IDENTIFICATION AND CHARACTERIZATION OF A TRANSIENT ANTIGEN IN THE CHICK EMBRYO

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JANE BOWEN OSOKO, B.Sc.

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AUTHOR: Jane Bowen Osoko, B.Sc. (University of Guelph)

SUPERVISOR: Dr. D.J. McCallion, Professor of Anatomy

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ABSTRACT

Transient embryonic antigens have been described in many systems in development. Although their function has not yet been elucidated their detection in the embryo but not in the adult state suggests a direct relationship with embryogenesis. The present investigation was undertaken in order to characterize and study a transient antigen (TEA) in chick embryo brain extracts.

TEA was identified by using specific antiserum prepared against 9 day embryonic chick brain extract. The antiserum was first absorbed with adult serum, liver and kidney extracts to remove non-neural antibodies. Then adult brain extract was added to remove antibodies directed against adult neural antigens. This antiserum (TAS) was then considered to be both embryo and neural specific. However, subsequent studies demonstrated TEA in extracts of 9 day embryonic liver, kidney and serum in concentrations similar to that found in 9 day brain extracts. TEA was therefore not specific to neural tissue as initially considered.

TEA demonstrated anodal migration in an electric field, similar to an alpha-globulin at pH 8.6. Ontogenic studies using immunoelectrophoresis and the quantitative technique of rocket immunoelectrophoresis were performed. TEA was present by 2 days incubation, and an initial peak at 4 days was noted. From day 6 on, TEA accumulated until a maximum concentration was reached at 12 days incubation. TEA levels then decreased until it could no longer be detected in 20 day embryo or adult brain extracts.

Molecular exclusion chromatography revealed the molecular weight of TEA to be 73,000 daltons. Isoelectric focusing demonstrated the isoelectric point at pH 4.8. The antigenic site of the TEA molecule was considered to be proteinaceous on the basis of its sensitivity to pronase; however it was not hydrolysed by either trypsin of chymotrypsin.

Based on the ontogenic pattern and the physical and chemical characteristics it was concluded that TEA was a chick alpha-fetoprotein. The possible role of TEA in embryonic development and the mechanisms of its regulation were also discussed.

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INTRODUCTION

Embryonic development includes the process of growth and differentiation. Although the mechanisms of cell division are well documented, the complex process of specialization is largely not understood. During differentiation two events occur; first there is the progressive accumulation of components specific for a given organ or tissue, and second there is the disappearance of certain constituents characteristic of the embryonic state. These embryonic substances, detectable only at a specific stage of development are the subject of this thesis.

Early cell biologists considered the possibility that differentiation was controlled by the qualitative division of the genome. The current theory is based on two concepts: that all cells contain the same complete genome present in the zygote, and that through the process of selective activation of specific gene sequences, cells acquire different properties and functional diversity (Davidson, 1976).

Since the characteristics of a cell depend on its components, the expression of specific genetic information is necessary for the synthesis of those substances. Some studies of the differentiation process have paid particular attention to those constituents that are antigenic in nature since they can be identified by precipitin reactions with antibodies raised against them. The initial appearance of these antigenic components

indicating the activation of the genetic mechanism controlling their production, and subsequent alterations in their characteristics reflecting changes at the gene level can be demonstrated both qualitatively and quantitatively by the antigen-antibody reaction.

The production and accumulation of antigens characteristic of the adult state can be considered within the concept of selective gene activation. However, the specific temporal existence of those substances detectable only in the embryo suggests that additional regulation is required. Genes coded for those stage-specific (Vylchanov et al, 1978), phase-specific (Holleman and Palmer, 1972) or transient (McCallion and Trott, 1964) embryonic or fetal antigens must be activated at a specific stage and subsequently inactivated or suppressed later in development. This highly controlled regulation suggests a specific role for these antigens in embryogenesis.

Transience may also be associated with processes other than embryonic development. Antigens not normally present in the organism may appear under certain conditions such as pregnancy (Tal et al, 1964; Bohn, 1979) and during tissue or cell culture (Ting et al, 1978). These antigens also exhibit a specific temporal existence supporting the concept that there is an organized feedback and control mechanism of regulation of gene products. In addition, transient antigens normally found during embryogenesis have been linked with the malignant process; they reappear in the adult in many instances of human and experimentally induced carcinoma (Holleman and Palmer, 1972). This suggests that

the capacity for the production of transient embryonic antigens is not lost, but rather is suppressed or masked during inactivation. Since these onco-developmental antigens have been studied primarily for use in the clinical diagnosis of malignancies their role during the normal course of development has not been extensively characterized.

Little is known of the function of transient embryonic antigens in general. Some appear to be associated with the onset of differentiation of a particular organ or tissue and may serve in some manner in the inductive process. Others have been described which cannot be directly related to induction. They may be precursors to the adult state either by structurally laying down the initial cytoarchitecture or by fulfilling an initial functional role with subsequent replacement by adult characteristics. Whether they play an active part in differentiation or are merely passive reflections of the process is not yet known, but their appearance is widespread.

1. Stage-specific Antigens

Transient antigens are found during periods of morphological change. For example, unique proteins are synthesized by bacteria cells about to undergo sporulation (Hanson et al, 1970). Life cycles that include metamorphic events such as insect (Roberts, 1971) and amphibian (Chen, 1968) development display differential uses of genetic information during the transition from one developmental phase to the next.

In embryonic development, transient antigens characteristic for particular developmental events such as blastula, gastrula, tailbud and larval stages have been described in Amphibia (Denis, 1961; Chen, 1968). In the mammal, studies of whole mouse embryos (Klose and von Wallenberg-Pachaly, 1976) using protein mapping demonstrated considerable differences in the composition of protein patterns in 9, 12 and 14 day embryo extracts. A group of proteins characterized by high molecular weight and similar isoelectric point were found to decrease in concentration and were no longer detectable in 14 day embryos.

Work from descriptive and experimental embryology has clearly established that during these early stages of development many of the organizational patterns are determined and organogenesis occurs. Although these transient antigens have not been linked with any one particular developmental event, their detection at this time and not at any other suggests an association with early differentiation processes occurring during development.

2. Transience in Erythrocytes

The most striking contrast between embryonic and adult characteristics is seen in polymorphic erythrocytes and the phase-specific hemoglobins they contain. In most species in which they exist, two distinct populations of erythrocytes make their appearance during development. The early red cell line is limited in duration and is morphologically recognizable by the presence of the nucleus at each stage of erythroid differentiation. The

later population has a self-perpetuating stem cell line which matures and becomes the characteristic red cell population.

a) Embryonic hemoglobins

Coincident with the appearance of the different erythrocytes are changes in the circulating hemoglobins. Studies in the mouse demonstrated three embryonic (E_1, E_{11}, E_{111}) and one adult (A) hemoglobin type with characteristic globin chains (Fantoni, 1970). HbE_1 contained x and y chains; HbE_{11} contained α and y chains; HbE_{111} contained α and z chains; and adult Hb contained α and β chains. The embryonic x, y and z chains were found only in nucleated erythroid cells derived from the yolk sac; adult globin chain α was shared with the yolk sac derived erythrocytes while the β chain was found only in liver erythroid cells and circulating reticulocytes of liver origin. The yolk sac erythroid cells did not synthesize adult β chains and the liver erythroid cells synthesized exclusively α and β chains of adult hemoglobin. The author therefore argued that the switch from embryonic to adult hemoglobins was due to the substitution of one erythroid cell line for another, specifically the substitution of erythroid cell differentiation in the liver for that occurring in the blood islands of the yolk sac.

A similar mechanism was once thought to occur in man; that is, each fetal site of erythropoiesis produced a different embryonic hemoglobin. However, human fetal liver and bone marrow cells were shown to synthesize both fetal and adult hemoglobin (Thomas et al, 1960). Both hemoglobin types are also synthesized

in the chick embryo by the yolk sac, the primary erythropoietic site until hatching (Ingram, 1972; Chapman and Tobin, 1979). Since both hemoglobin types can be synthesized by one organ, mechanisms other than cell lineage substitution must be considered for the hemoglobin switch.

Although functionally related to environmental changes at birth the decrease in embryonic hemoglobin synthesis and the increased production of adult hemoglobin begins well before. Studies in man have shown that the rates of synthesis are not affected by changes in environment and oxygen tension which occur at birth (Bard, 1973).

Since different hemoglobins are synthesized at different times during development and some populations contain both early and late hemoglobins (Chapman and Tobin, 1979) there seems to be an ordered sequence of change until the adult state is achieved. This control appears to be at the gene level where specific genes are selected at temporally determined stages of development.

b) Transient membrane antigens

In addition to changes in globin chain synthesis, transient antigens have been detected on human (Hakomori et al, 1972) and chicken (Sanders, 1968) peripheral red cell membranes.

In the White Leghorn chicken, antisera raised against three day post-hatching chicken red cells and reacted with red cells from chickens of various ages revealed the presence of a transient antigen by at least 18 days incubation; by 50 days post-hatching it began to disappear and was not detectable by 120 days

or in the adult (Sanders, 1968). The antigen was subsequently termed chick fetal-leukemic antigen (CFA) when it was found to reappear in adult peripheral red blood cells during AMV (Avian Myeloblastosis Virus) induced leukemia (Teplitz et al, 1974).

By absorbing anti-CFA serum with red cells from different avian species the antiserum was serologically dissected into thirteen CFA antigenic determinants (Dietart and Sanders, 1978). Comparison of hemagglutination properties among these species revealed specific patterns of CFA expression within the phylogenetic groups. The non-Gallus avian species studied and those chicken varieties most genetically dissimilar to the White Leghorn lost some CFA determinants during development but continued to express others in the adult. Hence CFA was embryo-specific only in the White Leghorn and closely related chicken species.

Chick fetal-leukemic antigen was also detected on erythrocyte precursor cells obtained from the bone marrow of adult chickens (Sanders and Kline, 1977) and reappeared in the adult in AMV-induced leukemia (Teplitz et al, 1974). This lead to the proposal that in the leukemic state an alteration of the normal differentiation-release pattern could result in the premature release of CFA-positive immature erythrocytes from the bone marrow into the peripheral blood. Similarly this could explain the detection of CFA in some adult birds, since red cell patterns of differentiation may differ among species. Higher levels of immature erythrocytes in peripheral blood than those in the White Leghorn adult chicken have been reported for some adult birds

(Lucas and Jamroz, 1961). Although there are insufficient numbers to account for the levels of CFA reactivity reported by Dietart and Sanders (1978), their presence suggests that factors controlling the selective release of red blood cells into the peripheral system differ between avian species.

Neither the mechanism of erythrocyte release nor the expression of CFA determinants on the red cell membranes themselves are understood. However, the heterogeneity in CFA expression among avian species suggests that control is at the gene level and dependent upon genotype.

3. Transient Lens Crystallins

The vertebrate lens is particularly suitable for studies of differentiation. It is an isolated avascular tissue and is composed entirely of an outer epithelial layer adherent to the capsule and the lens fibre cells derived from it. The bulk of the lens is composed of fibre cells which are continuously laid down; fibres in the innermost nuclear region are those formed during early postnatal and prenatal life while those in the outer cortex are added later. Since no cells are lost, the lens at any stage embodies its own developmental history.

