growth by preparing culture medium from different yolk fractions. These workers removed 11-13 somite embryos (40h incubation) with intact *areae vasculosae* and cultured them on agarose medium which contained either lipovitellinen (LDF), phosvitin,

lipovitellin (α and β) or livetin (serum protein fraction). Growth was quantified by measuring the protein nitrogen and DNA accumulation in individual embryos. Using these parameters as estimates of growth, they concluded that both livetin and lipovitellin supported embryo growth but phosvitin and lipovitellinen did not. Yager and Klein (1974) also tested various combinations of yolk proteins and found that optimal growth was achieved when lipovitellin was supplemented with 10% livetin. Lipovitellin is present in the granular fraction of yolk (Cook, 1968) and may be an important source of nutrient protein for the developing embryo. Yager and Klein (1974) proposed that this protein is used as a source of amino acids. Yolk granules containing lipovitellin may enter the lytic digestion pathway in EEC to provide raw materials for protein synthesis. As described previously, EEC take up latex spheres the size of yolk granules by endocytosis and transport them to intracellular yolk droplets (Mobbs and McMillan, 1979; 1981). Embryo growth is greatly enhanced by the livetin fraction of yolk which, as previously mentioned, contains serum proteins. These proteins are apparently important to the developing embryo. Yager and Klein (1974) suggested that they are transported intact by EEC from yolk to the vitelline circulation, presumably by the alternate intact transport pathway proposed by Mobbs and McMillan (1981).

D. Serum Protein Synthesis in the Chick Embryo

The proteins in plasma are often divided into three major groups, fibrinogen, albumin, and globulin, based on their solubility properties in sodium and ammonium sulphate. Fibrinogen is the precursor of fibrin, the clotting protein, and is removed along with blood cells and platelets when serum is prepared. Albumins and globulins are present in serum and when subjected to zone electrophoesis, separate into fractions with distinct boundaries. These fractions include albumin and several globulin groups named in order of decreasing mobility as follows: Albumin; α_1 and α_2 globulin, β globulin and γ globulin. Each of these fractions is a heterogeneous mixture of proteins. Serum proteins act as buffer molecules, nutrient molecules (albumin) and provide osmotic pressure in blood. In addition, they function in immunity (immunoglobulins) and transport insoluble substances, such as lipids. Other transport molecules are present in serum such as transferrin, an iron binding glycoprotein (Harper et al, 1977; Hall and Malia, 1984).

The serum of developing chick embryos contains proteins which are identical to adult serum proteins (prealbumin, albumin and transferrin) and proteins which are specific to the embryo (certain α globulins; Weller, 1976). Some proteins in the chick embryo may be transferred intact from the livetin fraction of yolk into blood whereas others may be synthesized from breakdown products of nutrient protein.

Kram and Klein (1976) examined serum protein synthesis in the chick embryo by culturing 11-13 somite embryos with intact *areae vasculosae* in medium containing radiolabelled valine. The culture medium containing secreted proteins was collected and proteins were separated using polyacrylamide gel electrophoresis. Densitometry was used to detect protein bands in gels which were then frozen and sliced. Incorporation of radiolabelled valine into protein was determined for each gel slice using a scintillation counter. By comparing newly synthesized secreted proteins with proteins in serum drawn with a syringe from similar embryos, these workers identified the newly synthesized proteins as transferrin, α globulins 'a' and 'b' and prealbumin. When the *area vasculosa* and the embryo proper were analyzed separately, it was determined that the *area vasculosa* was the site of serum protein synthesis. In subsequent experiments, microdissection was used to separate the endoderm (EEC), mesoderm and ectoderm of the *area vasculosa* of embryos incubated for 4 days (Young *et al*, 1980). The incorporation of labelled precursor into serum proteins was determined for each tissue layer and it was shown that the site of serum protein synthesis in the chick embryo is the EEC.

Transferrin is present in the livetin fraction of yolk but is also synthesized by EEC. Evidently the presence of serum proteins in yolk does not not preclude their synthesis by EEC. As Schechtman proposed (1952;1956), some of these

proteins may be transported intact from the yolk to the embryo, but the circulating serum proteins in the chick embryo are, at least in part, determined by serum protein synthesis in EEC.

E. Cell Sorting Experiments and Aggregation of Cells from the Extraembryonic Endoderm

When early chick embryos are dissociated and cultured in a rotating water bath, aggregates form which are composed of two distinct populations of cells (Zalik and Sanders, 1974; Sanders and Zalik, 1976). To analyze the process of cell sorting, these workers dissociated prestreak and early primitive streak embryos and allowed them to aggregate for various time intervals. When observed under the light microscope, aggregates cultured for 24h and 48h sorted into a population of densely packed cells surrounded by a layer of loosely packed cells. This outer layer of cells became folded after 5 days in culture. The yolk sac endoderm of intact embryos also becomes highly folded after several days of development (Rommanoff, 1960). Some cells in the dense phase underwent further development and differentiated into cells which resembled notocord and cartilage. A *basal lamina* was observed between the dense cells and the loose peripheral cells. These workers suggested that the densely packed cells originate from the epiblast of the prestreak or early streak embryo and the loose peripheral cells originate from the hypoblast. This was later confirmed in another

laboratory when separate groups of embryos were used to obtain epiblast cells and hypoblast cells (Eyal-Giladi *et al*, 1975). By radiolabelling one set of embryos with ^3H thymidine and then allowing cells to aggregate, these workers demonstrated that the epiblast cells and hypoblast cells sorted out into two histologically distinct populations.

Because cell sorting was observed in aggregates prepared from dissociated embryos, a technique was developed which allowed for isolation of a pure population of EEC so that selective cell adhesion could be studied in detail. Milos *et al* (1979) dissociated EEC from the *area opaca* of primitive streak embryos and observed that they aggregate rapidly in culture into solid spheres. Small cavities appeared within 24h and by 48h the aggregates were organized as vesicles composed of a large cavity surrounded by a thin layer of cells. The organization of EEC in aggregates resembles the thin endoderm layer in intact yolk sacs and these workers proposed that cavity formation is an intrinsic property of EEC. A detailed study using light and electron microscopy revealed that EEC in cultured aggregates and EEC in the intact *area opaca* have very similar intercellular junctions (Milos *et al*, 1984). It was also noted that similar cell shape changes occur in EEC aggregates and *in vivo*.

Serum Protein Synthesis in Cultured Aggregates of Cells from the Extraembryonic Endoderm

Chick embryo explants at the 11-13 somite stage of development require livetin, the serum protein fraction of yolk, for optimal growth (Yager and Klein, 1974). Embryo-specific serum proteins which are not present in livetin have been detected in the serum of 12 day embryos (Weller, 1966a) and it has been demonstrated that the EEC of the *area opaca* synthesize serum proteins (Young *et al*, 1980; Young and Klein, 1983). When EEC are cultured in rotating flasks they organize into structures which morphologically resemble the yolk sac endoderm (Milos *et al*, 1979; 1984). The purpose of the research reported here was to determine whether or not EEC aggregates also resemble the EEC of intact yolk sacs with respect to serum protein synthesis. The pattern of serum protein synthesis and secretion in cultured aggregates prepared from the EEC of primitive streak stage chick embryos was analyzed. This study also examined temporal changes in serum protein synthesis and secretion because serum proteins which are stage-specific may play an important role in embryogenesis. Aggregates were cultured for various time intervals and the proteins present in EEC and in the culture medium were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). These protein banding profiles were compared to reference proteins in 4 day embryo serum so that serum proteins present in EEC and in the culture medium could be

identified. In some experiments, radiolabelled amino acids were introduced into the medium so that serum proteins synthesized during the culture interval could be detected. Fluorography was used to determine which serum protein bands in SDS gels were labelled. Temporal changes in the pattern of serum protein secretion and synthesis in aggregates were detected.

II. MATERIALS AND METHODS

A. Preparation of Serum

Young *et al* (1980) reported a method for collecting serum from 8 day old chick embryos and this technique was modified for the purposes of this study. Serum was collected by dissecting out embryos incubated for 4 days after clamping the major vitelline arteries and veins which connect the embryo to the yolk sac. Embryos were rinsed in a modified Pannett and Compton (1924) saline (PCS; 82.8mM NaCl, 8.3mM KCl, 2.8mM $CaCl_2$, 2.5mM $MgCl_2$ and 1.5mM Hepes buffer (Sigma); adjusted to pH 7.5 with NaOH), and placed into a flask in a rotating water bath at 37°C and 30 revolutions/min for 1h. The embryos bled slowly into the flask and blood was collected by filtering the contents of the flask through several thicknesses of cheesecloth. The red blood cells were sedimented by centrifugation at 1000xg for 10 min at 4°C and the supernatant was collected and centrifuged again at 12,000xg for 30 min. The fluid fraction was removed and stored at -80°C.

B. Production of Aggregates from Cells of the Extraembryonic Endoderm

Fertile White Leghorn eggs were obtained from the University of Alberta Experimental Farm and incubated at 37°C and 55% humidity for 18h to the primitive streak stage (stage 4; Hamburger and Hamilton, 1951). Embryos and their

surrounding vitelline membranes were removed and transferred to a petri dish containing PCS. After 1h, the vitelline membranes with intact embryos were gently lifted off the yolk and placed into fresh PCS. Gentle swirling in the saline separated the embryos from their vitelline membranes. The wash was continued through several changes of PCS to remove as much yolk as possible.

Embryos were transferred into fresh PCS and examined under a dissecting microscope. Only those embryos at stage 4 (Hamburger and Hamilton, 1951) were selected and iridectomy scissors were used to separate the *area opaca* from the *area pellucida*. *Areae opacae* were suspended in 3ml of PCS for cell dissociation (approximately 35 embryos per 3ml PCS). Using a technique developed by Milos *et al* (1979), extraembryonic endoderm cells (EEC) were dissociated from overlying ectoderm by the following method: The *areae opacae* were pipetted using pipettes of decreasing bore size (internal diameters approximately 5mm-2mm). When the cell suspension was cloudy, it was filtered through 44μ Nitex mesh (B.& S.H. Thompson & Co. Ltd., Montreal). The sheets of ectoderm cells were retained in the mesh while clumps of endoderm cells passed through the mesh and were present in the filtrate. Endoderm cells were sedimented by centrifugation at 60xg for 4 min and the pellet was resuspended in Ca^{2+} Mg^{2+} free PCS (CMF-PCS). Cells were held on ice for 15 min and pipetted with a flamed pasteur pipette (internal diameter 0.5mm-1.0mm) to produce a suspension of

single cells. The cells were washed free of yolk through 3 cycles of suspension in saline followed by centrifugation at 60xg. The yolk remained in the supernatant and was discarded. The cells were resuspended in 3ml of Leibovitz (L-15) medium (GIBCO). The cell suspension was transferred to a 10ml Erlenmeyer flask and cultured in a rotating water bath at 37°C and 80-100 revolutions/min for 6-30h as required for each experiment. Approximately 35 embryos were dissociated for each flask and under the described conditions, 70 to 90 aggregates ranging in size from 0.3mm to 0.7mm formed within several hours.

C. Determination of Protein Secretion by Aggregates of Cells from the Extraembryonic Endoderm

Aggregates of EEC were prepared as described, cultured for 24h and separated from the culture medium by centrifugation at 19xg for 5 min. The medium was saved and aggregates were washed several times in Tris(hydroxymethyl)aminomethane buffer (Sigma; 0.0625M, pH 6.8) containing 0.25mM phenylmethylsulfonylfluoride (Sigma; Tris-PMSF). Aggregates were homogenized on ice in 200-300μl of Tris-PMSF and homogenates were centrifuged at 100,000xg for 1h to sediment particulate material. Supernatants which contained soluble proteins were collected and stored at -20°C and are herein called EEC extracts.

The culture medium from these experiments was centrifuged at 100,000xg for 1h and the supernatants were

collected. Because L-15 culture medium contained inorganic salts, amino acids, phenol red and other components, further preparation was required to remove these substances from culture medium samples. Because of the limited amount of protein available for analysis in this system, it was also necessary to concentrate the sample in order to allow for the detection of any secreted proteins by SDS-PAGE. This was accomplished in one of two ways: samples of culture medium were dialyzed against 4 changes of water (1000x sample volume) at 4°C (Spectrapor dialysis tubing, 8000 MW cutoff), lyophilized and reconstituted in 200-300μl of Tris-PMSF. Alternatively, samples of culture medium were concentrated in Centricon microconcentrators (Amicon; 10,000 MW cutoff) by centrifugation at 5000xg. Molecules retained in the concentrate were washed with Tris-PMSF and reconcentrated several times to a volume of 200-300μl. In some experiments, the secretion of serum proteins by EEC aggregates was studied at different time intervals of culture. For this purpose, freshly dissociated EEC were divided into four aliquots and three aliquots were cultured in L-15 medium for 6h, 18h or 30h respectively. The fourth aliquot of EEC was washed in L-15 medium and homogenized immediately. This EEC extract and the L-15 wash medium were treated as '0h' controls. All samples of aggregates and culture medium were collected and prepared for SDS-PAGE as described above.

D. Determination of Protein Synthesis in Aggregates of Cells from the Extraembryonic Endoderm

In preliminary experiments, the incorporation of various labelled precursors into protein was analyzed and compared. For this purpose, aggregates were prepared as described and cultured for 24h in the presence of 30μCi of ^{3}H labelled valine (New England Nuclear, specific activity 54.5 Ci/mM) or 30μCi of a mixture of amino acids labelled with either ^{3}H or ^{14}C (New England Nuclear); in the mixtures, specific activities varied for each amino acid. For the mixture labelled with ^{3}H the specific activites for each amino acid expressed in Ci/mM were: L-alanine, 70.1; L-arginine, 25.0; L-aspartic acid, 13.8; L-glutamic acid, 20.1; glycine, 41.2; L-histidine, 11.4; L-isoleucine, 75.1; L-leucine, 60.0; L-lysine, 88.7; L-phenylalanine, 21.0; L-proline, 36.8; L-serine, 7.1; L-threonine, 2.9; L-tyrosine, 48.0 and L-valine, 54.5. For the mixture labelled with ^{14}C the specific activities for each amino acid expressed in mCi/mM were: L-alanine, 176.0; L-arginine, 324.0; L-aspartic acid, 220.0; L-glutamic acid, 294.0; glycine, 110.0; L-histidine, 337.0; L-isoleucine, 331.0; L-leucine, 345.0; L-lysine, 317.0; L-phenylalanine, 494.5; L-proline, 273.0; L-serine, 169.0; L-threonine, 227.0; L-tyrosine, 492.0 and L-valine, 286.0.

Aggregates were separated from the culture medium by centrifugation at 19xg for 5 min. The medium was collected and the aggregates were washed 5 times in Tris-PMSF and

homogenized on ice in 500μl of Tris-PMSF. The resulting homogenates were centrifuged at 100,000xg for 1h to remove the particulate fraction and the supernatant which contained soluble proteins was removed. The culture medium was also centrifuged at 100,000xg for 1h. An aliquot of both the EEC extract and the culture medium were removed for protein determination and the volume of EEC extract was brought to 1ml using Tris-PMSF. Because the culture medium sample contained very small amounts of protein, 0.001% unlabelled bovine serum albumin was added to the sample as a carrier, to increase the total protein concentration and reduce the loss of labelled protein during the following procedure: Both the EEC extract and the medium sample were cooled on ice for 1 min. An aqueous mixture of ice cold 15% trichloroacetic acid (TCA) and 5% phosphotungstic acid (PTA) was added to each sample at a ratio of 10:1 (V/V) to precipitate proteins. Each tube was mixed on a vortex mixer and centrifuged at 1725xg. The supernatant was discarded and the protein precipitate was washed 3 times in cold 15% TCA - 5% PTA. The pellet was dissolved in 100μl of water and counted in 10ml of Aquasol (New England Nuclear) using an LKB Rackbeta Liquid Scintillation Counter. Incorporation was calculated as counts per minute (CPM) per μg of protein. These data were used to determine whether EEC aggregates incorporated labelled precursors into newly synthesized protein under the conditions used in this study, and to estimate the CPM of labelled protein loaded onto gels used

for fluorography.

For fluorography, aggregates were prepared as described and cultured for 24h in the presence of 30μCi of a mixture of amino acids labelled with either ^{3}H or ^{14}C. Both aggregates and culture medium were collected and prepared for SDS-PAGE as described for the experiments designed to detect protein secretion (Section C, Materials and Methods). In some experiments, the synthesis of proteins was compared at different time intervals of culture. For this purpose, freshly dissociated EEC were divided into four aliquots and three aliquots were cultured for 6h, 18h and 30h respectively. Exactly 6h prior to the end of each culture period, 30μCi of amino acid mixture labelled with either ^{3}H or ^{14}C was added to each sample; this corresponds to 0h, 12h and 24h after dissociation, respectively. Aggregates were cultured for the final 6h and both aggregates and culture medium were prepared for SDS-PAGE as described previously (Section C, Materials and Methods). The fourth aliquot of EEC was washed in unlabelled L-15 medium and homogenized immediately. This EEC-extract and wash medium were treated as unlabelled '0h' controls.

E. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Preparation of Samples

The concentration of protein in serum, EEC extracts and culture medium was determined by the method of Bradford

(1976) using the BioRad Kit and γ globulin as a standard. For SDS-PAGE, samples were diluted to appropriate concentrations with Tris-PMSF. Each sample was mixed with an equal volume of sample buffer (0.0625M Tris-pH 6.8, 4% SDS, 20% sucrose and .002% bromophenol blue) according to Payne (1976) and samples were heated at 60°C for 30 min prior to electrophoresis.

The following adult chicken serum and egg white proteins were purchased for use as reference standards to assist in the identification of proteins in 4 day serum: Serum immunoglobulin G (IgG; Calbiochem), egg white conalbumin (Sigma), serum albumin (Sigma) and egg white avidin (Sigma). Thick albumen was collected from eggs incubated for 24h, diluted in Tris-PMSF and prepared for SDS-PAGE as described for serum, EEC extract and culture medium. High and low molecular weight markers for molecular weight determination of proteins were obtained commercially (BioRad).

Electrophoresis

To provide a good comparison between various samples being analyzed in this study, a vertical slab gel electrophoresis system with an upper and lower buffer chamber was selected as described by Payne (1976; purchased from Tyler Research Corporation). The best separation of serum proteins was obtained using 12.5% acrylamide gels with a 5% stacking gel which were prepared from the following

stock solutions:

1. Acrylamide-bisacrylamide:

    30g acrylamide (Eastman Kodak)

    0.8g bisacrylamide (BioRad)

    Dissolve in 100ml water.

2. 10% Sodium dodecyl sulphate (SDS) (BioRad)

3. 1.5% Ammonium peroxydisulphate (APS; freshly prepared for each gel) (Fisher Scientific)

4. N,N,N',N'-Tetramethylenediamine (TEMED) (Eastman Kodak)

5. Stacking gel buffer:

    6g Tris (Sigma)

    48ml 1N HCl

    Add water to a total volume of 100ml.

    Adjust pH to 6.8 using HCl.

6. Separating gel buffer:

    36.3g Tris

    Add water to a total volume of 100ml.

    Adjust pH to 8.8 using HCl.

7. Electrode buffer:

    6g Tris

    28.8g glycine (Sigma)

    Add water to a total volume of 1000ml, pH 8.3.

The gel mould was prepared by clamping spacers (1.5mm

thickness) between 2 glass plates and sealing the plates with molten agarose (1.5%; Eastman Kodak). The separating gel was prepared by mixing the stock solutions as follows:

Acrylamide-bisacrylamide	16.7ml
Tris-HCl (pH 8.8)	5.0ml
10% SDS (aqueous)	0.4ml
TEMED	40µl
Water	15.9ml
1.5% APS (aqueous)	2.0ml

The APS was added after degassing the mixture for 2 min. The solution was poured between glass plates, overlayed carefully with a layer of aqueous 1% SDS and left to polymerize for 20 min. The stacking gel was prepared by mixing the stock solutions as follows:

Acrylamide-bisacrylamide	1.7ml
Tris-HCl (pH 6.8)	2.5ml
10% SDS (aqueous)	100µl
TEMED	10µl
Water	5.22ml
1.5%APS (aqueous)	0.5ml

The APS was added after degassing the mixture for 2 min. The surface of the polymerized separating gel was rinsed with water to remove the residual SDS. The stacking gel was

poured over the separating gel and a comb was inserted to mould sample wells. After polymerization, the comb was removed and the gel was secured in the running chamber. Molten 1.5% agarose was poured into the seam between the glass plate and the running chamber to ensure a leakproof seal. Electrode buffer (50mM Tris, 384mM glycine, pH 8.3) was added to the upper and lower buffer chambers and samples were loaded into the wells in 20μl or 30μl aliquots. Electrophoresis was carried out at 10-20mA overnight at 4°C (constant current). When the dye front ran to within 2 cm of the end of the gel, the gel was removed and fixed in the appropriate solution.

Gel Staining

Gels were initially stained for protein with Coomassie Brilliant Blue as described by Payne (1976) but this technique was not sensitive enough to detect protein in low concentration. Because greater sensitivity was required, a silver staining method (Oakley *et al*, 1980) was modified in order to enhance staining using glutaraldehyde, as described by Dion and Pomenti (1983). Gels were soaked overnight in aqueous 50% methanol (reagent grade) and 10% glacial acetic acid to remove SDS, rehydrated in aqueous 5% methanol and 7% acetic acid and soaked in aqueous 10% glutaraldehyde (BHD Chemicals) for 1h. In order to remove all the gluteraldehyde, gels were washed in a minimum of 4 changes of water over a 2h period with gentle agitation as

recommended by Giulan *et al* (1983). The silver staining protocol was carried out as follows:

1. Silver stain:

   Solution A: Dissolve 1.6g of silver nitrate (Fisher) in 8ml of water.

   Solution B: Mix 42ml of 0.36% sodium hydroxide with 2.8ml of 14.8 M ammonium hydroxide.

   Add solution 'A' dropwise to solution 'B' stirring constantly and add water to 100ml.

2. Stain gel for 15 min with constant agitation and wash for 5 min in distilled water.

3. Developer:

   2.5ml ~~1%~~ aqueous citric acid (Fisher)

   0.25ml 38% formaldehyde

   Add distilled water to 500ml

4. Place gel into developer with constant agitation until bands appear. Wash for 5 min in distilled water.

5. Stop development with aqueous 45% methanol and 10% acetic acid. Store gels in distilled water.

Occasionally an undesirable background developed during step 4. If this occurred, gels were destained in Kodak Rapid Fix for 5-30 min, washed in Kodak Hypoclear for 1h and placed in aqueous 45% methanol and 10% acetic acid for 30 min. Gels were stored in water and migration distances were measured for regression analysis (Mobility = Distance

migrated/ Total migration distance of dye front). All gels were photographed with Pan X film and printed on Kodak Rapid Contrast photographic paper.

Fluorography

Radiolabelled EEC extracts and medium samples for fluorography were assayed and diluted as described and CPM of labelled protein loaded onto each gel were estimated using incorporation data obtained in experiments described previously (Section D, Materials and Methods). Gels were run as described for SDS-PAGE, stained with silver and soaked in an autoradiography enhancing solution (Enhance, New England Nuclear) for 40 min. Gels were dried in a BioRad gel dryer under vacumm for 2h, placed in a Kodak X-Ray Film Holder with Kodak XAR-5 X-Ray film and exposed at -80°C for 21 days. X-ray films were developed with GBX Autoradiography Developer and Fixer (Kodak) and photographed as described above.

F. Autoradiography of Aggregates of Cells from the Extraembryonic Endoderm

Aggregates of EEC were cultured in unlabelled medium or in the presence of 30µl of ^{14}C labelled amino acid mixture (NEN). After harvesting, aggregates were fixed overnight in Bouins' fixative (Luna, 1968) and dehydrated through a graded series of alcohols (30%, 50%, 60%, 70% and 80%, 2 times each for 10 min; 95% and 100%, 3 times each for 10

min). Aggregates were cleared in chloroform and embedded in paraplast. Sections of 5μ thickness were cut on a Spencer "820" microtome and mounted on slides which had been precoated with 0.5% gelatin containing 0.01% chromium potassium sulfate.

For autoradiography, mounted sections were soaked in two changes of xylene to remove paraffin (5 min each) and rehydrated through a graded series of alcohols (100%, 90%, 70%, 50% 2 times each for 2 min). Slides were dipped into distilled water twice, transferred to the dark room and placed in a water bath at 42°C. Kodak Nuclear Track Emulsion (NTB-3) was melted at 42°C; slides were coated with emulsion and dried in a light tight box at 4°C overnight. The slides were exposed in the dark at 4°C for 4-14 days. Following this, they were developed in Kodak D-11 developer for 2.5 min, rinsed in distilled water and fixed in Kodak Rapid Fix for a minimum of 10 min. A thorough 15 min wash in running tapwater preceeded staining.

The staining protocol used here was a hemotoxylin and eosin stain (Luna, 1968) adapted for autoradiography. Slides were stained as follows:

1. Harris Hemotoxylin (Luna, 1968), 1 min.
2. 100ml 70% alcohol + 5 drops 1N HCl, rapid.
3. Distilled water, 30 sec.
4. 100ml tap water + 5 drops ammonium hydroxide, 30-60 sec.

5. Distilled water, 5 min.
6. Eosin Yale (Luna, 1968), 90 sec.
7. 96% alcohol, rapid, 2 changes.
8. Absolute alcohol, rapid, 2 changes.
9. Xylene, 2 min, 2 changes.

After drying briefly, slides were mounted, examined for silver grains and photographed using a Zeiss Photomicroscope III.

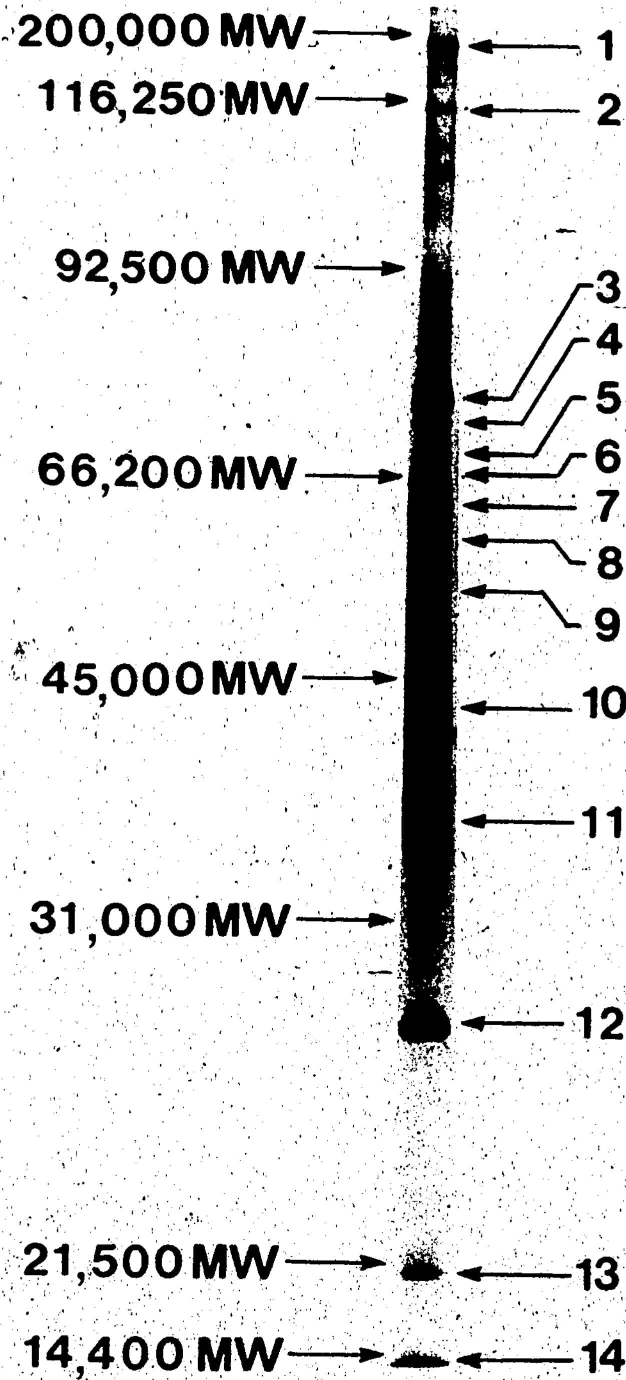
III. RESULTS

A. Preliminary Characterization of Serum From Embryos Incubated for Four Days

Serum Banding Profile in Sodium Dodecyl Sulfate Polyacrylamide Gels

Serum from 4 day chick embryos was analyzed using SDS-PAGE (herein referred to as 4 day serum); approximately 5µg of protein was loaded onto each gel. After gels were stained with silver, 14 distinct protein bands were observed (Figure 2). These proteins were labelled arbitrarily and consecutively from 1-14 representing high to low molecular weight proteins respectively. Proteins of known molecular weight were obtained from Biorad and used as standards to calculate the molecular weight of each protein band in serum. The molecular weight of each marker protein is indicated on the left side of the gel. When gels were stained with silver to detect proteins in 4 day serum, which were present in very small amounts, overstaining of marker proteins run in the same gel sometimes occurred. The bands produced by molecular weight marker proteins in overstained gels were not well discriminated in photographs. For clarity of presentation, gel lanes containing these markers were ommitted from figures. The log MW of marker proteins and their mobility in gels (Mobility = Distance migrated in gels/ Total migration of dye front) were used for regression

Figure 2. A 12.5% SDS-polyacrylamide gel stained with silver showing 4 day serum. Serum proteins are numbered arbitrarily and consecutively from 1-14 and are indicated on the right side of the figure. The mean molecular weights of these proteins (n=5) and the frequencies with which they appeared in 5 experiments are summarized in Table 1. Serum protein 7 may correspond to α globulins 'a' and 'b' and serum protein 12 may correspond to prealbumin, based on previously reported molecular weight data (Kram and Klein, 1976; see text). The position of BioRad molecular weight markers are indicated on the left side of the figure and include: myosin (200,000 MW), β-galactosidase (116,250 MW), phosphorylase B (92,500 MW), bovine serum albumin (66,200 MW), ovalbumin (45,000 MW), carbonic anhydrase (31,000 MW), soybean trypsin inhibitor (21,500 MW) and lysozyme (14,400 MW).