During the differentiation of lens epithelial cells to lens fibres there are both morphological and cytochemical changes in addition to significant changes in proteins. 95% of the proteins of the vertebrate lens are the crystallins which have no known enzyme function and are regarded as structural proteins. Three

groups of lens crystallins, the α, β and γ crystallins have been identified. Of these, the γ-crystallins have been shown to be involved in the differentiation of lens epithelial cells to fibre cells. In addition, at least one γ-crystallin present during embryonic life is no longer synthesized in the adult lens.

Papaconstantinou (1965) analysed the soluble proteins from adult and calf lens epithelial cells by DEAE-cellulose chromatography and showed that these cells contained α and β crystallins, but not the γ-crystallins. However, significant quantities of γ-crystallins could be found in the fibre cells of adult, calf and embryonic lenses. This suggests that the genes governing the synthesis of the γ-crystallins were activated either sometime during the differentiation of an epithelial cell to a fibre cell or soon after fibre formation is completed.

In addition, resolution of the γ-crystallins of adult and calf cortex and nucleus and embryonic lens demonstrated distinct elution profiles (Papaconstantinou, 1965). While the embryonic lens, calf cortex and nucleus, and adult nucleus demonstrated only one component the adult cortex γ-crystallins were eluted in three peaks. Furthermore, by electrophoresis of purified γ-crystallins it was found that the embryonic and adult nucleus proteins were similar, whereas the purified γ-crystallins from the adult cortex synthesized only in the adult demonstrated different electrophoretic mobilities. Similar results have also been shown in Xenopus laevis by starch gel electrophoresis; a γ-crystallin is one of the major bands lost during development (Clayton, 1970).

Thus in the lens the embryonic and early post-natal cytoarchitecture seems to be replaced by a more complex adult state.

Although the factors determining the regulation of lens crystalline synthesis are not understood the γ-crystallins demonstrate a structural component that is replaced during development and may also be related to lens fibre differentiation.

4. Transient Kidney Antigens

Studies of the development of the chick kidney have demonstrated a tissue-specific transient antigen that may be involved in the differentiation of the definitive kidney (Croisille, 1970). Antiserum was raised against 10 to 12 day mesonephros and absorbed with adult tissues to render it embryo and kidney specific. It was reacted by immuno-diffusion and a component in mesonephros extracts from 8 to 18 day embryos and in metanephros extracts from embryos of 12 to 20 days incubation precipitated. This antigen was specifically associated with the kidney during embryonic life and could not be detected in adult chicken kidney extracts.

The differentiation of the kidney is dependent on the inductive influence of the Wolffian duct and the ureter. Further immunofluorescent studies of adult kidney-specific antigens by Croisille (1970) demonstrated that adult kidney components appeared at the time of or shortly after the induction of the meso and metanephrogenic primordia. During the development of the mesonephros the first specific fluorescence was observed at the 36 somite stage (3 days incubation), when the first secretory

tubule differentiates under the inductive stimulus of the Wolffian duct. At 8 days incubation, similar fluorescence was shown to occur shortly after the induction of the metanephrogenic cells by the ureter.

Although the embryonic kidney-specific antigen could not be localized by immunofluorescent techniques utilized in this study, immunodiffusion experiments demonstrated its presence in mesonephric and metanephric tissues during definitive tubule differentiation. Studies of kidney antigens in human fetuses have revealed a similar fetal kidney antigen, detectable during the differentiation of the metanephrogenic mesenchyme, and present in the newborn but not in the postnatal or adult kidney (Linder, 1969).

Further studies using more sensitive techniques are necessary to determine whether the activation of gene sequences specific for this transient embryonic antigen is dependent on the induction process as the adult kidney antigens appear to be. In addition, the role of this antigen during differentiation of the kidney may be elucidated.

5. Transient Liver Antigens

Although neither ontogenic nor quantitative experimentation has been done, several studies have at least demonstrated the presence of transient embryonic antigens in liver tissue.

Antigenic changes in newborn rat liver were studied by means of immunodiffusion and immunoelectrophoresis combined with autoradiography (Raftell and Perlmann, 1968). Two day old rat

liver contained at least three transient antigens. They exhibited different electrophoretic mobilities (cathodal migration) from four antigens (anodal migration) unique for newborn serum and could not be detected in liver homogenates from adult rats prepared 24 hours after partial hepatectomy. Antiserum against adult liver contained no antibodies against them. However, since other tissues were not tested for cross-reactivity their specificity for liver was not confirmed.

Several transient antigens were also reported in the microsomal fraction of embryonic chick liver (Mutolo et al, 1965). Further separation by deoxycholate showed that they were bound to the deoxycholate-soluble fraction (membranes) and not to the ribosomes. At least two of these antigens could not be detected in adult microsome fractions or in other organs and they possessed different electrophoretic mobilities from the adult antigens. A complete ontogenic study was not performed but these transient liver antigens were demonstrated in at least 6, 10 and 15 day chick embryos.

Mutolo et al (1965) suggested that the differentiation of the antigenic pattern of the liver cells is masked by the sequential appearance of stage-specific protein changes in the course of development and that the differentiation of liver microsomes may take place by a quantitative modification of both the structural proteins and the ribosomal population. Although these studies do demonstrate the presence of a transient liver antigen(s) their function and possible mode of control still remain speculative.

6. Onco-developmental Antigens

Many investigations have demonstrated antigenic similarities between embryonic tissues and several tumor types. Because of this, many onco-developmental antigens have been studied on the basis of their clinical and diagnostic importance. Although the roles of the individual antigens in the normal course of development have not been extensively studied, a common gene-related process in development and carcinogenesis has been hypothesized.

a) Carcinoembryonic antigen

One of the most widely studied onco-developmental antigens from a clinical viewpoint is the carcinoembryonic antigen (CEA) first described by Gold and Freedman (1965). They studied a wide variety of human adult and fetal tissues to determine if they contained two tumor specific antigens previously found in human colonic cancer. Double immuno-diffusion experiments were performed using anticolonic tumor-specific antiserum. In adult tissues, identical antigens were present in all tested specimens of malignant tumors of endodermally derived epithelium of the digestive tract and pancreas, but were absent from all other tissues tested; they were not found in patients with cancers of non-digestive tract origin nor in any with non-malignant disease. In fetal tissues these same antigens were present in the fetal gut and its functionally related derivatives, the liver and pancreas, but were not detected in any other tissues. They were found in fetuses between two and six months of gestation.

Initially CEA was considered to be specific for the endodermal tissue of the embryonic digestive system and endodermally derived malignant lesions in the adult and the possibility that it could be used as a serologic screening test for digestive cancer was considered. Although it has not been reported in other fetal tissues recent studies using more sensitive techniques have demonstrated the presence of CEA in patients with non-neoplastic disease (Wegener et al, 1979) and in those with tumors of extra gastrointestinal origin (Ryatsep et al, 1979). Although CEA may be of little value as a diagnostic tool its continued use in the prognosis and assessment of therapy in established malignancies has been suggested (Chu et al, 1979).

b) Alpha-fetoprotein

Studies of mammalian (Gitlin and Boesman, 1966), amphibian (Chen, 1970), and avian (Weller, 1966) sera have indicated an increase in the complexity of serum proteins as development proceeds. In each species studied the fetal serum contained a protein that was not detectable in the normal adult. This transient serum protein demonstrated an electrophoretic mobility of an alpha-globulin at pH 8.6 and was subsequently termed alpha-fetoprotein (AFP).

AFP has been extensively studied in the human because of its many and diverse clinical manifestations. Abelev et al (1963), were the first to demonstrate the recurrence of AFP in cancers. It was found in mouse hepatomas and in fetal and newborn serum but was undetectable in the adult serum or tissues by their techniques.

AFP was found in sera of patients with primary hepatocellular cancer (Ruoslahti et al, 1974) as well as in those with gonadal and extragonadal germ cell tumors (Norgaard-Pederson et al, 1972). In addition, a variety of congenital fetal disorders are associated with high-for-dates amniotic AFP levels (Milunsky et al, 1975); AFP levels in amniotic fluid are used in screening for neural tube defects (Brock, 1979).

In the human (Ruoslahti and Seppala,1972a) and in animal models (Masopust et al, 1970) AFP has been characterized as a glycoprotein with a molecular weight of approximately 70,000. In addition, rather than being restricted to a short temporal appearance AFP is present throughout the gestational period in all species studied. For example, in the human fetus AFP synthesis was evident as early as 29 days gestation, the earliest age studied (Gitlin and Biascucci, 1969). It was found to increase in concentration until the fourteenth week when the serum concentration of AFP began to fall exponentially. AFP was thought to disappear from the circulation a few weeks after birth but studies using sensitive radioimmunoassay techniques have demonstrated its presence in very low concentrations in normal adult serum (Ruoslahti and Seppala,1972b); the difference in AFP concentrations between human fetal and adult sera is almost a millionfold (Hirai, et al, 1973).

An embryonic serum protein(s) has also been demonstrated in chick embryo serum (Weller, 1966; Gitlin and Kitzes, 1967; Lindgren et al, 1974). Although chick AFP does not cross-react

with anti-mammalian serum nor does anti-chick fetoprotein antiserum react with mammalian AFP's (Lindgren et al, 1974), similar ontogenic patterns (Weller and Bowdon, 1974a) and chemical characteristics (Weller and Bowdon, 1974b) suggest homology with mammalian AFP.

In the chick the major site of AFP synthesis is the yolk sac. However in those animals where the yolk sac becomes atretic early in development AFP synthesis continues in the liver. Since AFP is not merely a by-product of yolk sac function and its presence during much of the gestational period is ensured, it must be of functional importance throughout embryonic and fetal development. As a serum protein it may serve in a transport capacity or in a protective role. In addition, the recurrence of AFP as well as other onco-developmental antigens in certain carcinomas supports the concept of a similar if not common mode of genetic regulation in these two systems.

7. Transient Brain Antigens

Studies of human fetal brains have demonstrated neurospecific transient antigens (Vylchanov et al, 1978). Immunodiffusion reactions of absorbed 8 to 10 week embryonic human brain antiserum with extracts of human brain from various developmental stages demonstrated three or four brain antigens. One or two embryonic brain antigens (EBA) were present only between 8 and 10 weeks of gestation; another was characteristic of embryonic and fetal brain up to the thirtieth week of gestation; the last was shared by embryonic and adult brain. Confirmation of the immunodiffusion

data by immunoelectrophoresis was reported; EBA demonstrated an electrophoretic mobility of standard human albumin (Kehoyov et al, 1976).

To determine if EBA was an onco-developmental antigen similar to alpha-fetoprotein and carcinoembryonic antigen the absorbed embryonic brain antiserum was tested against extracts of the following human tumors: meningiomas, glioblastomas, astrocytomas, neurinomas and brain metastasis of lung carcinoma (Kehoyov et al, 1976). Immunodiffusion demonstrated a reaction of complete identity with an antigen shared by embryonic brain and meningioma; no other tumor extracts cross-reacted. After absorption of the antiserum with twenty-two week embryonic brain extract, the antiserum still demonstrated a precipitin line common to both EBA and meningioma. Immunoelectrophoresis showed that these two antigens shared identical electrophoretic mobilities. In addition, absorption of the 8 to 10 week embryonic brain antiserum with meningioma extract lead to complete saturation of the antibodies directed against the transient neural antigens.