200,000 MW
116,250 MW
92,500 MW
66,200 MW
45,000 MW
31,000 MW
21,500 MW
14,400 MW
1
2
3
4
5
6
7
8
9
10
11
12
13
14

analysis and the log MW of serum proteins was calculated from mobility data. Molecular weight determinations from 5 separate analyses were used to calculate the mean molecular weight ± standard error (SE) for each serum protein band. The results are summarized in Table I. Because serum protein 13 was absent in one gel, the frequency with which the different serum proteins were detected in gels is also reported (Frequency = Number of times a protein band was observed/ Total number of experiments). It should be noted that determination of the molecular weight of proteins at the dye front is limited to the molecular weight range of the standards used. In this study, the smallest protein used as a standard is 14,400 MW (lysozyme) so any proteins at the dye front could, in principle, be smaller than this.

Putative Identification of Serum Proteins

An attempt was made to identify as many as possible of the 14 proteins observed in 4 day serum. For this purpose adult chicken serum proteins and egg white proteins that were available from commercial sources were purchased. Conalbumin is a protein component of egg white which corresponds to serum transferrin; both have the same amino acid sequence but differ in their carbohydrate moieties (Williams, 1962b). Because it was the only transferrin molecule which was available as a relatively pure protein preparation, conalbumin was selected as a reference standard for embryonic serum transferrin in this study. Adult chicken

Table 1. Mean molecular weights (n=5) and relative frequencies of proteins in 4 day serum. Proteins were separated by their molecular weight using SDS-PAGE (Figure 2). The proteins were named arbitrarily and consecutively from 1-14 and data from 5 experiments were pooled. Mean molecular weight ± SE was calculated for each protein and the relative frequency was calculated for each protein as follows: Number of times protein band was observed/ Total number of experiments.

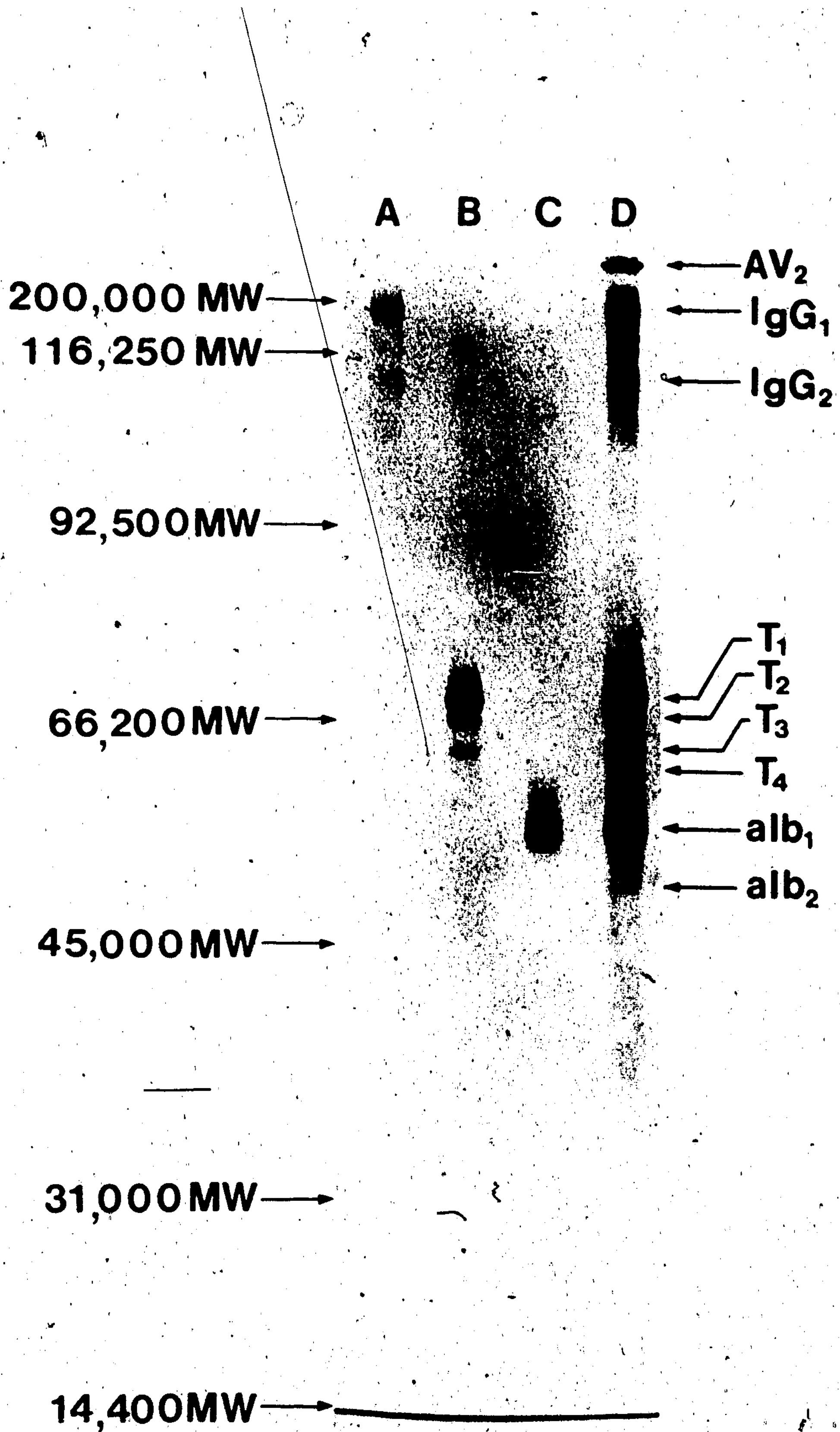
TABLE 1.

PROTEINS	MEAN MOLECULAR WEIGHT ± SE	RELATIVE FREQUENCY
1	148,000 ± 2600	1.0
2	134,200 ± 1900	1.0
3	79,500 ± 1300	1.0
4	76,600 ± 1400	1.0
5	72,300 ± 1500	1.0
6	69,800 ± 1800	1.0
7	66,500 ± 1400	1.0
8	62,200 ± 1200	1.0
9	56,500 ± 1600	1.0
10	47,200 ± 1600	1.0
11	38,600 ± 600	1.0
12	26,500 ± 1100	1.0
13	17,600 ± 300	0.8
14	15,400 ± 800	1.0

serum albumin, adult chicken serum IgG and egg white avidin were also available commercially and were selected as reference standards. Figure 3 is a representative gel showing IgG (lane A), conalbumin (lane B) and serum albumin (lane C) run on a 12.5% SDS-polyacrylamide gel both separately and mixed (lane D). Mixed reference markers are also present in Figure 4 (lane A).

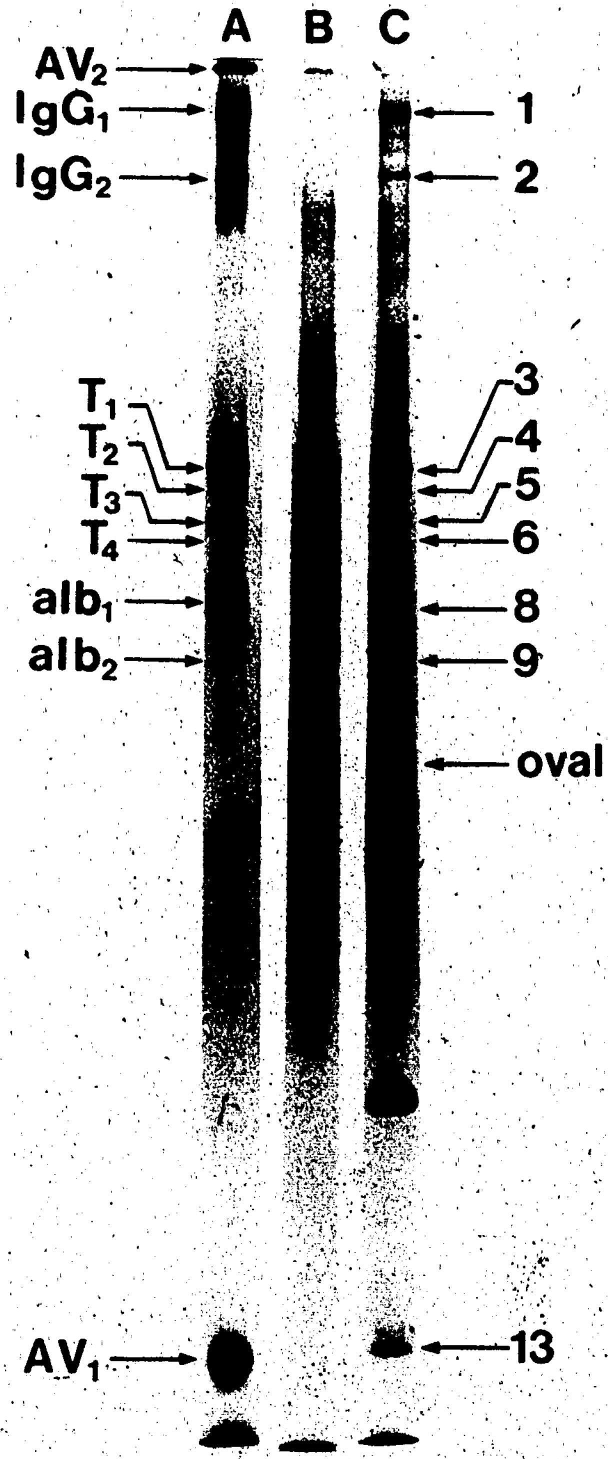
In the gel shown in Figure 3, each lane was loaded with 0.5μg of protein; the lane with mixed sample contained 0.5μg of each protein. Chicken serum IgG has a reported molecular weight of approximately 180,000 (Gallagher and Voss, 1964). Under the conditions of SDS-PAGE used in this study, it was detected as one dark staining band, IgG_1 (148,000 MW) and one light staining band, IgG_2 (134,200 MW, lanes A and D). An additional faint band was sometimes observed between IgG_1 and IgG_2. Conalbumin separated electrophoretically into 4 bands (lanes B and D), one major band, two light staining bands and one faint band. Because transferrin and conalbumin are apparently identical proteins (Williams, 1962b) these bands were named T_1 (79,500 MW), T_2 (76,600 MW), T_3 (72,300 MW) and T_4 (69,800 MW) from high to low molecular weight respectively. Although band T_4 was often faint and sometimes absent, it was detected in many gels and its position is marked here. Adult chicken serum albumin migrated as a single distinct band, Alb_1 (62,200 MW) and occasionally a faint second band, Alb_2 (56,500 MW), was observed (lanes C and D). This heterogeneity has been observed in other

Figure 3. A 12.5% SDS-polyacrylamide gel stained with silver showing reference marker proteins. These proteins are labelled on the right side of the figure and include: Lane A, chicken serum IgG (IgG_1, 148,000 MW and IgG_2, 134,200 MW); Lane B, egg white conalbumin (T_1, 79,500 MW; T_2, 76,600 MW; T_3, 72,300 MW and T_4, 69,800 MW); Lane C, chicken serum albumin (Alb_1, 62,200 MW and Alb_2, 56,500 MW); Lane D contains a mixture of these proteins and also egg white avidin (AV_2) which appears to be an aggregate of avidin (AV_1; 17,600 MW) in this gel (see text). The position of BioRad molecular weight markers are indicated on the left side of the figure and include: myosin (200,000 MW), β-galactosidase (116,250 MW), phosphorylase B (92,500 MW), bovine serum albumin (66,200 MW), ovalbumin (45,000 MW), carbonic anhydrase (31,000 MW) and lysozyme (14,400 MW).



A
B
C
D
200,000 MW
116,250 MW
92,500 MW
66,200 MW
45,000 MW
31,000 MW
14,400 MW
AV2
IgG1
IgG2
T1
T2
T3
T4
alb1
alb2

Figure 4. A 12.5% SDS-polyacrylamide gel stained with silver showing reference marker proteins, egg white albumen and 4 day serum. Lane A contains a mixture of reference marker proteins including chicken serum IgG (IgG_1 and IgG_2), egg white conalbumin (T_1, T_2, T_3 and T_4), chicken serum albumin (Alb_1 and Alb_2) and avidin as both an aggregate (AV_2) and dissociated into subunits (AV_1, 17,600 MW). Lane B contains a sample of egg white albumen and presumptive ovalbumin is indicated on the right of the figure (oval). Lane C contains 4 day serum and those proteins in serum which comigrate with reference markers are indicated on the right side of the figure. These include serum proteins 1, 2, 3, 4, 5, 6, 8, 9 and 13. The protein in serum (lane C) which comigrates with ovalbumin in egg white albumen (lane B) is serum protein 10.


A
B
C
AV2
IgG1
IgG2
T1
T2
T3
T4
alb1
alb2
AV1
1
2
3
4
5
6
8
9
oval
13

albumin preparations and has been attributed to a number of factors including isomerization, impurities and binding of buffer salts (reviewed by Peters, 1969). Avidin is composed of 4 identical subunits and has a molecular weight of 66,000 (Green, 1964). This protein, Av_1, ran as a single band at 17,600 MW indicating that it dissociated into subunits (AV_1; Figure 4, lane A). After avidin was stored at -20°C for several months it produced a dark staining high molecular weight band (Av_2; Figure 3, lane D and Figure 4, lane A) in addition to the 17,600 MW band, Av_1 (Figure 4, lane A). It is possible that some aggregation of avidin subunits took place during storage. Polymerization of protein after long term storage has been reported for human serum albumin (Finlayson, 1965).

When reference proteins (Figure 4, lane A) were compared to protein bands in 4 day serum (lane C), a number of comigrating bands were observed. Those serum proteins which comigrated with reference standards are indicated by arrows on the right side of Figure 4. Based on their comigration with IgG_1 and IgG_2, serum proteins 1 and 2 were putatively identified as IgG_1 and IgG_2 respectively. Serum proteins 3, 4, 5 and 6 comigrated precisely with the 4 components of conalbumin (T_1 - T_4) and were putatively identified as serum transferrins. They are herein referred to as T_1, T_2, T_3 and T_4 respectively. Chicken serum albumin, Alb_1 and Alb_2, comigrated with serum proteins 8 and 9 respectively. These proteins were putatively identified as

components of albumin and are herein referred to as Alb_1 and Alb_2 respectively. Serum protein 13 was putatively identified as avidin based on its comigration with Av_1 and is herein referred to as Av_1. Lane B (Figure 4) is egg white albumen in which one of predominant proteins is ovalbumin. Based on its molecular weight (45,000), ovalbumin was identified in this lane and is indicated on the right side of the figure (oval). The protein in 4 day serum (lane C) which comigrates with ovalbumin is serum protein 10. This protein, which is also prevalent in serum samples, may represent ovalbumin. This protein band is very broad and is overstained in most gels but attempts to stop stain development any earlier resulted in the loss of other faint staining serum protein bands. Eight subfractions of ovalbumin have been identified which differ in their carbohydrate content (Kato *et al*, 1984) and three fractions of ovalbumin which differ in phosphate content can be resolved using electrophoresis (Perlman, 1952). These factors likely contribute to the broadness of the putative ovalbumin band in the gels presented here.

A second criterion used for putative identification of proteins in 4 day serum was molecular weight data reported previously for serum proteins synthesized by explanted 3 day old chick embryos (Kram and Klein, 1976; Young *et al*, 1980). These workers reported that transferrin was synthesized by yolk sac endoderm cells (EEC) and had a molecular weight of 78,000. In this study, four transferrin molecules were

detected in 4 day serum (Figure 4, lane C). The major protein band, T_1 (serum protein 3), had a molecular weight of 79,500 ± 1300 and is likely equivalent to the transferrin reported by Kram and Klein (1976). These workers also reported the presence of a broad band in gels (55,000 - 62,000 MW) which they identified as embryo-specific α globulins 'a' and 'b'. In this study, serum protein 7 has a mean molecular weight of 66,500 ± 1400. Although this is higher than the molecular weight data for the α globulins reported by Kram and Klein, the mean molecular weight is close to that reported by these authors and serum protein 7 may correspond to α globulins 'a' and 'b' (serum protein 7, Figure 2 and Table 1). Finally, these workers identify a protein with a molecular weight of 23,000 as prealbumin. In this study, protein 12 had a mean molecular weight of 26,500 ± 1100 and for the same reasons outlined above for serum protein 7, it is putatively identified as and herein referred to as prealbumin (PA) (serum protein 12, Figure 2 and Table 1).

B. Secretion of Serum Proteins by Aggregates of Cells from the Extraembryonic Endoderm

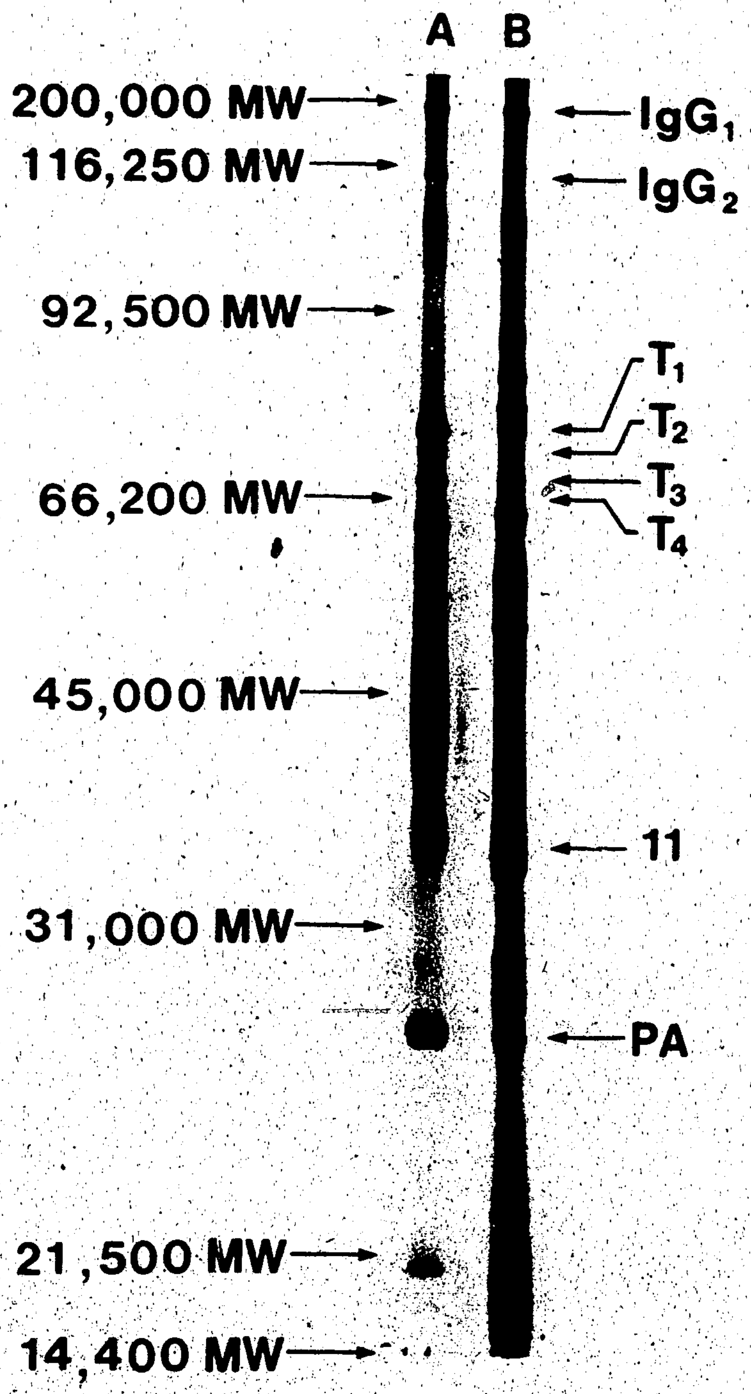
Serum proteins which are to be secreted by EEC may be synthesized by these cells. Alternatively, these proteins may be transported into EEC from albumen or yolk and stored in cytoplasmic vesicles to be released into the vitelline circulation. In order to detect any serum proteins present

in EEC after experimental dissociation and after various time intervals of culture, extracts of cells or aggregates were prepared in the presence of protease inhibitor (PMSF). These extracts are, in principle, enriched with water soluble proteins contained in the various intracellular compartments. The culture medium from these experiments was dialyzed, concentrated and prepared for SDS-PAGE as described previously in order to detect any proteins that were secreted into the medium by EEC aggregates.

Detection of Serum Proteins in Aggregates and Culture Medium Using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Preliminary experiments were conducted to establish whether or not serum proteins could be detected in EEC extracts and culture medium based on their comigration with proteins in serum from 4 day embryos on SDS gels. For this purpose, EEC were cultured in L-15 medium for a total of 24h and cell extracts and medium were analyzed using SDS-PAGE. Approximately 3-5μg total protein was loaded in each lane of the gel and samples from 3 separate experiments were analyzed. Figure 5 is a representative gel comparing 4 day serum (lane A) to EEC extract from aggregates cultured for 24h (lane B). The position of BioRad molecular weight markers are indicated on the left side of the gel and the position of protein bands in EEC extract which comigrate with serum proteins in lane A are indicated on the right

Figure 5. A 12.5% SDS-polyacrylamide gel stained with silver showing soluble proteins present in EEC extracts prepared from aggregates cultured for 24h. Lane A contains 4 day serum and lane B contains EEC extract. Protein bands in lane B which comigrate with serum proteins in lane A are indicated on the right side of the figure and include: IgG (IgG_1), transferrin (T_1, T_2, T_3 and T_4), serum protein 11 (11) and prealbumin (PA). The frequencies with which these proteins appeared in 3 experiments are summarized in Table 2. IgG_2 was observed in one experiment so its position is marked here. Many other protein bands were observed but they are unidentified at this time. The position of BioRad molecular weight markers are indicated on the left side of the ~~figure and~~ include: myosin (200,000 MW), β-galactosidase (116,250 MW), phosphorylase B (92,500 MW), bovine serum albumin (66,200 MW), ovalbumin (45,000 MW), carbonic anhydrase (31,000 MW), soybean trypsin inhibitor (21,500) MW), and lysozyme (14,400 MW).


A
B
200,000 MW
116,250 MW
92,500 MW
66,200 MW
45,000 MW
31,000 MW
21,500 MW
14,400 MW
IgG$_1$
IgG$_2$
T$_1$
T$_2$
T$_3$
T$_4$
11
PA

side of the gel. Proteins were observed in EEC extracts which comigrate with the following serum proteins: IgG_1, IgG_2, T_1, T_2, T_3, T_4, serum protein 11 (11) and prealbumin (PA). The frequency with which these serum proteins appeared in 3 experiments is summarized in Table 2.

In the gel shown in Figure 5, IgG_2 was not detected but because it was detected in one replicate its position is marked here. T_4 was only observed in one gel (Figure 5) but it stained very faintly and may have been present in such small amounts that it could not be detected by the methods used in this study. In addition, the band in EEC extracts which comigrated precisely with serum protein 11 in two experiments was detected as a protein band of slightly lower molecular weight in the gel shown in Figure 5. Because it was observed in other gels, this band likely represents serum protein 11 as indicated (Figure 5). Many other protein bands appeared in EEC extracts in the molecular weight range of 80,000 to 116,000 and 14,000 to 65,000 but at the present time, none of these bands are identified. In summary: using comigration with serum proteins on SDS gels as the criterion for identifying serum proteins in extracts, the results indicate that IgG_1, IgG_2, T_1, T_2, T_3, T_4, serum protein 11 and prealbumin (PA) are present in extracts prepared from EEC aggregates after a 24h culture period.

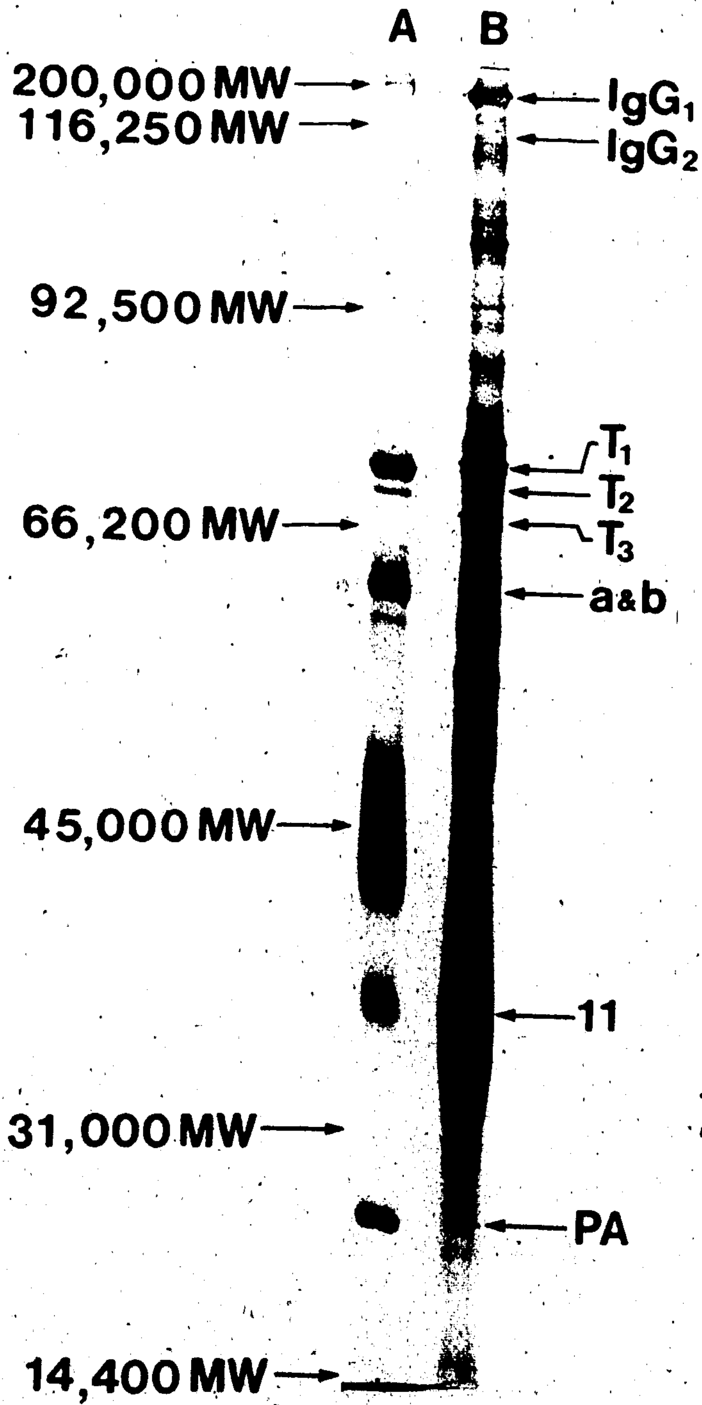
Figure 6 is a representative polyacrylamide gel comparing 4 day serum (lane A) to the medium in which aggregates were cultured for 24h (lane B). The position of

Table 2. Relative frequencies of proteins observed in gels using different samples of EEC extracts prepared from aggregates cultured for 24h. Proteins were separated by their molecular weight using SDS-PAGE (Figure 5). Data from 3 experiments were pooled and the relative frequency was calculated for each protein as follows: Number of times protein band was observed/ Total number of experiments.

TABLE 2.

PROTEIN	RELATIVE FREQUENCY
IgG_1	1.00
IgG_2	0.34
T_1	1.00
T_2	1.00
T_3	1.00
T_4	0.34
Protein 11	1.00
Prealbumin	1.00

Figure 6. A 12.5% SDS-polyacrylamide gel stained with silver showing proteins released into the culture medium by EEC aggregates cultured for 24h. Lane A contains 4 day serum and lane B contains culture medium from EEC aggregates. The proteins in culture medium (lane B) which comigrate with proteins in serum (lane A) are indicated on the right side of the figure and include IgG (IgG_2 and IgG_2), transferrin (T_1, T_2, T_3) and serum protein 11 (11). The frequencies with which these proteins appeared in 3 experiments are summarized in Table 3. α globulins 'a' and 'b' as well as prealbumin appeared some experiments so their positions are marked here. Many other protein bands were observed but they are unidentified at this time. The position of BioRad molecular weight markers are indicated on the left side of the figure and include: myosin (200,000 MW), β-galactosidase (116,250 MW), phosphorylase B (92,500 MW), bovine serum albumin (66,200 MW), ovalbumin (45,000 MW), carbonic anhydrase (31,000 MW) and lysozyme (14,400 MW).



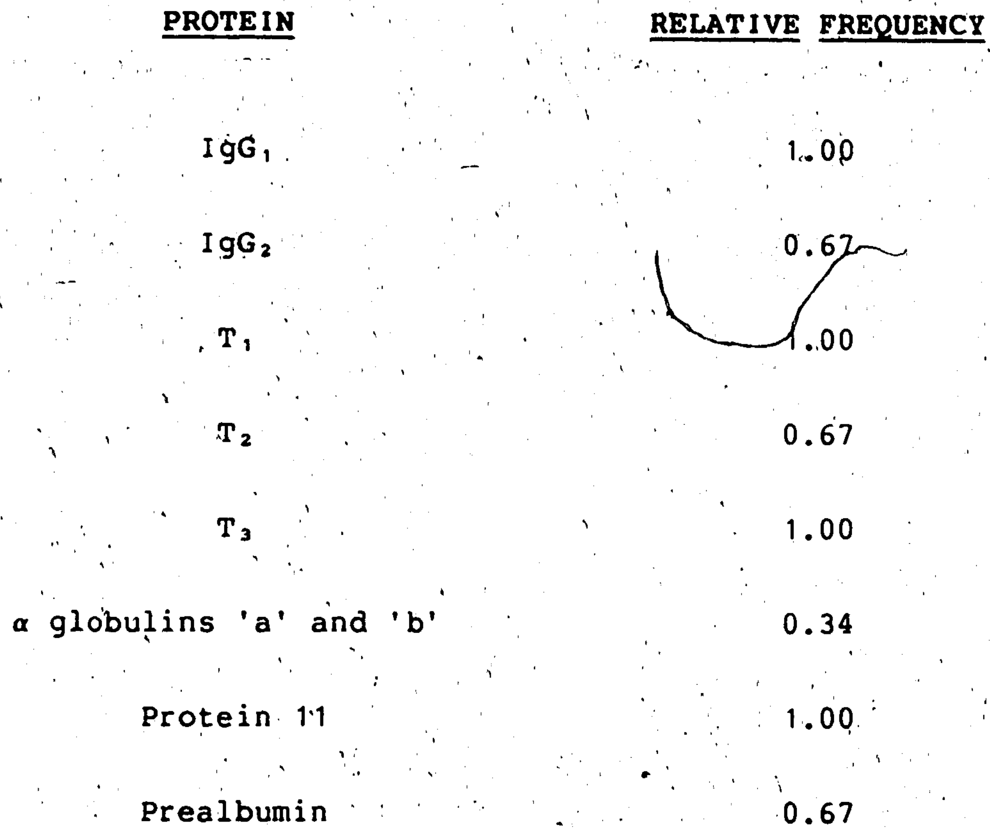
A
B
200,000 MW
116,250 MW
92,500 MW
66,200 MW
45,000 MW
31,000 MW
14,400 MW
IgG1
IgG2
T1
T2
T3
a&b
11
PA

BioRad molecular weight markers are indicated on the left side of the gel and the position of protein bands in culture medium which comigrated with serum proteins in lane A are indicated on the right side of the gel. The quality of this gel is poor but regrettably, the photographic record of two other replicates was destroyed when a film was ruined by a bad batch of Kodak D11 developer. Gels were destained slightly for photography and this resulted in fading of bands. An attempt was made to restain these gels so they could be rephotographed but this was unsuccessful. The frequency data reported here is based on a written record of the data from these experiments.