This meningioma-associated embryonic neural antigen was thus considered analogous to alpha-fetoprotein and carcinoembryonic antigen. However, the lack of cross-reactivity of the embryonic meningioma antigen with either of these, determined by immunoelectrophoresis and immunodiffusion and the different embryonic origin of the meningioma tumor tissue suggested that EBA was a separate antigen.

Studies in the chick embryo have also demonstrated an

embryonic brain-specific antigen. When 9 day chick embryo brain extract was tested against homologous antiserum by immunodiffusion, two neural-specific antigens were found (McCallion and Trott, 1964). One was present in six day brain extracts and persisted in the adult; the other could be demonstrated by the 8th day of incubation and disappeared just before hatching. There was no evidence of neurospecific antigens at earlier developmental stages. One other transient antigen was reported but was common to several other embryonic organs tested.

Further immunoelectrophoretic studies using 9 day embryonic chick brain antiserum demonstrated four groups of precipitin bands in 9 day incubation chick brain with different electrophoretic mobilities (McCallion and Trott, 1965). One group which displayed little,if any electrophoretic migration contained transient embryonic antigens. Four precipitin bands within this group were found to represent common tissue antigens while the other was neurospecific when compared with other embryonic organs. These findings were in agreement with the earlier immunodiffusion studies (McCallion and Trott, 1964), but with the further refinement of electrophoretic techniques more precipitin bands were found.

Since the earliest detection of this transient neural antigen was at eight days incubation its appearance does not appear to be related to the neural inductive process occurring in the chick within the first twenty-four hours of incubation. Its role may be related then to the formation of the definitive cyto-architecture of the brain. Since no immunofluorescent studies

were performed, localization and correlation with the development of a particular neural cell type could not be determined.

Although transient embryonic antigens have been studied in various tissues and organs in many species and are known to recur in certain instances of carcinoma, individually most have not been extensively characterized. Because many studies have been concerned with the qualitative descriptions of their existence comparisons of other characteristics to elucidate a possible common role in development has been difficult.

The purpose of the present investigation was to verify the presence of a transient embryonic antigen in saline soluble embryonic chick brain extracts, to quantitate its ontogenic pattern and to characterize it using immunochemical techniques. From these findings more knowledge of the quantitative aspects of a particular transient antigen would be gained. This, in turn, may aid in elucidating the function of transient embryonic antigens during development and further our understanding of the differentiation process itself.

MATERIALS AND METHODS

1. Preparation of Antigen Extracts.

Fertilized eggs from White Leghorn chickens were incubated in a humidified, forced air incubator (model 2940I; Jamesway) at 38°C. Adult tissues were also obtained from White Leghorn chickens.

Whole embryos from day 1 to 4 were removed and washed in cold 0.85% saline to remove yolk. Whole brains from day 5 to 20 of incubation, and adult brains, were removed, freed from surrounding membranes and washed in cold 0.85% saline. Brains were kept cold on ice throughout the extraction procedure.

All samples were homogenized for 60 seconds in an equal volume of 0.85% NaCl in a Brinkman Polytron tissue homogenizer and centrifuged at 2600 xg (Sorval RC-5 superspeed refrigerated centrifuge) for 30 minutes. The supernatant was stored at 4°C, while the precipitate was resuspended in an equal volume of 0.85% saline and centrifuged again. This procedure was repeated twice. The three supernatants thus obtained were pooled and centrifuged at 100,000 xg (IEC/B-6 ultracentrifuge) for 1 hour.

The final clear supernatant was concentrated by positive pressure ultrafiltration (Amicon Diaflow, PM 10 filter) to a total protein concentration of 30.0 to 60.0 mg/ml and stored at -25°C in 1.0 ml aliquots.

Tissue extracts of adult brain, liver and kidney, and 9 day liver and kidney were prepared in the same manner.

Blood from several 9 and 15 day embryos was collected from the

chorioallantoic or vitelline vessels. The vessel was teased away from membranes and secured over the shell opening; it was then cut, and blood was collected directly into capillary tubes, minimizing contamination from other embryonic fluids. The blood was then spun in a hematocrit for 5 minutes. The sera from embryos of the same age were pooled and stored at -25°C, while the pooled red blood cells were lysed in 75.0 µl cold buffer (0.03 M KCl, 0.02M $MgCl_2$, 0.01M Tris-HCl, pH 7.4) to which 0.1% Triton X-100 was added (Tobin et al, 1976). The lysate was stirred for 5 minutes on ice and centrifuged (Brinkman 3200 Eppendorf centrifuge) for 15 minutes. The supernatant was tested against antisera immediately.

2. Preparation of Antiserum.

a) Production of antibodies.

Two New Zealand White rabbits were used to obtain antiserum to 9 day brain extract (9BE). The 0.5 ml 9BE (30.0-40.0 mg/ml) sample was prepared as described above, centrifuged, and suspended in an equal volume of Freund's Complete Adjuvant. Half of this suspension was injected on each side intramuscularly in the gluteal region. This procedure was repeated weekly for four weeks. The rabbits were then boosted every six weeks for 4 to 5 months until the antibody response was found to be optimal as judged by immunodiffusion. At this time the rabbits were anaesthetized with sodium pentabarbitol and exsanguinated by carotid catheter.

The blood was placed in a 37°C incubator for 2 to 3 hours to allow

clot formation, refrigerated overnight and centrifuged for 15 minutes at 2600 xg the next day. The clot was removed and the serum was further centrifuged for 15 minutes at 10,000 xg. The clear serum (AS) was collected, pooled and stored at -25°C.

b) Absorption of antisera

The antiserum (AS) was absorbed with adult serum, liver and kidney extracts to remove common tissue antigens and render it neurospecific (AAS). The solution was incubated at 37°C for 1 hour with occasional mixing and centrifuged for 15 minutes at 10,000 xg. Absorption was monitored by immunodiffusion, and the procedure was repeated until there was no cross-reactivity with these adult tissue extracts. In some cases as much as 0.5 ml absorbing extract was added to 1.0 ml antiserum. The absorbed serum (AAS) was further absorbed with adult brain extract to remove all adult neural antibodies using the method described above. The resulting antiserum (TAS) was then considered to contain only neurospecific embryonic antibodies.

c) Ammonium sulphate precipitation

This procedure was used to isolate crude IgG from AAS and TAS. The antisera were diluted with an equal volume of borate buffer (250.0 ml 0.2 M Boric Acid, 87.5 ml 0.05 M Sodium Borate, diluted to 1000.0 ml with distilled water). To this, an equal volume of 80% saturated ammonium sulphate was added dropwise, with continuous stirring. After adjusting the pH to 8.0 with 0.1 M ammonium hydroxide the solution was mechanically stirred for 30

minutes, and then centrifuged for 30 minutes at 5,000 xg. The supernatant was discarded; the pellet was resuspended in the original volume of borate buffer and the precipitation procedure was repeated twice. The final pellet was resuspended in a small amount of distilled water, dialysed overnight in borate buffer and centrifuged for 20 minutes at 10,000 xg the next day.

d) IgG purification

The crude IgG fractions of AAS and TAS were further purified using Affinity Chromatography. Diethylaminoethyl (DEAE) Affini-Gel Blue (Bio-Rad Laboratories) was the matrix used. The procedure produces an IgG fraction from the serum by stepwise elutions of increasing ionic strength buffers. This fraction is purified of all major serum contaminants and free of proteolytic activity.

Ammonium sulphate precipitated AAS was prepared for column application by dialysing against two changes of Tris-HCl buffer, pH 8.0 with 0.02% NaN_3 for 24 hours and centrifuging for 20 minutes at 10,000 xg.

The column (45.0 cm x 1.5 cm ID) was packed according to specifications, regenerated with two bed volumes of 8M Urea and equilibrated with Tris-HCl buffer, overnight. The AAS sample was applied, followed by 250.0 ml Tris-HCl buffer. The stepwise elutions were obtained by adding 250.0 ml of 0.04 M NaCl in Tris-HCl buffer, and then 250.0 ml 0.5 M NaCl in Tris-HCl buffer. The 4.0 ml fractions were collected and the absorbance was monitored at 280 nm on a Gilford 620 spectrophotometer.

The three major protein peaks eluted were concentrated by positive pressure ultrafiltration and tested by immunodiffusion against 9BE. The same procedure was used to isolate IgG from TAS.

3. Immunodiffusion

Double immunodiffusion analyses were performed by the method of Ouchterlony (1953). In addition to the demonstration of antigen-antibody reactions, this qualitative technique differentiates between reactions of complete, partial and non-identity.

Microscope slides (25.0 x 75.0 mm) were acid washed with a commercial preparation (Nochromix; Godax Laboratories), soaked in running tap water for 24 hours, rinsed in deionized water and dried.

One and one half gm of agarose (Type I Low EEO; Sigma) in 50.0 ml electrophoresis buffer and 50.0 ml distilled water was mixed and heated in a boiling water bath until the agarose was completely dissolved and evenly dispersed. The slides were pre-coated with 1.0 ml of hot agarose applied with a pipette and cooled. They were then covered with filter paper, soaked with distilled water to prevent uneveness and dried overnight at 37°C. Each precoated slide was then coated with 3.0 ml hot 1.5% agarose, cooled and stored in gel buffer at 4°C. Wells were punched and antigen extracts were placed in peripheral wells around a central well containing antibody; the antigen-antibody reaction was allowed to proceed in a closed, humidified plexiglass container for 16 to 24 hours at 37°C. The slides were then washed in several changes of 0.85% NaCl for 24 hours and photographed.

Slides were preserved in the following manner. Whatman

No. 1 filter paper was carefully laid flat over the slide. Absorptive material (paper towels) and a weight (books) were placed over this to compress the gel into a thin flat sheet. The plates were then dried under a stream of hot air from a hairdryer, stained and destained.

The stain was made by adding 1.0 gm Coomassie Brilliant Blue G to 90.0 ml 95% ethanol and 200.0 ml acetic acid and left overnight at room temperature. The solution was then filtered before 90.0 ml distilled water was added. The destainer consisted of 250.0 ml 95% ethanol and 100.0 ml acetic acid made up to 800.0 ml with distilled water. Slides were stained for 5 minutes, destained until the background colour was removed and hot air dried.

Dried agarose was prevented from peeling off the slides by coating them with varnish as follows: The plates were heated in a 60°C oven. While still hot, they were dipped in a solution of 2.0 ml decanted shellac, 2.0 ml glycerol and 96.0 ml 95% ethanol and immediately returned to the 60°C oven until dry.

4. Immunoelectrophoresis

Immunoelectrophoresis was run according to the method of Grabar and Williams (1953), modified by Scheidegger (1955). The antigen sample was subjected to electrophoresis so that proteins were distributed according to their electrophoretic mobilities. After the completion of the electrophoresis, a trough parallel to the direction of migration was filled with antiserum. Diffusion of both antigens and the antiserum results in the formation of characteristic precipitin bands at the point of equivalence.