Protein bands were observed in culture medium which comigrated with the following serum proteins: IgG_1, IgG_2, T_1, T_2, T_3, α globulins 'a' and 'b' (a&b), protein 11 (11) and prealbumin (PA). The frequency with which these serum proteins appeared in 3 experiments is summarized in Table 3. In the gel shown in Figure 6, the region in lane B where transferrin bands appear in 24h medium is fuzzy and the bands are indistinct. The position of T_1, T_2 and T_3 are indicated by arrows because they were clearly present in 2 replicates of this experiment. A protein band which comigrated with α globulins 'a' and 'b' was observed in one experiment and its position is indicated in Figure 6 (a&b). Similarly, the region where prealbumin would appear in culture medium is indicated here because it was present in two experiments. It may be present in this gel but staining

Table 3. Relative frequencies of proteins observed in gels using different samples of culture medium from EEC aggregates cultured for 24h. Proteins were separated by their molecular weight using SDS-PAGE (Figure 6). Data from 3 experiments were pooled and the relative frequency was calculated for each protein as follows: Number of times protein band was observed/ Total number of experiments.

TABLE 3.



PROTEIN	RELATIVE FREQUENCY
IgG_1	1.00
IgG_2	0.67
T_1	1.00
T_2	0.67
T_3	1.00
α globulins 'a' and 'b'	0.34
Protein 11	1.00
Prealbumin	0.67

in this region is indistinct. In summary: using comigration with serum proteins as the criterion for identifying serum proteins, the results show that IgG_1, IgG_2, T_1, T_2, T_3, α globulins 'a' and 'b', protein 11 and prealbumin are secreted into the culture medium by EEC aggregates cultured for 24h.

C. Temporal Changes in Serum Protein Secretion by Aggregates of Cells from the Extraembryonic Endoderm

Preliminary experiments demonstrated that serum proteins could be detected in both EEC extracts and in culture medium using SDS-PAGE (Figures 5 and 6). In order to determine whether the pattern of serum protein secretion changed during culture, EEC aggregates and medium were harvested at various time intervals for analysis using SDS-PAGE. Qualitative information about the relative abundance of these proteins was obtained by observing the relative staining intensity of bands produced by these proteins after different time periods in culture.

Detection of Serum Proteins in Aggregates and Culture Medium at Various Time Intervals

EEC were dissociated as described and divided into 4 aliquots. Approximately 140 embryos were used for each experiment. One aliquot of cells was washed in L-15 medium and homogenized immediately; this sample was used as a time 0h control. The remaining 3 aliquots were used to prepare

aggregates as described and were cultured for 6h, 18h or 30h. All samples were prepared for SDS-PAGE as described previously and results from two separate experiments were analyzed.

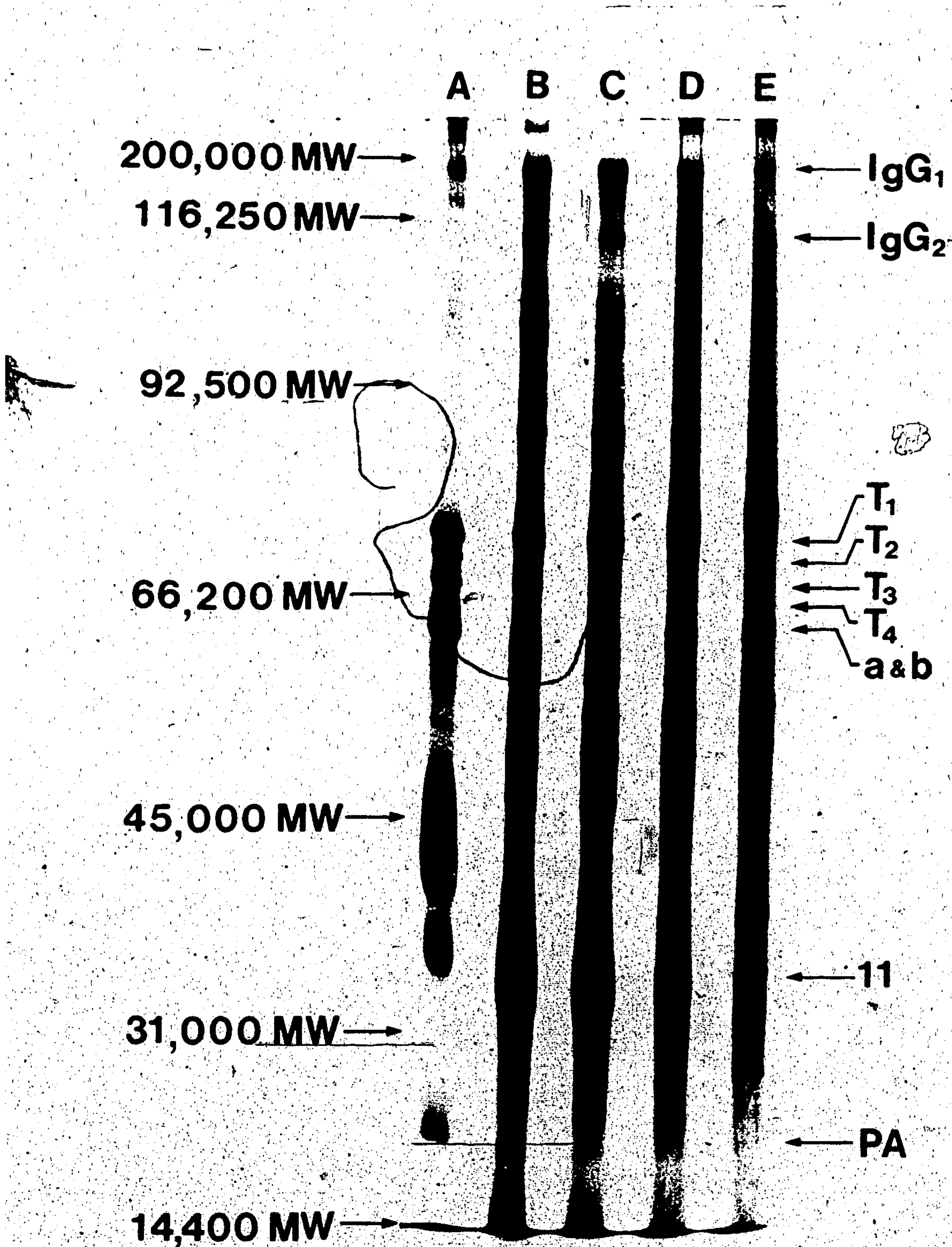
In order to make meaningful comparisons of the protein banding profile for each time interval studied, it was important to ensure that for each sample a comparable amount of protein was loaded onto gels. Two approaches were taken to this problem. After dissociation, EEC were pooled and an identical volume of cell suspension was distributed between 4 flasks. In one experiment, EEC aggregates were harvested, homogenized and the protein concentration of each sample was determined. The volume of each sample was adjusted with Tris-PMSF so the protein concentrations were similar for each extract (3-5µg depending on the experiment). A second approach was used in a repeat of this experiment; EEC extracts were prepared then they were concentrated in Centricon-100 concentrators to a dead stop volume of 35µl. An identical volume of extract was loaded onto gels for each time period studied and because only small amounts of sample were available, the protein content of samples was not determined. It was assumed that in this second approach, proteins were present in concentrated extracts in the same relative abundance as in the original medium. The protein banding profile comparing the relative abundance of proteins in EEC extracts from aggregates cultured for various times was similar for each approach.

Figure 7 is a representative gel showing 4 day serum (lane A), EEC extract 0h control (lane B), EEC extract from aggregates cultured for 6h (lane C), for 18 h (lane D) and for 30h (lane E). The positions of BioRad molecular weight markers are indicated on the left side of the gel and those proteins in EEC extracts which comigrate with serum proteins in lane A are indicated on the right side of the gel. IgG_1 was detected in the 0h control (lane B) as a dark staining broad band and the staining intensity of this band decreased gradually in 6h and 18h EEC extracts (lanes C, D and E). After 30h in culture, only a very faint band corresponding to IgG_1 was detectable in gels (lane E). This protein may be degraded by cells or it may be secreted into the medium.

T_1 was observed in the extracts prepared from uncultured cells (0h control) as a broad band which diminished in staining intensity over time (lanes C, D, and E). Faint protein bands which comigrated with T_2 and T_3 and T_4 were observed in 0h control samples (lane B) and these bands disappeared from aggregates during the culture period (lanes C, D and E). T_1 may be degraded by cells or it may be secreted into the medium. At the same time as T_1 is disappearing from aggregates, a protein band of slightly higher molecular weight stains more intensely. The identity of this protein is unknown but it could represent a modification of T_1, perhaps through glycosylation.

The serum protein identified as α globulins 'a' and 'b' appeared as a broad band in 4 day serum (lane A) and in EEC

Figure 7. A 12.5% SDS-polyacrylamide gel stained with silver showing proteins present in EEC extracts prepared from aggregates cultured for various time intervals. Lane A contains 4 day serum and lanes B, C, D and E contain EEC extracts: uncultured cells (lane B; 0h control), aggregates cultured for 6h (lane C), for 18h (lane D) and for 30h (lane E). Proteins in extracts (lanes B, C, D and E) which comigrate with serum proteins in lane A are indicated on the right side of the figure and include: IgG (IgG_2 and IgG_2), transferrin (T_1, T_2, T_3 and T_4), α globulins 'a' and 'b' (a&b), serum protein 11 (11) and very faint bands corresponding to prealbumin (PA). Those serum proteins indicated by arrows are not necessarily present at every time interval of culture (see text). Many other protein bands were observed but they are unidentified at this time. The position of BioRad molecular weight markers are indicated on the left side of the figure and include: myosin (200,000 MW), β-galactosidase (116,250 MW), phosphorylase B (92,500 MW), bovine serum albumin (66,200 MW), ovalbumin (45,000 MW), carbonic anhydrase (31,000 MW) and lysozyme (14,400 MW).


A
B
C
D
E
200,000 MW
116,250 MW
92,500 MW
66,200 MW
45,000 MW
31,000 MW
14,400 MW
IgG1
IgG2
T1
T2
T3
T4
a&b
11
PA

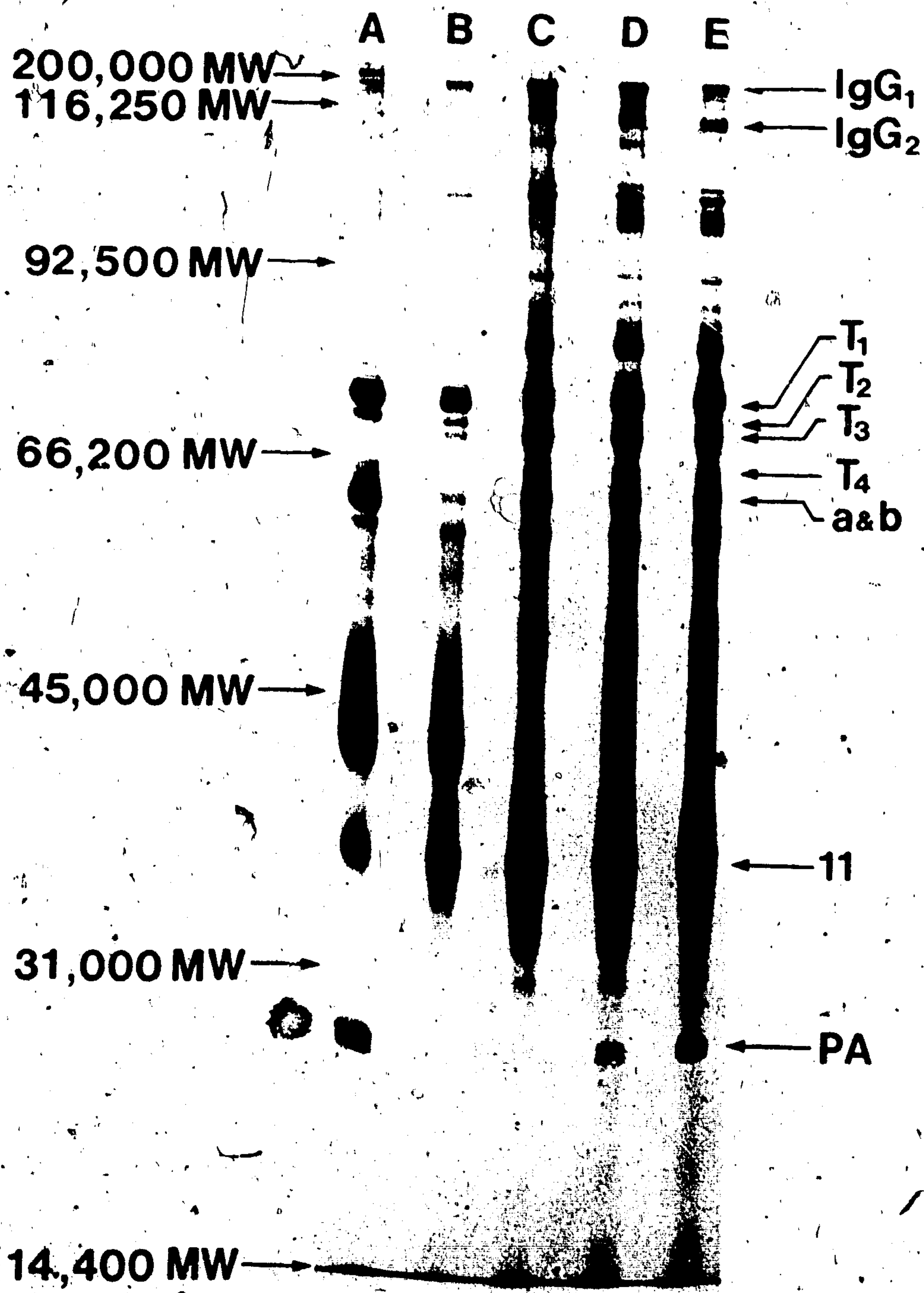
extracts, a doublet was observed which comigrated approximately with this protein. This doublet may represent the α globulins and it was very faint in 0h control extracts (lane B) but appeared to increase in concentration over the time intervals studied here (lanes C, D and E). Kram and Klein (1976) reported that 3 day old embryo explants cultured on whole egg medium incorporated radiolabelled amino acids into α globulins 'a' and 'b' and it was later shown that this protein was synthesized by EEC (Young *et al*, 1980).