Microscope slides were prepared as for immunodiffusion and wells and troughs were made with a template. Electrophoresis was carried out at 4°C for 2 hours using a constant current of 6 mA per slide. The electrophoresis buffer was 0.1 M barbitone acetate, pH 8.6 (Oxoid) with 0.01% NaN_3 added as a preservative. Development of the antigen-antibody reaction and all procedures following were the same as those described for immunodiffusion.

5. Quantitative Immunoelectrophoresis

a) Crossed immunoelectrophoresis

Crossed immunoelectrophoresis (Weeke,1973a) provides greater resolution than the classical immunoelectrophoretic technique of Grabar and Williams (1953) due to the combination of electrophoretic separation of proteins in agarose gel followed by perpendicular electrophoresis into an antibody containing gel.

Glass plates (70.0 x 100.0 mm) were precoated with 5.0 ml of 1.5% agarose and then coated with 12.0 ml 1.5% agarose as described for immunodiffusion. The sample well was filled with 15.0 µl 9BE diluted to 4.0 mg/ml and the first dimension electrophoresis was performed at 20 mA per plate for 2 hours. All but 15 mm of the gel at the cathodal end was discarded and replaced with 10.0 ml of 1.5% agarose to which 500.0 µl TAS had been added after cooling to 50°C. The second dimension electrophoresis was then performed by turning the plate 90° in the electrical field. Electrophoresis was continued for 16 hours at 10 mA per plate. After electrophoresis, the plates were dried, stained and destained as described for immunodiffusion. Since the dried agarose did not peel off the large plates shellac was not applied.

b) Rocket immunoelectrophoresis

Rocket electrophoresis (Weeke, 1973b) is used to compare the concentration of a given antigen in a number of samples. The samples were electrophoresed into an antibody-containing gel. The resulting antigen-antibody reaction was in the form of a rocket-shaped precipitin line where the heights of the "rockets" were used for quantitative comparison.

Glass plates (70.0 x 100.0 mm) were precoated with 5.0 ml 1.5% agarose as described for immunodiffusion. Then 12.0 ml 1.5% hot agarose was cooled to 50°C and 250.0 µl TAS was added. The solution was mixed and quickly poured onto the plates on a levelling table. After cooling a 15.0 µl aliquot of each brain extract, diluted to 4.0 mg/ml total protein was added to wells punched into the cathodal end.

Electrophoresis at 20mA per slide at 4°C was begun prior to sample loading and proceeded for 8 hours. After electrophoresis, the plates were dried, stained and destained as described for immunodiffusion.

Some rocket electrophoreses were performed on 50.0 x 75.0 mm glass slides. The same protocol described above was used except that the slides were precoated with 2.0 ml 1.5% agarose and coated with 6.0 ml 1.5% agarose containing 125.0 µl TAS. Electrophoresis was at 10 mA per slide for 8 hours.

c) Fused rocket electrophoresis

Fused rocket electrophoresis (Svendsen, 1973), a modification

of rocket electrophoresis, is used to identify antigens following separation experiments. Samples were allowed to diffuse briefly in an agarose gel prior to electrophoresis into an antibody-containing gel, resulting in a continuous precipitin line for each antigen contained in the sample. The amount of antigen precipitated and its distribution among fractions could thus be determined.

For these experiments 70.0 x 100.0 mm glass plates were precoated with 1.5% agarose and 12.0 ml 1.5% agarose was then poured onto the plate and cooled. With a ruler and razor blade the gel was cut to a final width of 15.0 mm; the rest was discarded. Ten ml agarose mixed with 100.0 ul TAS was poured onto the rest of the glass plate and cooled. The 15.0 µl aliquots from fractions obtained in separation experiments were added to wells in the antibody-free gel and left to diffuse for 15 minutes prior to electrophoresis.

Electrophoresis at $4^{o}C$ was at 20 mA per slide for 8 hours. Drying, staining and destaining procedures were the same as those described for immunodiffusion.

6. Protein Determination.

Total protein concentrations of extracts were determined according to the method of Lowry et al. (1951). Bovine serum albumin (BSA) was used as a standard and samples were read at 750 nm.

7. Enzyme Digestion.

Treatment of antigens with enzymes of different proteolytic pro-

perties aids in the elucidation of the nature of the antigenic determinants. In this study, pronase, chymotrypsin and trypsin were used.

One mg samples of trypsin, chymotrypsin and pronase were each dissolved in 1.0 ml 50.0 mM Tris-HCl, pH 7.6 containing 20.0 mM $CaCl_2$. Then 10.0 µl enzyme and 10.0 µl 9BE (25.0 mg/ml) were mixed and incubated at 37°C for 2, 4 or 6 hours. At that time 20.0 µl aprotinin was added to terminate the digestion. A control sample containing 10.0 µl buffer rather than enzyme was included. The hydrolysed samples were tested for activity against TAS by immunodiffusion.

To verify the enzyme activity of the proteases and to further ensure enzyme inhibition by aprotinin prior to immunodiffusion, a sensitive assay for the detection of these enzymes was used (Protease Detection Kit; Bio-Rad). Ten µl samples of the extract-enzyme-aprotinin mixture were added to wells punched into a 1.0% agarose plate containing a bovine casein preparation and incubated 12 to 18 hours at 37°C. Enzyme activity was demonstrated by the digestion of the casein and the formation of a transparent ring around the wells.

8. Molecular Weight Determination.

Molecular exclusion chromatography provides a simple method for molecular weight determination based on the molecular sieving properties of the gel filtration media.

In this study a 90.0 cm x 2.5 cm (ID) column was packed with Sephacryl S-200 (Pharmacia Fine Chemicals) according to specifications and washed and equilibrated with 3 volumes of 50.0 mM K_2HPO_4 buffer containing 0.15 M NaCl, 2.0 mM EDTA and 0.1 mM 2-mercapto-ethanol, pH 7.2.

The column was calibrated as follows. First, 1.0 mg blue dextran 2000 dissolved in 1.0 ml K_2HPO_4 buffer was applied to the column. Then 5.0 mg ribonuclease A (MW 13,700), 5.0 mg ovalbumin (MW 45,000) and 20.0 mg aldolase (MW 158,000) dissolved in 1.0 ml K_2HPO_4 buffer was applied. The final application consisted of 5.0 mg each of chymotrypsinogen (MW 25,000) and bovine serum albumin (MW 67,000) dissolved in 1.0 ml K_2HPO_4 buffer. Two ml fractions were collected; samples were not applied to the column until peaks from the previous run were eluted and their absorbance at 280 nm determined.

9BE (40.0 mg/ml) was dialysed overnight in two changes of K_2HPO_4 buffer. A 1.25 ml aliquot was applied to the column after the standards were eluted. Again, 2.0 ml fractions were collected and the absorbance at 280 nm was determined. Fractions were assayed by fused rocket electrophoresis using TAS in the gel.

The distribution coefficient (Kav) of each standard was calculated using the formula: $Kav = \frac{Ve-Vo}{V_t-Vo}$ where

Kav = fraction of the stationary gel volume available for a given source

V_t = total volume of packed gel = $\pi r^2 h$

Vo = elution volume of molecules distributed in the mobile phase; determined by the void volume of blue dextran 2000

Ve = elution volume of solvent

The Kav of the standards were plotted against their log molecular weight. The molecular weight of the unknown was determined by comparing the calculated Kav to the standard curve.

9. Isoelectric Focusing

The isoelectric point (pI) was determined in the following manner:

Precision bored glass tubes (12.5 x 0.5 cm) were soaked in chromic acid overnight and rinsed in changes of water, deionized water and 95% ethanol. They were then soaked in Photo-Flo 200 (Kodak) diluted 1:200 and dried in a 150°C oven.

Ten gm acrylamide (Bio-Rad Laboratories) and 0.4 gm bisacrylamide (Bio-Rad Laboratories) were added to 50.0 ml distilled water. For each tube, solution A, consisting of 0.625 ml acrylamide-bisacrylamide mixture, 0.313 ml 80.0% glycerol, 0.125 ml ampholyte (Biolyte 3/10, Bio-Rad Laboratories) and 0.186 ml distilled water was mixed and deaerated for 2 minutes. Solution B, containing 1.5 µl tetramethylethylenediamine (TEMED; Bio-Rad Laboratories) in 1.25 ml freshly made 50% ammonium persulfate was mixed and deaerated separately. Solutions A and B were then mixed and 2.1 ml was quickly poured into each tube. Care was taken to ensure an even flow of gel solution into the tubes to prevent air entrapment. Each gel was carefully topped with approximately 100 µl distilled water and polymerized

under ultraviolet light at room temperature for 20 to 30 minutes.

The distilled water was then discarded and the top of the gel was rinsed three times with 5.0% sucrose. The tube was filled to the top with 5.0% sucrose and lowered into the cathode vessel of the gel electrophoresis cell (model 150, Bio-Rad Laboratories), containing 0.04 M NaOH, deaerated before use. The anode vessel was filled with 0.01 M H_3PO_4. Electrodes were connected to an LKB power supply set at: 30 watts and 500 volts, and run at 1 mA per gel for one-half hour.

After the prerun, the 5.0% sucrose was discarded from the top of the gel. Twenty-five µl 9BE (20.0 mg/ml) with 5.0 µl 80% glycerol and 5.0 µl ampholyte (3/10) was loaded onto the top of the gel; 5.0 µl ampholyte (3/10) was added to 100.0 µl 5.0% sucrose and carefully layered over the sample. Twenty-four µl BSA (2.2 mg/ml), 1.0 µl 5.0% bromophenol blue and 3.0 µl red blood cell lysate were used as standards and applied to the gels in a similar manner. The LKB power supply was set at: 30 watts, 1000 volts, and 1 mA per gel. The electrophoresis was run until focusing of the standards was obtained, 2 to 3 hours.

After the run, the gels were immediately released by squirting water down the sides of the tubes with a syringe. Gels were cut into 2 mm discs and each disc was cut in half. One half was used for pH determination, while the other was prepared for electrophoresis.

To determine pH the gel pieces were placed in test tubes containing 0.5 ml previously boiled distilled water. Ampholytes were eluted by shaking in a water bath at room temperature overnight.

The pH for each disc piece was measured.

The gel segments were placed in micro-tubes and homogenized in 100.0 µl 50.0 mM K_2HPO_4 buffer, pH 7.0 containing 0.1 mM 2-mercaptoethanol, 2.0 mM EDTA and 0.15 M KCl to prepare them for electrophoresis. Proteins were eluted by shaking in a water bath at room temperature overnight. The samples were then tested against TAS by fused rocket electrophoresis.

RESULTS

1. Production of Monovalent Antiserum

The non-specific nature of the rabbit anti-9 day chick embryo brain serum (AS) was demonstrated by immunodiffusion against extracts of both neural and non-neural tissue (Fig. 1A). Extensive precipitin reactions were noted with all tissues tested. Several precipitin lines were also demonstrated when AS was reacted with adult (ABE) and 9 day embryonic (9BE) brain extracts by immuno-electrophoresis (Fig. 1C).

To prepare a neural specific antiserum, AS was absorbed with adult liver and kidney extracts and adult serum until no cross-reactivity could be detected by immunodiffusion (not shown). This absorbed neural specific antiserum (AAS), cross-reacted by immunoelectrophoresis with ABE and 9BE demonstrated precipitin lines with both extracts (Fig. 1D). These antigens showed a slight anodal migration. At least one appeared to be common to both ABE and 9BE.

Adult brain extract was then added to AAS so the antiserum would react with only embryonic neural antigens. Immunodiffusion of this antiserum (TAS) with various adult extracts demonstrated an absence of cross-reactivity with both neural and non-neural tissue extracts, but a precipitin line was present against 9BE (Fig. 1B). Immunoelectrophoresis of ABE and 9BE developed with TAS demonstrated no reaction with ABE, but a single precipitin band formed against

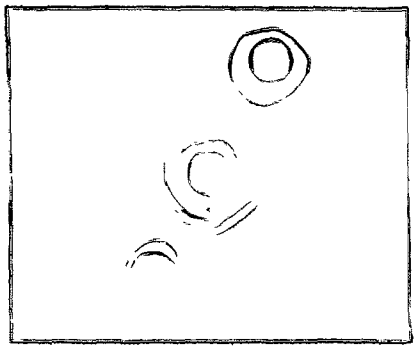
Figure 1A Photograph of double immunodiffusion plate containing unabsorbed anti-9 day chick embryo brain serum (AS) in the central well and the following tissue extracts in the peripheral wells: adult liver (AL), adult brain (AB), adult serum (ASe), adult kidney (AK) and 9 day embryonic brain (9B).
Note numerous precipitin lines common to both neural and non-neural tissue extracts.

Figure 1B Photograph of double immunodiffusion plate containing TAS in the central well and the following tissue extracts in the peripheral wells: adult liver (AL), adult brain (AB), adult serum (ASe), adult kidney (AK) and 9 day embryonic brain (9B).
Note cross-reaction with embryonic brain extract only and no precipitin lines with adult extracts of either neural or non-neural origin.

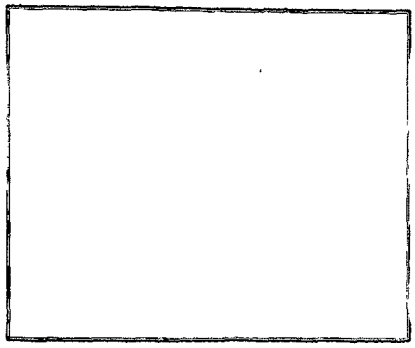
Figure 1C Photograph of immunoelectrophoresis plate with adult brain extract (A) in the upper well, 9 day embryonic brain extract (9) in the lower well and the central trough containing unabsorbed serum AS.
Note numerous precipitin arcs displaying anodal migration at pH 8.6.

Figure 1D Photograph of immunoelectrophoresis plate with adult brain extract (A) in the upper well, 9 day embryonic brain extract (9) in the lower well and AAS in the central trough, dried and stained.
Note precipitin bands are displayed with both 9 day and adult brain extract. One appears to be common to both extracts.

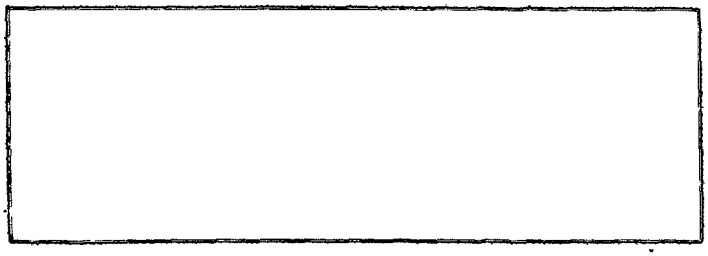
Figure 1E Photograph of immunoelectrophoresis plate with adult brain extract (A) in the upper well, 9 day embryonic brain extract (9) in the lower well and TAS in the central trough.
Note the single precipitin band against 9 day brain extract; no cross-reactivity is demonstrated with adult brain extract.



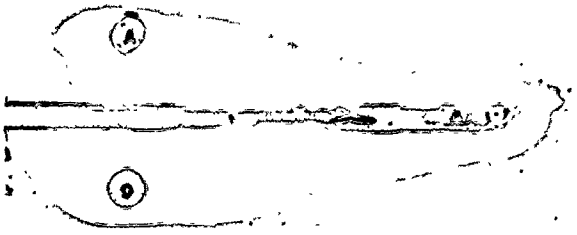
1A



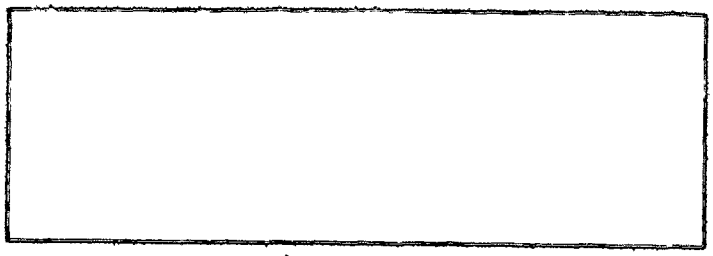
1B



1C



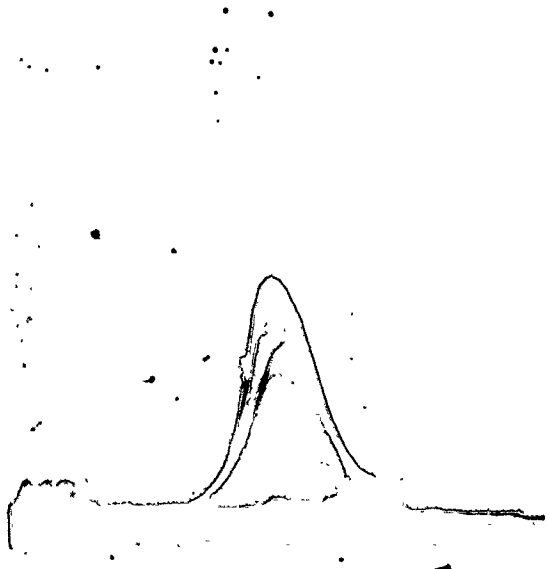
1D



1E

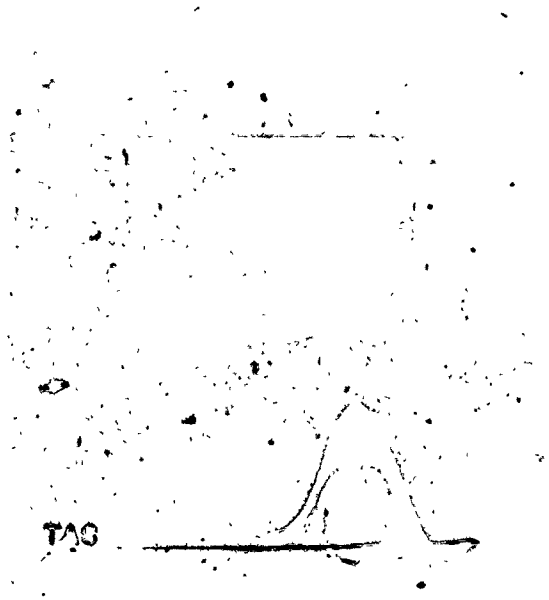
Figure 2A Photograph of crossed immunoelectrophoretic analysis of 9 day embryonic brain extract (9). The antibody-agarose gel contained AAS.
Note two precipitin peaks demonstrating slight anodal migration.

Figure 2B Photograph of crossed immunoelectrophoretic analysis of 9 day embryonic brain extract (9). The antibody-agarose gel contained TAS.
Note single precipitin peak.



(9

2A



(9

2B

9BE (Fig. 1E).

Since immunoelectrophoresis identifies only the minimum number of components, the more sensitive technique of crossed immunoelectrophoresis was employed to ensure the monospecificity of TAS. Crossed immunoelectrophoresis of 9BE in gels containing either AAS or TAS are shown in Figure 2. Two precipitin peaks of similar electrophoretic mobility were demonstrated using AAS (Fig. 2A), while only one of these peaks precipitated when TAS was utilized (Fig. 2B). This parallels the results obtained by immunoelectrophoresis (Fig. 1D and Fig. 1E). Thus while AAS contained antibodies directed against two neural components, one of which is an adult antigen, TAS was considered monospecific for a transient embryonic neural antigen (TEA).

TAS and AAS gamma globulins were isolated from contaminants in the antiserum by ammonium sulphate precipitation and affinity chromatography. These procedures did not affect the immuno-electrophoretic profile.

2. Ontogenic Appearance of TEA

a) Immunoelectrophoretic studies

The ontogeny of TEA was investigated immunoelectrophoretically using TAS. Whole chick embryo extracts from days 1 to 4, embryonic brain extracts from days 5 to 20 and adult brain extract were tested for the presence of TEA. The resulting pattern is shown in Figures 3 to 6. No precipitin reaction could be visualized with day 1 (Fig. 3A) and day 2 (Fig. 3B) extracts. A very faint

Figure 3A Photograph of immunoelectrophoretic plate with extract of whole embryos of 1 day incubation (1) in the upper well, and the trough containing TAS. Note absence of precipitin bands.

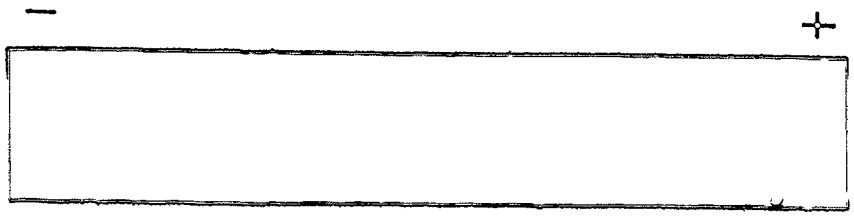
Figure 3B Photograph of immunoelectrophoretic plate with extract of whole embryos of 2 days incubation (2) in the upper well and the trough containing TAS. Note absence of precipitin bands.

Figure 3C Photograph of immunoelectrophoretic plate with extract of whole embryos of 3 days incubation (3) in the upper well, and the trough containing TAS. Note appearance of a faint precipitin arc indicated by the arrow.

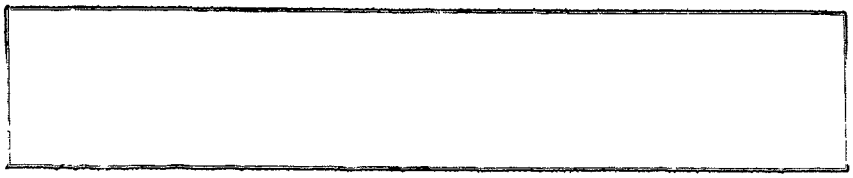
Figure 3D Photograph of immunoelectrophoretic plate with extract of whole embryos of 4 days incubation (4) in the upper well, and the trough containing TAS. Note appearance of a distinct precipitin band.

Figure 3E Photograph of immunoelectrophoretic plate of brain extract from embryos of 5 days incubation (5) in the upper well and the trough containing TAS. Note single precipitin band.

Figure 3F Photograph of immunoelectrophoretic plate of brain extract from embryos of 6 days incubation (6) in the upper well and the trough containing TAS. Note single precipitin band.