Protein 11 is present in large amounts in 0h control extracts (lane B) and based on staining intensity, its concentration decreased rapidly in aggregates so that by 30h in culture, only a small amount remained (lanes C, D and E). Again, this protein may be degraded by cells or it may be secreted into the medium. Finally, a very faint protein band was observed in all extracts (lanes B, C, D and E) which comigrated with prealbumin (PA) in lane A. In summary, the results show that IgG_1, IgG_2, T_1, T_2, T_3, T_4, α globulins 'a' and 'b', serum protein 11 and prealbumin are present in EEC extracts. The protein banding profiles and changes in staining intensity of bands described here (Figure 7) were consistent when extracts from two separate experiments were analyzed using SDS-PAGE. Many other protein bands were observed in these extracts and based on their staining intensity in gels, many proteins appeared to decrease in concentration over time and several appeared to increase in

concentration over time. These proteins have not yet been identified. Changes in the banding profile of proteins in EEC extracts cultured for various time intervals may represent changes in the pattern of protein synthesis, degradation of protein or secretion of protein by these cells.

Analysis of the culture medium from these experiments provided information about proteins that were secreted by EEC over the time intervals studied here. Culture medium was prepared as described for SDS-PAGE analysis. The same two approaches described for EEC extracts were used in these studies. Figure 8 is a representative gel showing 4 day serum (lane A), wash medium (0h control; lane B), medium from aggregates cultured for 6h (lane C), 18h (lane D) and 30h (lane E). The position of BioRad molecular weight markers are indicated on the left side of the gel and the position of those proteins in medium samples which comigrate with serum proteins in lane A are indicated on the right side of the gel. In the gel shown in Figure 8, samples of medium were assayed for protein and concentrations of 6h, 18h and 30h samples were adjusted accordingly (3-5μg depending on the experiment). The protein concentration in the 0h control sample (lane B) was very low but it was included in this analysis in order to determine whether any proteins were secreted or released by EEC into the medium during the brief wash period used here. Based on their comigration with serum proteins in lane A, a number of serum

Figure 8. A 12.5% SDS-polyacrylamide gel stained with silver showing proteins released into the culture medium by EEC aggregates cultured for various time intervals. Lane A contains 4 day serum and lanes B, C, D and E contain culture medium from EEC aggregates; wash medium (lane B; 0h control), medium from aggregates cultured for 6h (lane C), for 18h (lane D) and for 30h (lane E). Proteins in medium (lanes B,C,D and E) which comigrate with serum proteins in lane A are indicated on the right side of the figure and include: IgG (IgG_2 and IgG_2), transferrin (T_1, T_2, T_3 and T_4), α globulins 'a' and 'b' (a&b), serum protein 11 (11) and prealbumin (PA). Those serum proteins indicated by arrows are not necessarily present at every time interval of culture (see text). Many other protein bands were observed but they are unidentified at this time. The position of BioRad molecular weight markers are indicated on the left side of the figure and include: myosin (200,000 MW), β-galactosidase (116,250 MW), phosphorylase B (92,500 MW), bovine serum albumin (66,200 MW), ovalbumin (45,000 MW), carbonic anhydrase (31,000 MW) and lysozyme (14,400 MW).


A
B
C
D
E
200,000 MW
116,250 MW
92,500 MW
66,200 MW
45,000 MW
31,000 MW
14,400 MW
IgG1
IgG2
T1
T2
T3
T4
a&b
11
PA

proteins were detected in medium samples (lanes B, C, D and E). IgG_1 was observed in the wash medium and in medium after aggregates were cultured for 6h, 18h and 30h (lanes B, C, D and E). The concentration of this protein in extracts prepared from EEC aggregates appeared to decrease over the time intervals studied here (Figure 7). It is possible that this protein is secreted or released by EEC into the culture medium.

In the gel shown in Figure 8, T_1 migrated as a broad band in 4 day serum (lane A). In 0h and 6h samples (lane B and C), the band which comigrated with T_1 was resolved as a doublet. In medium from aggregates cultured for 18h (lane D), the staining intensity of this band increased and after 30h in culture the same band stained as a single broad band (lane E). T_1 disappeared in EEC extracts over the same time period (Figure 7) and over the same culture period its concentration in medium appeared to increase suggesting that it was secreted into the medium by EEC. Protein bands were observed in medium samples which comigrated approximately with T_2 and T_3 and a very faint band comigrated with T_4 (lanes C, D, and E).

A distinct band which comigrated with the α globulins in 4 day serum was observed in culture medium at all time intervals studied (lanes B, C, D and E). In addition, protein 11 appeared in all samples of medium in large amounts (lanes B, C, D and E). It disappeared in EEC extracts over time (Figure 7), suggesting again that it was

secreted into the medium by EEC. Finally, a band which comigrated with serum prealbumin (PA) in lane A was observed in medium samples (lanes C, D and E). It was very faint in 6h medium (lane C) but the staining intensity of this band increased in 18h and 30h medium (lanes D and E). This band occasionally appeared as a doublet (Figure 8) but was more frequently observed as a single band in gels. Prealbumin appeared to increase in concentration in medium over the time intervals studied here but was present at all stages in EEC extracts as a very faint band (Figure 7). It is possible that it is synthesized by EEC and released rapidly into the medium. Based on these results, it was concluded that IgG_1, T_1, T_2, T_3, T_4, α globulins 'a' and 'b', protein 11 and prealbumin are secreted by EEC into the culture medium over the time intervals studied. Based on staining intensity, both T_1 and prealbumin appeared to increase in concentration in medium as the time interval of culture was increased.

D. Synthesis of Serum Proteins by Aggregates of Cells from the Extraembryonic Endoderm

Previous experiments demonstrate that serum proteins are present in EEC extracts and in culture medium and that the amount of protein present at different time intervals of culture varies for some proteins (Figures 5, 6, 7 and 8). These proteins may be present in EEC at the time of dissociation and secreted or released into the medium during the culture interval. Alternatively, these proteins may be

synthesized by EEC and secreted or released into the medium. In order to determine whether serum protein synthesis takes place in cultured EEC aggregates, radiolabelled amino acids were introduced to the culture medium so that newly synthesized proteins could be detected using SDS-PAGE and fluorography.

Incorporation of Radiolabelled Amino Acid into Protein

Experiments were conducted in order to determine whether EEC aggregates incorporated radiolabelled amino acids into proteins under the conditions used in this study. Cells were dissociated as described and cultured for 24h in the presence of a mixture of ^{3}H labelled amino acids or a mixture of ^{14}C labelled amino acids (New England Nuclear). Because serum proteins are apparently secreted into the culture medium by EEC aggregates, both EEC extracts and culture medium were assayed for labelled protein using TCA precipitation and scintillation counting. The extent of incorporation was quantified and is reported here as CPM/μg protein. Table 4 shows that labelled amino acid was detected in the proteins precipitated by TCA in both EEC extracts and culture medium.

Under the conditions used in this study, EEC synthesize protein and secrete it into the culture medium. Radiolabelled amino acid mixtures produced a greater number of CPM/μg protein than labelled valine and the amino acid mixtures were selected for further labelling experiments.

Table 4. Incorporation of ^{3}H valine and ^{3}H or ^{14}C amino acid mixture into TCA precipitable protein. Aggregates were cultured for 24h in the presence of label, EEC extracts were prepared and the culture medium was collected. Proteins were precipitated using 15% TCA and 5% PTA as described in Materials and Methods. Incorporation was calculated as counts per minute (CPM) per μg protein.

TABLE 4.

RADIOLABEL	EEC EXTRACTS (CPM/μg protein)	CULTURE MEDIUM (CPM/μg protein)
^{3}H Valine	13.44	7.79
^{3}H Amino Acid Mixture	242.79	20.88
^{14}C Amino Acid Mixture	156.48	45.69

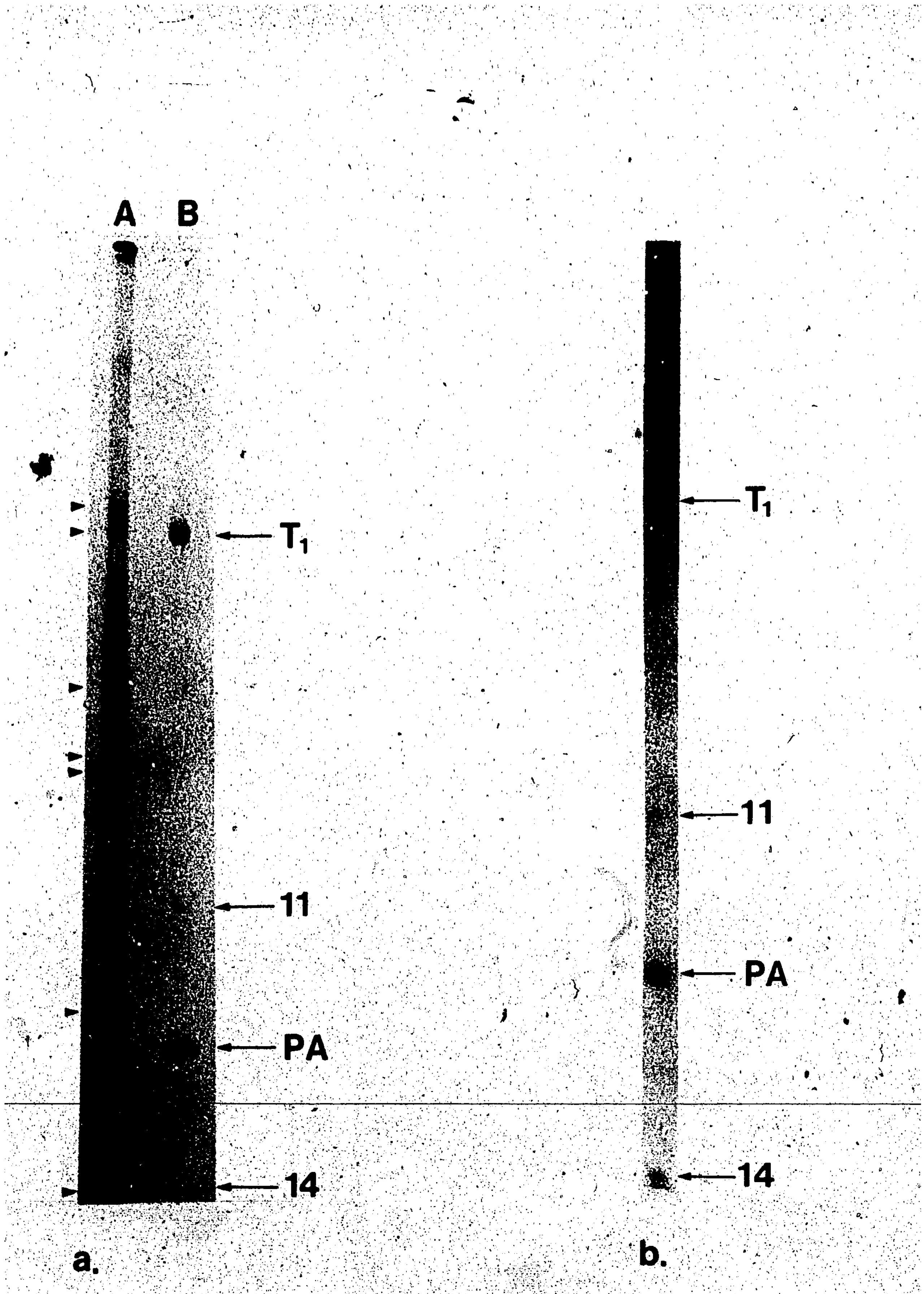
Synthesis and Secretion of Serum Proteins in Aggregates of Cells from the Extraembryonic Endoderm Cultured for 24 Hours

In order to determine whether any of the proteins synthesized by EEC aggregates were serum proteins, preliminary experiments were conducted using radiolabelled amino acid mixture. EEC were cultured in the presence of 30µCi of ^3H or ^{14}C labelled amino acid mixture for 24h. EEC extracts and culture medium were prepared for SDS-PAGE as described. For cultures labelled with ^3H amino acid mixture, approximately 80µg of total EEC extract protein (19,400 CPM) and 40 µg of total culture medium protein (840 CPM) were loaded onto gels. For cultures labelled with ^{14}C labelled amino acid, approximately 80µg total protein (12,600 CPM) for EEC extracts and 70µg total culture medium protein (3200 CPM) were loaded onto gels. Approximately 5µg of 4 day serum was run on each gel so that any proteins in extracts or medium which contained labelled amino acid could be identified in SDS gels based on their comigration with serum proteins. Gels were dried and exposed to X-ray film at -80°C.

After 21 days of exposure, gels containing ^3H labelled samples produced no detectable bands on X-ray film (data not shown). The same exposure time for gels containing ^{14}C labelled samples produced distinct bands on the film (Figure 9a). EEC extracts produced a number of radiolabelled proteins (lane A). With the exception of a broad band at the dye front which appeared to comigrate with serum protein 14,

Figure 9. a. A fluorogram prepared by exposing X-ray film to dried 12.5% SDS-polyacrylamide gels containing EEC extracts and culture medium prepared from aggregates cultured for 24h in the presence of ^{14}C labelled amino acid mixture. Lane A contains EEC extract and lane B contains culture medium. Some unidentified radiolabelled protein bands in lane A are indicated on the left side of the figure by arrowheads. None of these bands comigrated with proteins in 4 day serum except the intensely labelled band at the dye front. This band comigrated with serum protein 14. The radiolabelled protein bands in the culture medium (lane B) which comigrated with proteins in 4 day serum are indicated on the right side of the figure and include transferrin (T_1), a very faint band corresponding to serum protein 11 (11), prealbumin (PA). Serum protein 14 (14) migrated at the dye front. The X-ray film was exposed at -80°C for 21 days.

Figure 9 b shows a fluorogram of culture medium prepared in the same manner as the sample shown in Figure 9 a (lane B). In this gel, serum protein 14 migrated to a position close to the dye front. The X-ray film was exposed -80°C for 21 days.


A
B
T1
11
PA
14
a.
T1
11
PA
14
b.

none of these bands comigrated with proteins in 4 day serum. Lane B contains proteins secreted into the culture medium and these proteins comigrated precisely with proteins in 4 day serum. A single radiolabelled band comigrated with T_1, a very faint band was observed which comigrated with serum protein 11 and a distinct band comigrated with prealbumin. These protein bands are indicated on the right side of the X-ray film. These bands were not labelled in EEC extracts but they may be synthesized by these cells and then rapidly secreted. It is possible that they are present in aggregates in very small amounts and may be detectable on X-ray films if exposure time is increased. In one repeat of this experiment a radiolabelled protein was observed close to the dye front which comigrated with serum protein 14 (Figure 9b).

Temporal Changes in Serum Protein Synthesis in Aggregates of Cells from the Extraembryonic Endoderm

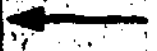
When EEC aggregates were cultured for various time periods, the pattern of serum protein synthesis varied at different time intervals of culture. Experiments were designed in exactly the same manner as described previously for the analysis of secretion/release of proteins by EEC aggregates into the culture medium. A mixture of amino acids labelled with either ^3H or ^{14}C was introduced into the culture medium 6h prior to harvest. Samples were prepared for SDS-PAGE as described. In order to ensure that samples

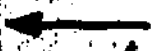
from each time interval studied could be compared quantitatively, identical volume of cell suspension was introduced into each flask and both EEC extracts and medium were concentrated in a Centricon-100 concentrator to dead stop volume, 35μl. Because only small quantities of sample were obtained in each experiment, these samples were not assayed for protein content. Based on previous assay results, it was estimated that 100-150μg total protein was loaded onto gels for both EEC extract and medium samples. Because the labelling period used here was 6h and the labelling period used in incorporation experiments was 24h, it was estimated that extracts and culture medium labelled for 6h contained only 25% of the CPM/μg protein calculated for extracts and culture medium labelled for 24h. Based on these estimates, CPM loaded onto gels were as follows: ^{3}H labelled EEC extracts, 520-780: ^{3}H labelled medium samples, 3900-5900; ^{14}C labelled EEC extracts, 1140-1720 and ^{14}C labelled medium samples, 1140-1720.