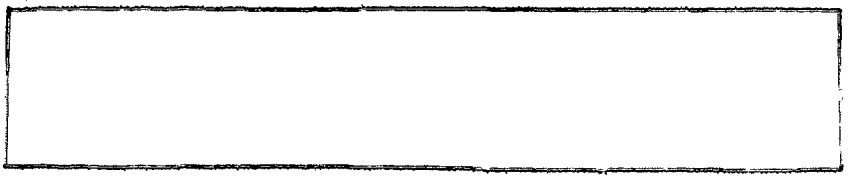


3A

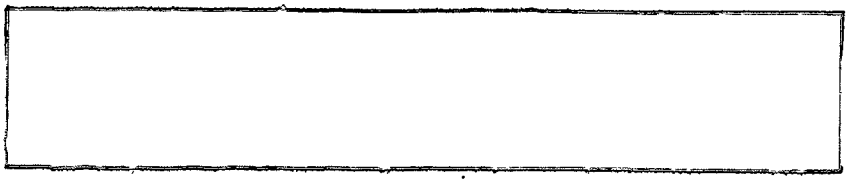


3B

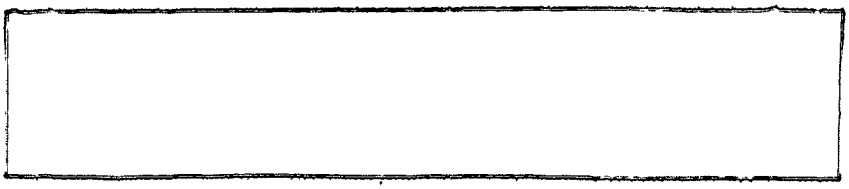
3C



3D



3E



3F

Figure 4A Photograph of immunoelectrophoretic plate of brain extract from embryos of 7 days incubation (7) in the upper well and the trough containing TAS. Note single precipitin band.

Figure 4B Photograph of immunoelectrophoretic plate of brain extract from embryos of 8 days incubation (8) in the upper well and the trough containing TAS. Note single precipitin band.

Figure 4C Photograph of immunoelectrophoretic plate of brain extract from embryos of 9 days incubation (9) in the upper well and the trough containing TAS. Note single precipitin band.

Figure 4D Photograph of immunoelectrophoretic plate of brain extract from embryos of 10 days incubation (10) in the upper well and the trough containing TAS. Note single precipitin band.

Figure 4E Photograph of immunoelectrophoretic plate of brain extract from embryos of 11 days incubation (11) in the upper well and the trough containing TAS. Note single precipitin band.

Figure 4F Photograph of immunoelectrophoretic plate of brain extract from embryos of 12 days incubation (12) in the upper well and the trough containing TAS. Note single precipitin band.

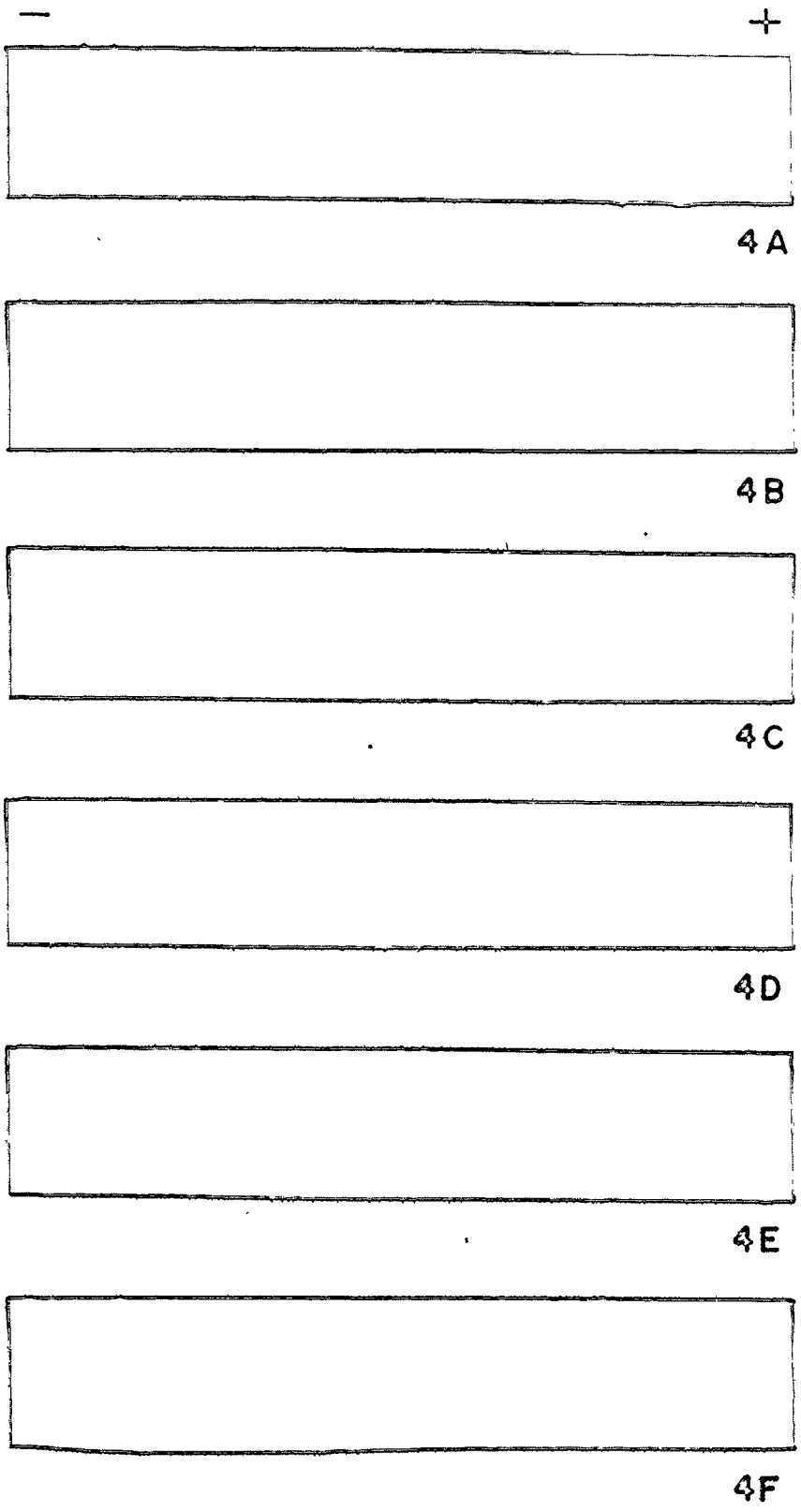

−
+
4A
4B
4C
4D
4E
4F

Figure 5A Photograph of immunoelectrophoretic plate of brain extract from embryos of 13 days incubation (13) in the upper well and the trough containing TAS. Note single precipitin band.

Figure 5B Photograph of immunoelectrophoretic plate of brain extract from embryos of 14 days incubation (14) in the upper well and the trough containing TAS. Note single precipitin band.

Figure 5C Photograph of immunoelectrophoretic plate of brain extract from embryos of 15 days incubation (15) in the upper well and the trough containing TAS. Note single precipitin band.

Figure 5D Photograph of immunoelectrophoretic plate of brain extract from embryos of 16 days incubation (16) in the upper well and the trough containing TAS. Note single precipitin band.

Figure 5E Photograph of immunoelectrophoretic plate of brain extract from embryos of 17 days incubation (17) in the upper well and the trough containing TAS. Note single precipitin band.

Figure 5F Photograph of immunoelectrophoretic plate of brain extract from embryos of 18 days incubation (18) in the upper well and the trough containing TAS. Note absence of precipitin band.

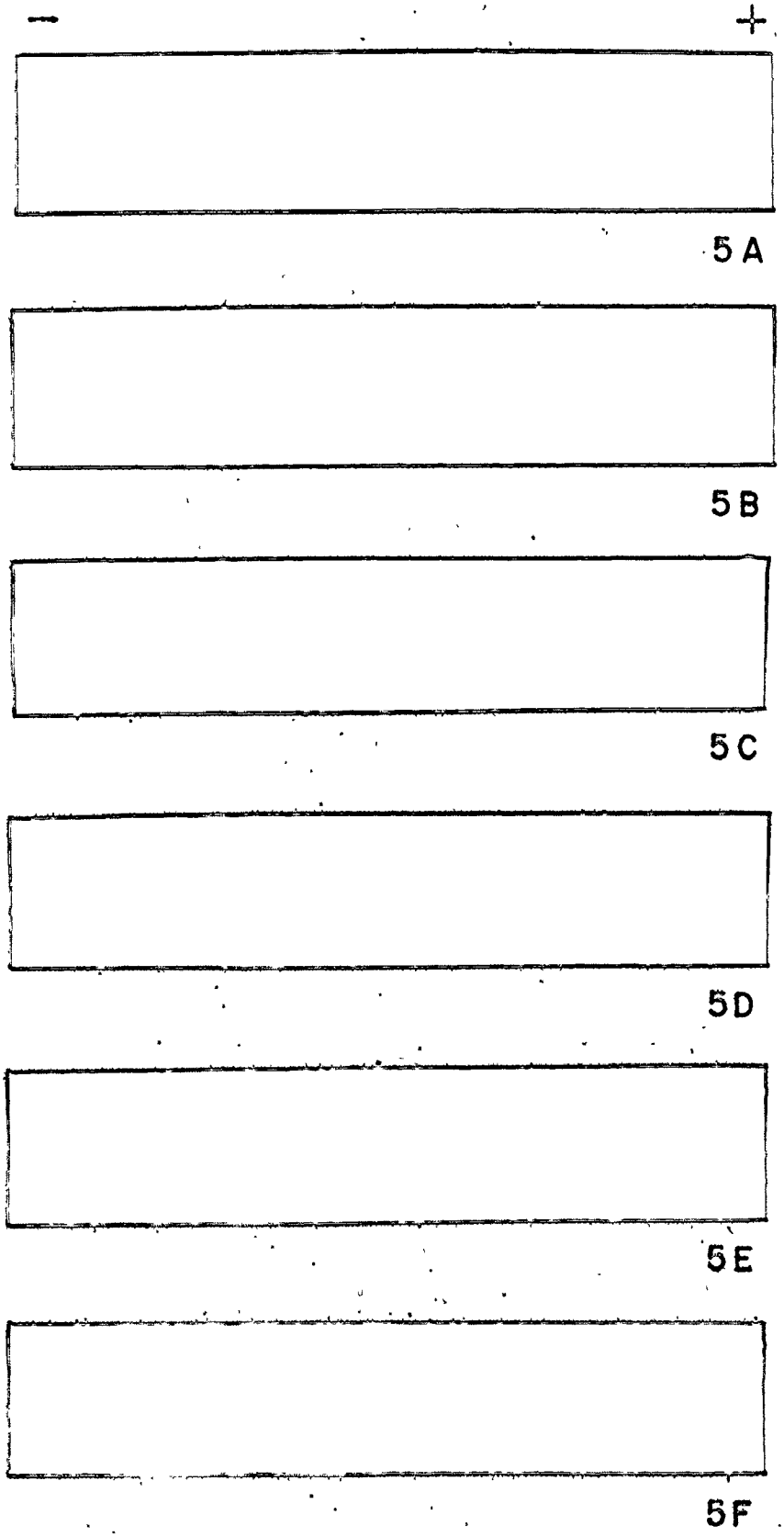
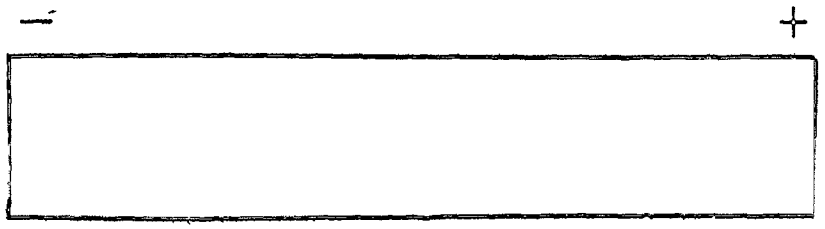

−
+
5A
5B
5C
5D
5E
5F

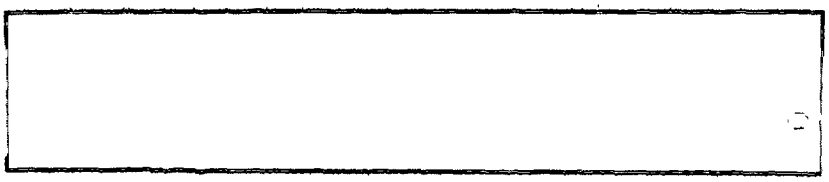
Figure 6A Photograph of immunoelectrophoretic plate of brain extract from embryos of 19 days incubation (19) in the upper well and the trough containing TAS.
Note absence of precipitin band.

Figure 6B Photograph of immunoelectrophoretic plate of brain extract from embryos of 20 days incubation (20) in the upper well and the trough containing TAS.
Note absence of precipitin band.

Figure 6C Photograph of immunoelectrophoretic plate of adult brain extract (A) in the upper well and the trough containing TAS.
Note absence of precipitin band.



6A



6B

6C

precipitin band could be detected with 3 day embryo extracts (Fig. 3C). From day 4 to day 17 (Fig. 3D to Fig. 5E) a precipitin line could be seen, corresponding to TEA. No precipitin bands could be detected in day 18 (Fig. 5F), day 19 (Fig. 6A), day 20 (Fig. 6B) or adult (Fig. 6C) brain extracts.

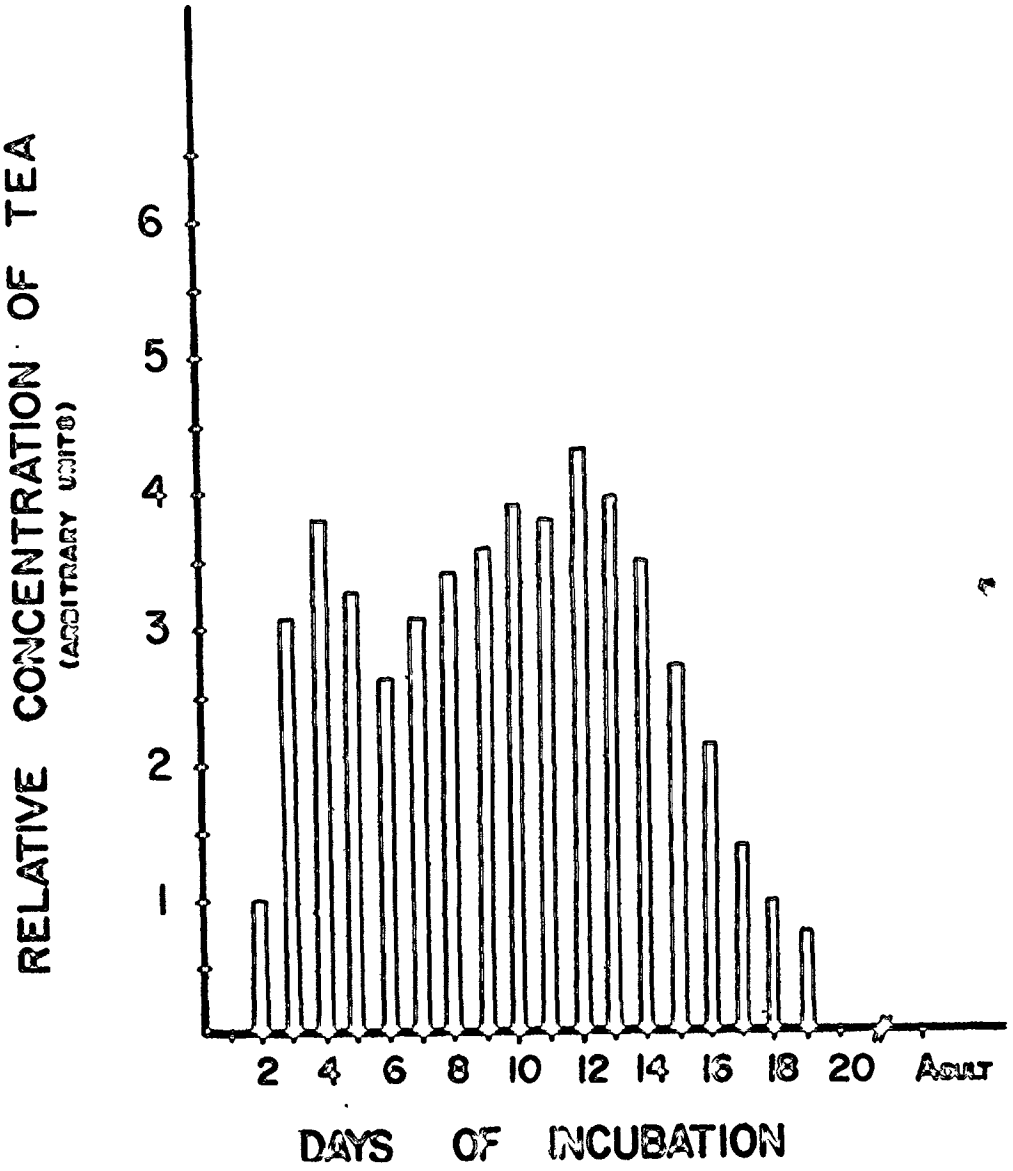
b) Rocket immunoelectrophoresis

Quantitation of TEA was carried out using rocket immunoelectrophoresis against TAS. All extracts contained equal total protein concentrations and since TAS is specific for TEA, the heights of the "rockets" demonstrate the relative concentration of TEA at each stage of development, and in the adult. The results are summarized in Fig. 7 as a normalized graph indicating relative amounts of TEA during incubation.

Contrary to results obtained by immunoelectrophoresis (Fig. 3B and Fig. 5F) TEA was shown by the more sensitive rocket immunoelectrophoresis to be present in very low concentrations in 2 day whole embryo and 18 day brain extracts and just detectable in 19 day embryonic brain extracts.

Quantitative comparisons demonstrated an initial peak of TEA at day 4 with decreasing concentrations at days 5 and 6; by day 7 a second increase began with a maximum concentration achieved at day 12. Subsequently TEA demonstrated a progressive decrease in concentration until it could no longer be detected by day 20.

Figure 7 Diagrammatic representation of rocket electrophoresis of whole embryo extracts from 1 day to 4 days incubation, brain extracts from 5 days to 20 days incubation and adult brain extract (A) into TAS containing gel.
Note the relative concentration of TEA within each of these extracts is depicted.


RELATIVE CONCENTRATION OF TEA
(ARBITRARY UNITS)
1
2
3
4
5
6
2 4 6 8 10 12 14 16 18 20 ADULT
DAYS OF INCUBATION

3. Enzyme Digestion of TEA

9 day embryonic brain extract was incubated with the enzymes pronase, trypsin and chymotrypsin and then reacted with TAS by immunodiffusion to determine the nature of the antigenic site (Fig. 8). Precipitin lines displaying a reaction of identity with the control 9BE were present in both the trypsin (Fig. 8B) and chymotrypsin (Fig. 8C) digested preparations even after 6 hours of incubation. However no precipitation was demonstrated with 9BE treated with pronase (Fig. 8B). Since the reactivity of TEA was only inhibited by pronase, the antigenic site although apparently proteinaceous in nature was not composed of trypsin or chymotrypsin-sensitive amino acids.

4. Molecular Weight of TEA

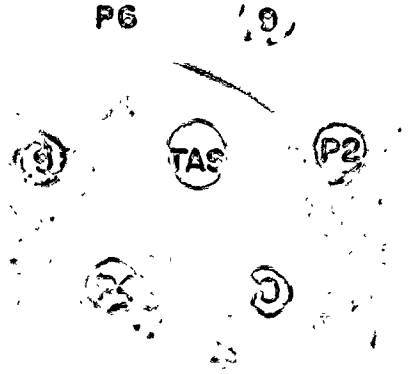
9 day embryonic brain extract components were separated on the basis of their molecular weight by molecular exclusion chromatography. Fractions eluted from the column were tested for TEA by fused rocket electrophoresis in a TAS-containing agarose gel. Figure 9A is a diagram of the elution profile of TEA determined by fused rocket electrophoresis.

The total volume of the column was calculated to be 442.0 ml; the void volume (Vo), the elution volume of blue dextran was estimated to be 176.0 ml. Since the major TEA peak was eluted from 226.0 ml to 240.0 ml the mean elution volume (233.0 ml) of TEA was used to calculate its corresponding Kav. After Kav determination and comparison to the standard curve (Fig. 9B), the molecular weight of TEA was estimated to be 73,000 daltons.

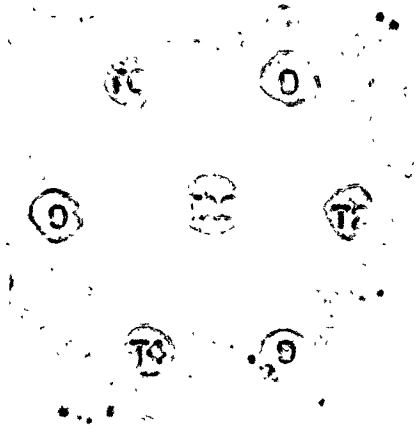
Figure 8A Photograph of immunodiffusion plate containing TAS in the central well and control 9 day embryonic brain extract (9), and 9BE hydrolysed with pronase for 2 (P2), 4 (P4) and 6 (P6) hours in peripheral wells.
Note absence of precipitin lines in digested extracts and the precipitin line demonstrated by control extracts.

Figure 8B Photograph of immunodiffusion plate containing TAS in the central well and control 9 day embryonic brain extract (9), and 9BE hydrolysed with trypsin for 2 (T2), 4 (T4) and 6 (T6) hours in peripheral wells.
Note reaction of identity of control extracts and trypsin digested extracts for all incubation times.

Figure 8C Photograph of immunodiffusion plate containing TAS in the central well and control 9 day embryonic brain extract (9), and 9BE hydrolysed with chymotrypsin for 2 (C2), 4 (C4) and 6 (C6) hours in peripheral wells.
Note reaction of identity of control extracts and chymotrypsin digested extracts for all incubation times.