When gels containing ^{3}H labelled EEC extracts were exposed to X-ray film for 21 days, no bands were observed (data not shown). When gels containing ^{14}C labelled EEC extracts were exposed for the same time period, faint bands were observed in the 6h sample (Figure 10, lane B) but these bands became less distinct at 18h (lane C) and 30h (lane D). None of the bands observed in this fluorogram comigrated with proteins in 4 day serum. When samples of medium were analyzed using fluorography, a band which comigrated with T_1

Figure 10. A fluorogram prepared by exposing X-ray film to dried 12.5% SDS-polyacrylamide gels containing EEC extracts prepared from aggregates labelled with ^{14}C amino acid mixture for 6h prior to harvest. Lane A contains uncultured unlabelled EEC extract (0h control), lane B contains extract from aggregates cultured for 6h, lane C contains extract from aggregates cultured for 18h and lane D contains extract from aggregates cultured for 30h. The arrows on the right side of the figure indicate some very faintly labelled unidentified protein bands, none of which comigrated with the proteins in 4 day serum. The labelled bands at the dye front comigrated with serum protein 14 but may also represent degraded labelled protein. The X-ray film was exposed to at -80°C for 21 days.

A B C D

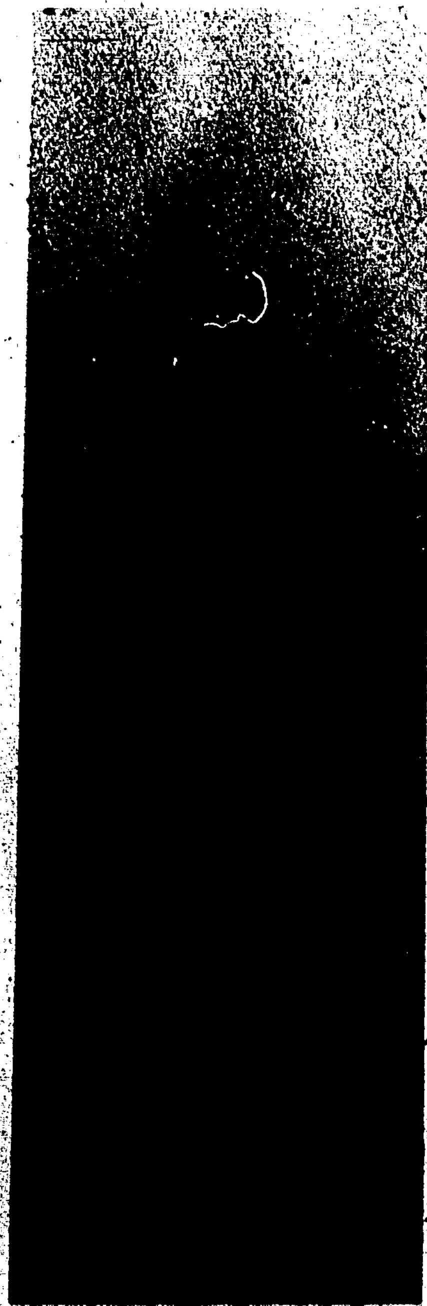




in 4 day serum was observed in medium from aggregates cultured for 18h (Figure 11, lane C) and 30h (lane D). These results suggest that transferrin is synthesized by EEC aggregates then secreted into the medium. Because it is absent in medium from aggregates cultured for 6h, it is possible that this protein was not synthesized by EEC until between 6h and 18h in culture. A second band which comigrated with prealbumin in 4 day serum was detected at all time intervals studied (lanes B, C and D). This protein is apparently synthesized and secreted by EEC prior to 6h in culture and it continues to be produced through the time intervals studied here. A labelled band was observed which migrated at the dye front and this band may represent serum protein 14. All samples were labelled for 6h prior to harvest ensuring that any protein which was observed on fluorograms was synthesized within this 6h period. This eliminated the possibility that changes in the concentration of labelled protein were due to accumulation of labelled protein in the medium.

A band was detected in fluorograms of 24h medium which comigrated with serum protein 11 (Figure 9a, lane B; Figure [illegible]). This band was not observed in 6h, 18h and 30h medium but this may be due to the length of the labelling period. In preliminary experiments, aggregates were cultured in the presence of label for 24h but in the experiment just described, a 6h label period was used for each time interval. It is possible that serum protein 11 is

Figure 11. A fluorogram prepared by exposing X-ray film to dried 12.5% SDS-polyacrylamide gels containing culture medium from EEC aggregates labelled with ^{14}C amino acid mixture for 6h prior to harvest. Lane A contains wash medium from uncultured unlabelled EEC (0h control), lane B contains medium from aggregates cultured for 6h, lane C contains medium from aggregates cultured for 18h and lane D contains medium from aggregates cultured for 30h. The arrows on the right side of the figure indicate those labelled protein bands in medium samples which comigrated with the proteins in 4 day serum and includes transferrin (T_1) and prealbumin (PA). The labelled bands at the dye front comigrated with serum protein 14. The X-ray film was exposed at -80°C for 21 days.


A
B
C
D
T_1
PA

synthesized in very small amounts but accumulates in the medium over 24h. A 6h labelling period may not be sufficient to produce the quantity of serum protein 11 required for detection on fluorograms when films are exposed for 21 days.

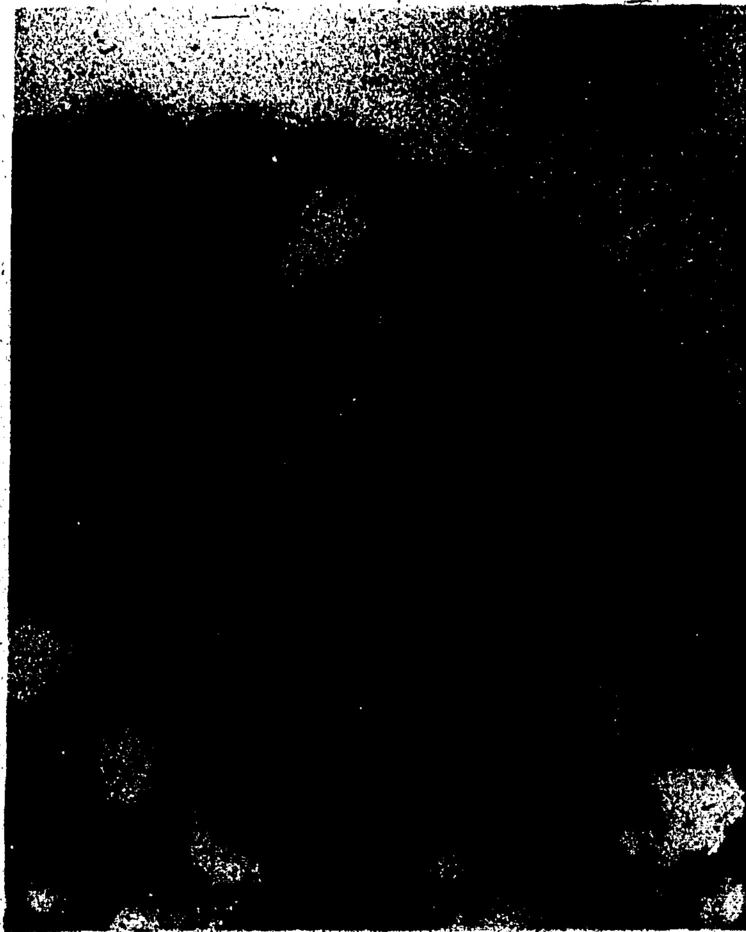
In conclusion, aggregates cultured for 0-30h synthesize and secrete prealbumin within the first 6h of the culture interval and these same aggregates begin to synthesize transferrin between 6h and 18h in culture. Serum protein 11 and other proteins may be produced in small amounts by the aggregates since they were not observed after a 6h labelling period but were present after a 24h labelling period. Serum protein 14 may also be synthesized and secreted by aggregates at all time intervals studied but it should be noted that the protein at the dye front may also represent degradation product resulting from protein breakdown. Figure 9b shows that serum protein 14 can be detected as a discrete band after a 24h labelling period.

E. Detection of Protein Synthesis in Aggregates of Cells from the Extraembryonic Endoderm Using Autoradiography

Serum proteins are synthesized by EEC aggregates and secreted into the culture medium. Aggregates of EEC begin to cavitate after several hours in culture (Milos *et al*, 1979) and proteins may be secreted into cavities by these cells in addition to being secreted into the medium. In order to determine whether any newly synthesized proteins are secreted into cavities, aggregates were cultured for various

time periods in the presence of ^{14}C then they were fixed and prepared for autoradiography. Figure 12 shows a representative autoradiogram of an aggregate cultured for 18h. Silver grains were observed both in cavities within the aggregate and at the periphery of the aggregate. No silver grains were detected in an unlabelled control aggregate prepared in the same manner. These results suggest that newly synthesized proteins are secreted by cells at the periphery of the aggregate and also into the cavities which form in the aggregate. The major radiolabelled protein bands observed in fluorograms of culture medium from EEC aggregates (Figures 9a, 9b and 11) are transferrin, prealbumin and possibly serum protein 14. It is likely that at least some of the silver grains observed in autoradiograms are serum proteins synthesized by EEC aggregates.

Figure 12. An autoradiogram of a 5μ paraffin section from an aggregate cultured in the presence of ^{14}C labelled amino acid mixture for 18h. Labelled slides were coated with emulsion and exposed for 9 days. Silver grains in the cavities and at the periphery of the aggregate are indicated by arrows. Silver grains were not detected in an unlabelled control aggregate prepared in the same manner. Scale: 1cm = 13 μm.



IV. DISCUSSION

The results of this study show that several serum proteins, including transferrin and prealbumin, were synthesized by EEC aggregates and secreted into the culture medium (Figure 11). The synthesis of transferrin was initiated sometime between 6h and 18h in culture whereas prealbumin synthesis was detected within the first 6h of the culture period. This is the first time that serum protein synthesis and secretion have been studied in EEC at this early developmental stage.

Proteins in serum can be transported through the circulatory system from their site of synthesis to any other tissues in the embryo. By interacting with differentiating tissues and organ systems at specific sites in the embryo, these molecules may function as biochemical signals in differentiation and embryogenesis. For example, transferrin is a serum protein synthesized and secreted by the EEC of the *area opaca* in the chick embryo (Young *et al*, 1980). It is presumably transported into the vitelline circulation where it becomes a major component of embryonic serum (Weller, 1966a; Kram and Klein, 1976). The site of transferrin synthesis is extraembryonic but transferrin receptors are synthesized by various cell types in the chick embryo including spinal neurons (Markelonis *et al*, 1985) and primitive embryonic erythrocytes (Schmidt *et al*, 1986). Transferrin molecules are a class of iron-binding glycoproteins which are important for the transport of

extracellular iron into cells (reviewed by Aisen, 1980) but may also play other roles in embryogenesis such as stimulating growth and differentiation. In this study, the pattern of serum protein synthesis and secretion in EEC from primitive streak stage chick embryos was characterized because these molecules may be very important in embryogenesis.

The *area opaca* of chick embryos at the primitive streak stage is already engaged in serum protein synthesis (Kram and Klein, 1976) and the site of synthesis in embryos incubated for 4 days is the endoderm layer of the *area opaca* (Young *et al*, 1980). The pattern of protein synthesis in these cells may be affected by mechanical and temperature stress which cells undergo during the dissociation procedure used here. In addition, aggregates are produced from EEC by culturing them in a synthetic medium containing amino acids but no macromolecules. Indeed, it has been demonstrated that chick embryos utilize proteins in preference to amino acids for protein synthesis (Walter and Mahler, 1958). Some time in culture may be necessary for cells to recover from stress and adapt to new culture conditions before protein synthesis is resumed. In addition, the synthesis of individual proteins may be affected to a different extent.

Nevertheless, the detection of prealbumin synthesis after a 6h culture period indicates that EEC are capable of adapting to culture conditions and utilizing amino acids from the medium for protein synthesis within a relatively

short time interval. Whether protein synthesis patterns in aggregates are the same as those which take place *in ovo* has not yet been established but Zagris (1985) observed that erythroblasts isolated from blood islands of prestreak embryos produce embryonic and adult hemoglobins in the same sequence as they appear *in ovo*. In other embryonic systems such as the rabbit embryo at preimplantation stages, it has been demonstated that protein synthesis patterns are identical *in vivo* and *in vitro* (Van Blerkom *et al*, 1970).

The EEC aggregates used in this study resemble the EEC of intact *area opacae* morphologically. Aggregates cavitate and form thin-walled vesicles in the absence of other cell types (Milos *et al*, 1979). *In ovo*, the endoderm layer of the *area opaca* spreads to surround the yolk, forming a thin-walled sphere (Rommanoff, 1960). In addition, intercellular junctions form in EEC aggregates during cavitation which resemble those in the endoderm of the *area opaca in vivo* (Milos *et al*, 1984). If biosynthesis is required in EEC for the morphological process of cell spreading *in ovo*, or cavitation *in vitro*, then EEC aggregates may also resemble the endoderm of the *area opaca* biochemically. Other workers have suggested that specific regions in the chick embryo may undergo chemical differentiation and diverge to different developmental fates prior to the manifestation of associated morphological changes (Zagris and Matthopoulos, 1985). While primitive streak formation was once considered the first significant

morphological event in chick development, Eyal-Giladi and Kochav (1976) characterized prestreak development in detail. These workers observed that the formation of the *area pellucida* and the hypoblast represent important morphogenetic changes that occur prior to primitive streak formation. It is possible that associated biochemical signals are present at these early stages. Regional differences in protein synthesis patterns have been detected in the prestreak chick embryo by Zagris and Matthopoulos (1985). These workers propose that new proteins appear as a result of chemical differentiation which preceeds morphological differentiation. Cavitation in EEC aggregates is a morphogenetic process which resembles the spreading of EEC around the yolk *in vivo*. This morphogenesis may take place in response to chemical differentiation in EEC, such as the synthesis of new proteins. *In ovo*, proteins synthesized in these cells may also influence morphogenesis in other tissues if the proteins are secreted into the vitelline circulation and transported through the serum.

The EEC in the *area opaca* of the chick embryo synthesize serum proteins and the pattern of synthesis changes at different stages of development (Young *et al*, 1980; Young and Klein, 1983). Serum protein profiles were originally studied by analyzing serum obtained by direct sampling of blood (Weller and Schechtman, 1962; Weller, 1966a; 1966b; 1976; Gitlin and Kitzes, 1967; Weller and Bowden, 1974). In these studies, embryos incubated for at

least 5 days were used because their circulatory structures were well developed and accessible to experimental manipulation. Kram and Klein (1976) observed that explanted chick embryos maintained in culture synthesized and secreted serum proteins into the medium. This technique allowed for analysis of serum protein synthesis in chick embryos prior to the formation of a functional circulatory system. Nevertheless, much of this work focused on embryos incubated for at least 3 days (Kram and Klein, 1976; Young *et al*, 1980; Young and Klein, 1983). In the study reported here, the differential dissociation technique developed by Milos *et al* (1979) was utilized to obtain a pure population of EEC from primitive streak stage embryos. Serum protein synthesis was analyzed in aggregates produced from these cells over a 30h culture period.

The first approach used in this study was to determine whether or not serum proteins could be detected in extracts prepared from EEC aggregates. Presumably, these aggregates contain newly synthesized secretory proteins, proteins involved in the maintenance of cellular metabolism and proteins taken up by EEC from the nutrient source for either degradation, storage or secretion. Because EEC aggregates were cultured in medium which contained no macromolecules, any proteins which fall into the latter category would have to be present in EEC at the time of dissociation. The proteins in the medium of the cultured aggregates represent those proteins which are released from intracellular

reserves, including stored yolk proteins or proteins that are synthesized then secreted by EEC aggregates during the culture interval. Radiolabelled amino acids were introduced into the culture medium to determine which of those proteins in EEC extracts and culture medium were synthesized by cells during the course of the experiment.

In this study, two IgG molecules (IgG_1 and IgG_2) were detected in the culture medium and in EEC extracts (Figures 7 and 8). IgG is present in the livetin fraction of yolk (Williams, 1962a) and it is transported across the EEC in the yolk sac to confer passive immunity to the developing chick (Kowalczyk *et al*, 1985). Previous studies have shown that there are at least two subpopulations of IgG in the yolk of the chicken oocyte which differ in their susceptibility to digestion by the enzyme papain (Loeken and Roth, 1983). Linden and Roth (1978) showed that exogenous IgG binds directly to receptor molecules on the chick yolk sac and proposed that IgG is transported intact across the yolk sac endoderm via receptor mediated endocytosis. IgG binding and uptake has been demonstrated in cultures of chick EEC and the process is similar to that which takes place *in ovo* (Reinhold *et al*, 1984; Donaldson *et al*, 1985).

The findings reported here show that IgG_1 and IgG_2 are present in EEC at the time of dissociation (Figure 7, lane B). These molecules are likely transported into EEC from yolk prior to dissociation. Under culture conditions, they gradually dissappear from EEC (lanes C, D and E). These

molecules may be released into the culture medium where they were detected at every time interval studied (Figure 8). IgG_1 and/or IgG_2 may be synthesized by EEC aggregates and then released but no synthesis was detected in fluorography studies and there are no previous reports of IgG synthesis in early chick embryos. Contaminating yolk may account for the presence of some IgG observed in the culture medium, but embryos and cells were washed extensively to reduce this problem. The medium used to wash freshly dissociated EEC was analyzed and a relatively small amount of IgG was detected in this sample (Figure 8, lane B). These proteins were either rapidly released from EEC during the wash interval or they represent proteins derived from yolk contaminant. Based on the staining intensity of bands, more IgG_1 and IgG_2 were detected in medium during the culture intervals studied (Figure 8, lanes C, D and E) than in the medium used to wash freshly dissociated EEC (lane B) indicating that at least some IgG was released from EEC. Kowalczyk *et al* (1985) propose that the bulk of IgG in yolk may be utilized by EEC as a nutrient and degraded because only a small fraction of the IgG present in yolk is detected in embryonic circulation. This could also account for the observed depletion of IgG_1 and IgG_2 in EEC extracts.

Transferrin is synthesized and secreted by EEC (Young *et al*, 1980; Young and Klein, 1983) but it is also present in the livetin fraction of yolk (Williams, 1962a). In this study, four transferrin molecules, T_1, T_2, T_3 and T_4 were

detected in extracts prepared from freshly dissociated EEC and all of these proteins dissappeared from the aggregates over the culture intervals studied here (Figure 7, lanes B, C, D and E). All four transferrins were detected in the culture medium over the same time interval (Figure 8, lanes C, D and E) and T_1, T_2 and T_3 were detected in the medium used to wash freshly dissociated EEC (lane B). As described for IgG, these proteins may be rapidly released from EEC or they may be present in residual yolk contaminant. Based on staining intensity, T_1 increased in concentration in the medium over time. Fluorography results showed that T_1 was the only transferrin molecule synthesized and released into the medium by EEC aggregates (Figures 9a and 11).

In the rat embryo, transferrin is transferred intact across the yolk sac (Huxam and Beck, 1984). It is possible that some yolk derived transferrin was present in EEC at the time of dissociation and then released by aggregates into the culture medium. At the same time, T_1 was synthesized and secreted by aggregates sometime after 6h in culture leading to an increase in the relative concentration of T_1 in the medium. Williams (1962b) reported that yolk transferrin and chick serum transferrin have identical amino acid sequences but different carbohydrate moieties. These molecules could, in principle, interact with different transferrin receptors and therefore play unique roles in the embryo. Because T_1 synthesis is initiated between 6h and 18h in culture it may be a stage specific protein. It is a major component of

embryonic serum and may be important in tissues distant from the EEC in the yolk sac. As previously mentioned, spinal neurons and primitive embryonic erythrocytes synthesize transferrin receptors (Markelonis *et al*, 1985; Schmidt *et al*, 1986). In addition, transferrin is required by embryonic chick neurons grown in culture at a specific time period (Aizenman *et al*, 1986) and it also has myotropic effects on chick embryo skeletal muscle (Oh and Markelonis, 1980; 1982).

The role of transferrin in cultured EEC aggregates is unclear but it may be synthesized by these cells because they are already committed to transferrin synthesis at the time of dissociation. Zagris (1985) showed that erythroblasts from blood islands of prestreak embryos synthesized hemoglobin even when the morphological development of explanted embryos was inhibited by culturing them ventral side down on vitelline membranes. This author suggested that the erythroblasts were committed to differentiation at the time of explantation. Indeed, specific cell types can be removed from the embryo and their phenotypic development under culture conditions can be assessed to determine whether or not these cells are committed to certain differentiation pathways in the absence of a normal embryonic environment. This approach has been used by a number of workers to study committment in vertebrate neural crest cells (reviewed by Weston, 1986).

The synthesis of α globulins 'a' and 'b' has been reported for the *area opaca* of chick embryos at the primitive streak stage (Kram and Klein, 1976). In the present study, the synthesis of these α globulins (55,000-62,000 MW; single broad band) was not detected. Serum protein 7 (66,500 MW) was putatively identified here as the pair of proteins that Kram and Klein refer to as α globulins 'a' and 'b'. The rationale for this was that it was the only unidentified protein band detected in 4 day serum close to the molecular weight reported by these workers for α globulins 'a' and 'b'. A doublet which comigrated with this protein was detected in EEC extracts (Figure 7). It appeared to increase in concentration over the time interval studied, suggesting that it may be synthesized by EEC aggregates. A dark staining band which comigrated with serum protein 7 was also detected in culture medium at all time intervals (Figure 8).

Nevertheless, it is possible that serum protein 7 does not correspond to α globulins 'a' and 'b'. Alternatively, these proteins may be synthesized in amounts which are not detectable by the methods used here. It is also possible that the synthesis of these proteins by EEC ceases under the culture conditions used here. Young and Klein (1983) reported that EEC from the *area vitellina* of 3 day old chick embryos stop synthesizing α globulins 'a' and 'b' after one day in culture. This is in contrast to the pattern of synthesis reported for these proteins in embryos incubated

in ovo (Sanders and Klein, 1977). Young and Klein (1983) suggest that some factor or factors are required for α globulin synthesis which may be absent under the culture conditions used here.

The α globulins 'a' and 'b' are embryo specific proteins which are not present in adult serum (Kram and Klein, 1976). In the chick, α globulin 'a' corresponds to mammalian α fetoprotein (AFP) (Young *et al*, 1980); chick AFP is a glycoprotein and recent reports have estimated the molecular weight to be 71,000 (Ido and Matsumo, 1982). Although its role in development is unclear, the observation that AFP reappears in adults with certain types of cancer suggests that it is involved in the induction of cell proliferation (Hirai, 1982). EEC proliferate *in ovo* (Bellairs, 1963) but not *in vitro* (Young and Klein, 1983). If AFP is required for cell proliferation then EEC may stop dividing in culture because α globulin 'a' synthesis stops under culture conditions. Further studies on EEC aggregates using modified labelling protocols and increased exposure time for X-ray films may determine whether or not these proteins are synthesized. It would be interesting to investigate some of the factors which may influence α globulin synthesis in culture, such as the presence of other cell types.

Serum protein 11 (38,600 MW) was present in large amounts in EEC at the time of dissociation (Figure 7, lane B) but was depleted from cells over the time interval

studied here (lanes C, D and E). It was detected in culture medium at all time intervals (Figure 8) and was apparently synthesized by EEC aggregates which were subjected to a relatively long labelling period (24h; Figures 9a and 9b). The synthesis of serum protein 11 was not observed in temporal studies where a shorter 6h labelling period was used (Figures 10 and 11). This observation lends support to earlier suggestions that some proteins may be synthesized in amounts which were not detected in this study. Because such a large amount of serum protein 11 is present in aggregates prepared from freshly dissociated EEC, it may be stored in EEC prior to dissociation and then released into the medium where large amounts were also detected at all time intervals studied (Figure 8). It is possible that EEC also synthesize small amounts of this same protein or small amounts of a different protein with the same approximate molecular weight. Two dimensional PAGE studies may help to distinguish between these two possibilities.

Apolipoproteins are molecules involved in lipid transport which are synthesized by the endoderm in mammalian yolk sacs (Shi and Heath, 1984; Shi *et al*, 1985). These proteins have been detected in the serum of rat (Wu and Windmeuller, 1979) and mouse (LeBoeuf *et al*, 1983). Various apolipoproteins have been reported with a molecular weight range of 28,000 to 46,000 (Bieseigel and Utermann, 1979; Zannis *et al*, 1982; Shi *et al*, 1985). Because the synthesis of serum protein 11 was detected here and it is in the same

molecular weight range reported for mammalian apolipoproteins, it may represent a previously undescribed chick embryo apolipoprotein.

Prealbumin was detected in this study as a very faint band in EEC extracts (Figure 7) and based on staining intensity, the concentration of prealbumin in the culture medium increased over the time interval studied (Figure 8). Fluorography results show that prealbumin is synthesized and released into the culture medium at all time intervals studied (Figures 9a, 9b and 11). This observation is consistent with previous reports (Kram and Klein, 1976; Young *et al*, 1980; Young and Klein, 1983). In humans, prealbumin is involved in thyroxine transport (Blake and Oatley, 1977) and vitamin A transport (Smith and Goodman, 1979). The role of prealbumin in development is unclear but antibodies against human embryonic prealbumin stain various human cancer cell lines (Page *et al*, 1985) indicating it may play a role in cell proliferation.

Serum protein 14 is synthesized and secreted by EEC (Figure 9b). It may also be present in other gels but because it usually migrates at or near the dye front, this cannot be determined by the methods used here. This protein may represent a soluble endogenous lectin previously described by Cook *et al* (1979). This lectin has β-D-galactose binding activity and it has an approximate molecular weight of 11,000 (Zalik *et al.*, 1983). Under rotary culture conditions, the aggregation of EEC is inhibited by

addition of extracted endogenous lectin (Milos and Zalik, 1982). These authors propose that this molecule promotes cell detachment at high concentrations. Lectin activity has also been detected in the fluid filled cavities of aggregates and it has been suggested that it plays a role in cell rearrangement and cavity formation (Milos *et al*, 1985). The source of this lectin activity is unknown but little lectin activity has been detected in preparations of yolk from the subgerminal cavity (Cook *et al*, 1979). It is possible that EEC aggregates synthesize and secrete lectin during cavity formation. At least some of the proteins synthesized by EEC aggregates are released into cavities as shown by silver grains in autoradiograms (Figure 12). Although further experiments are necessary to determine whether serum protein 14 represents the endogenous soluble lectin described by Cook *et al* (1979), affinity purified antibody against this lectin binds to EEC in sections of primitive streak stage embryos (S.E. Zalik, personal communication).

Other proteins were detected in EEC extracts and culture medium which did not comigrate with proteins in 4 day serum. Some of these proteins may represent nutrient reserves stored in EEC prior to dissociation. Previous studies have shown that yolk droplets in EEC decrease in size when aggregates are cultured for 24h and that some yolk is extruded into the culture medium by cells at the periphery of aggregates (Milos *et al*, 1984). The culture

medium may therefore contain some yolk derived proteins which are released from EEC. Some of the proteins observed in EEC extracts which did not comigrate with serum proteins were synthesized by these cells (Figure 9a, lane A; Figure 10). Because none of these proteins were secreted into the culture medium (Figure 11), they likely represent intracellular proteins involved in the maintenance of cellular metabolism. They could also represent structural proteins which were not tightly bound to membrane and therefore not removed by ultracentrifugation. None of these proteins have been identified at this time.

This investigation has shown that serum proteins are synthesized and secreted by EEC aggregates in culture. These aggregates also undergo morphological changes during cavitation which resemble those which take *in ovo* as the EEC spread to surround the yolk (Milos *et al*, 1979; 1984). When chick embryos with intact *areae opacae* are cultured on medium containing radiolabelled ovalbumin, label is detected in newly synthesized protein throughout the embryo (Hassel and Klein, 1971). In the study reported here, ovalbumin was detected in the medium used to wash uncultured EEC (Figure 8, lane B) but not in 6h, 18h or 30h culture medium (lanes C, D and E). Ovalbumin was not detected in EEC extracts (Figure 7), suggesting that it was utilized by EEC as a nutrient protein as reported for intact *areae opacae* (Hassel and Klein, 1971). The EEC aggregates used in this study exhibit features which resemble those described for EEC in

the intact embryo and it is likely that the pattern of serum protein synthesis and secretion in cultured aggregates is similar to that which takes place *in ovo*.

In other embryonic systems, investigators have tried to correlate the patterns of protein synthesis in embryos with particular morphogenetic events. Summers *et al* (1986) identified 3 morphogenetic periods in *Drosophila* development which coincide with rapid changes in protein synthesis, both qualitative and quantitative. Lovell-Badge *et al* (1985) detected stage specific and tissue specific proteins in the chick embryo and identified one of these proteins as a potential marker of mesodermal cell differentiation. Similar approaches have been used to analyze sea urchin embryogenesis (Bedard and Brandhorst, 1983) and the processes of meiotic maturation (Schulz and Wasserman, 1977) and germinal vesicle breakdown (Richter and McGaughey, 1983) in the mammalian oocyte.

By studying temporal changes in protein synthesis, stage specific molecules can be identified which may be associated with morphogenetic processes occurring at that particular stage. Once putative markers of differentiation and embryogenesis are identified, these molecules can be isolated and further experiments can be designed to test their effects on various cell types at different stages of development. Milos *et al* (1982) have used purified endogenous chick lectin to study the role this molecule plays in cavitation. By isolating individual serum proteins

and testing their effects on different tissues *in vitro*, the specific role they play in embryogenesis may eventually be elucidated.

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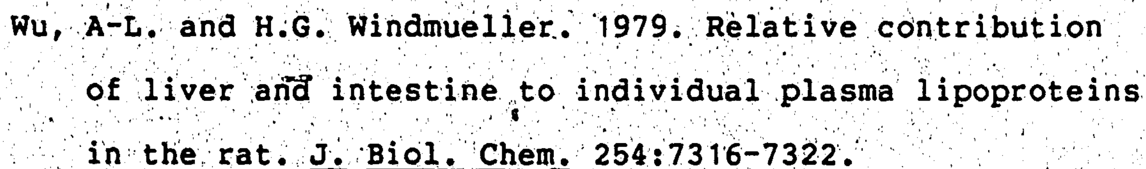

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