8A

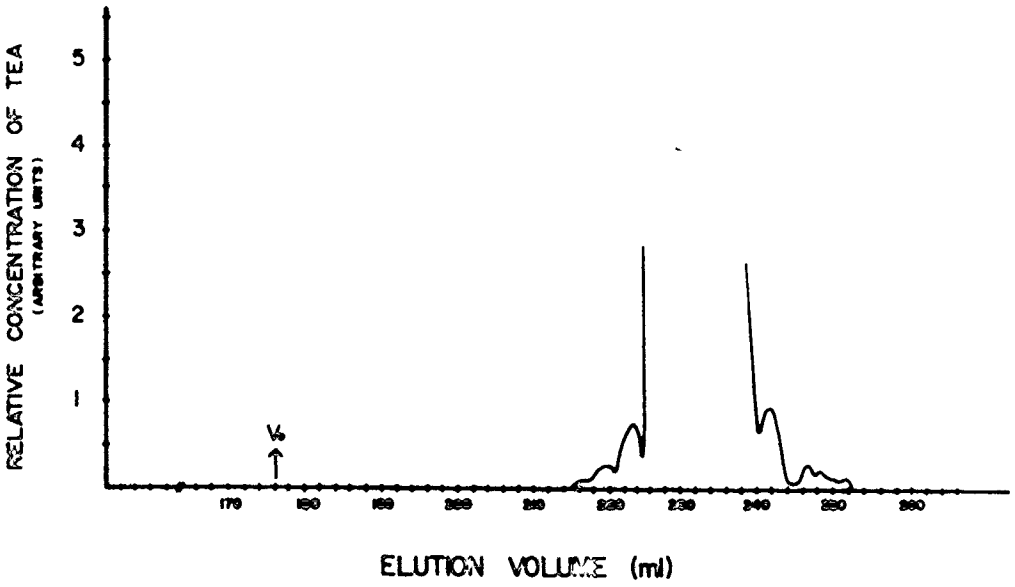


8B

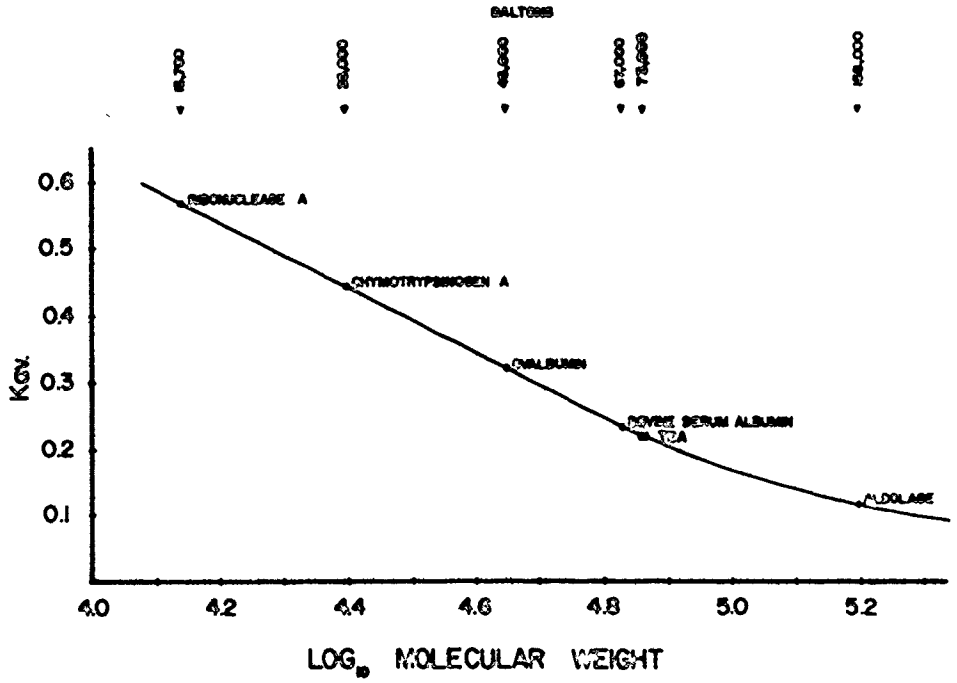
8C

Figure 9A Diagram of elution profile of TEA after separation of 9BE on Sephacryl S-200SF. Fractions containing TEA were identified by fused rocket electrophoresis in agarose containing TAS.

Figure 9B Estimation of molecular weight of TEA after gel filtration in Sephacryl S-200SF. Standard proteins used were ribonuclease A, chymotrypsinogen A, ovalbumin, bovine serum albumin and aldolase.


RELATIVE CONCENTRATION OF TEA
(ARBITRARY UNITS)
5
4
3
2
1
V_e
170
180
190
200
210
220
230
240
250
260
ELUTION VOLUME (ml)

9A


0.6
0.5
0.4
K_{GV}
0.3
0.2
0.1
4.0
4.2
4.4
4.6
4.8
5.0
5.2
LOG_{10} MOLECULAR WEIGHT

9B

5. Isoelectric Point

Polyacrylamide tube gel isoelectric focusing was used to separate the components of 9 day embryonic brain extract according to their isoelectric point (pI). The pH determination at 2.0 mm intervals demonstrated a linear pH gradient in the focused gels (Fig. 10A). Proteins eluted from the 2.0 mm fractions were applied to fused rocket electrophoresis in TAS-containing agarose in order to identify TEA (Fig. 10B). The pI of TEA was estimated to be at pH 4.8.

6. Embryonic Tissue Specificity

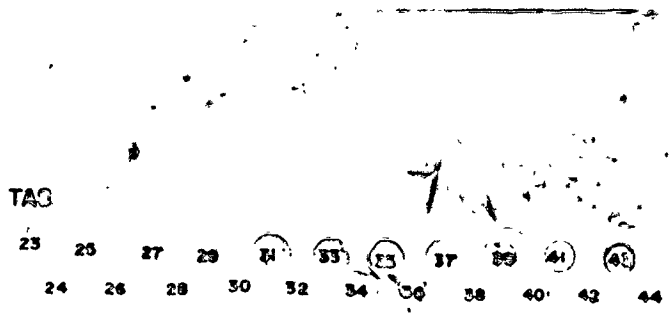
a) Immunodiffusion and Immunoelectrophoresis

During the antiserum preparation, anti-embryonic chick brain serum (AS) was absorbed with adult liver and kidney extracts and adult serum to remove non-neural antibodies from the serum. The resulting antiserum was considered to be neural specific. Upon further absorption with adult brain extract TAS was considered to be embryo as well as neural specific.

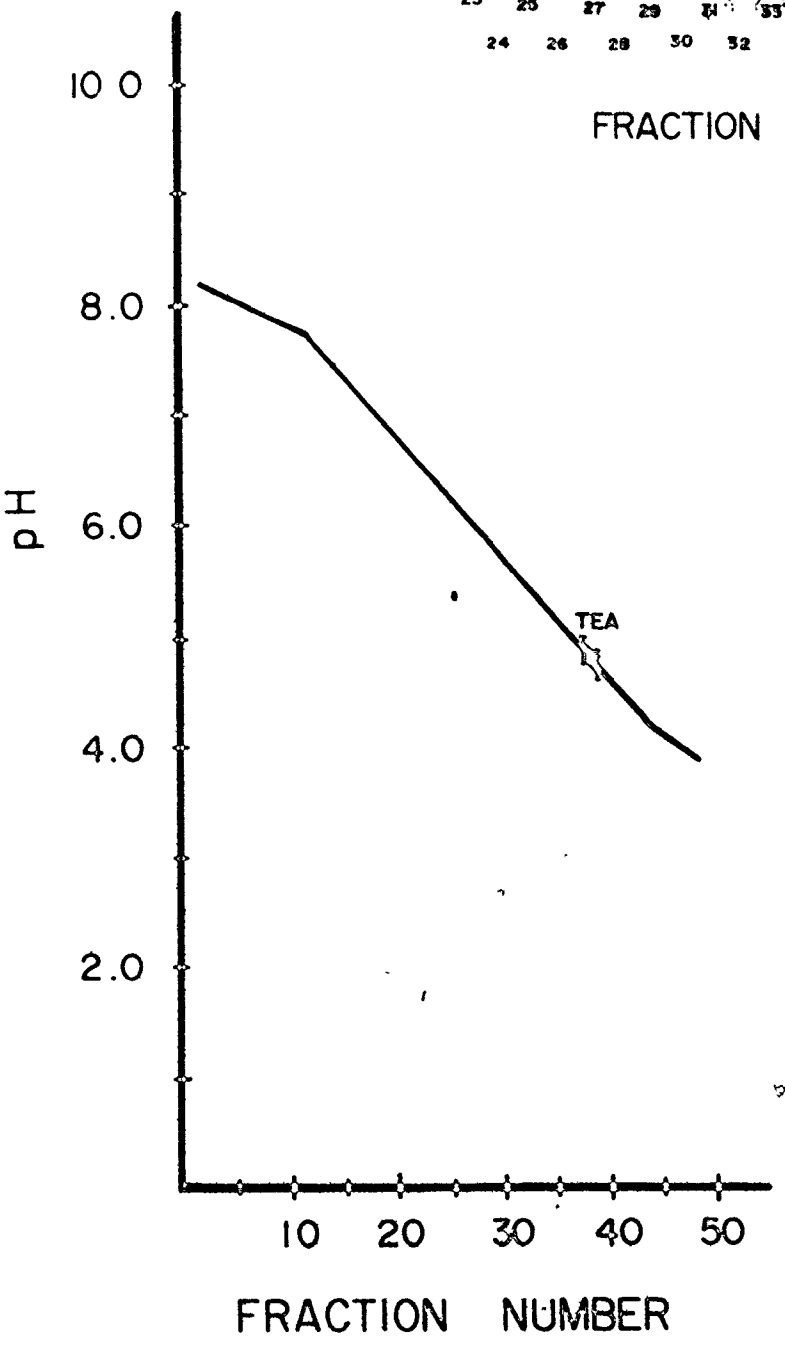
However when embryonic extracts of 9 day liver (Fig. 11C) and kidney (Fig. 11D) were tested by immunoelectrophoresis against TAS a precipitin band corresponding to TEA was demonstrated. In addition, when TAS was further absorbed with 9 day embryonic liver or kidney, no precipitation was demonstrable even after the antiserum was concentrated by positive pressure ultrafiltration. Thus TEA was shown to be common to embryonic brain, liver and kidney extracts.

Figure 10A Diagram of linear pH gradient achieved by polyacrylamide gel tube isoelectric focusing. The bar represents those fractions found to contain TEA by fused rocket electrophoresis.

Figure 10B Photograph of fused rocket electrophoresis plate. Proteins eluted from the focused gel fractions were electrophoresed into agarose containing TAS. Note TEA was demonstrated to be eluted from two consecutive 2.0 mm fractions.


TAG
23 25 27 29 31 33 35 37 39 41 43
24 26 28 30 32 34 36 38 40 42 44
FRACTION NUMBER

10A


10 0
8.0
6.0
4.0
2.0
pH
TEA
10 20 30 40 50
FRACTION NUMBER

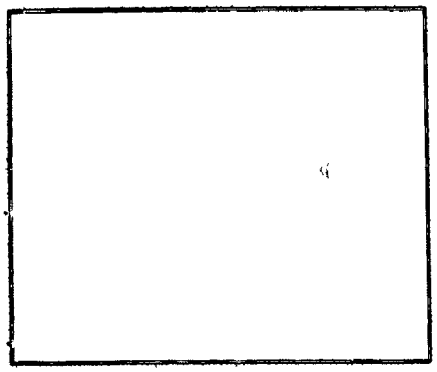
10B

Figure 11 A Photograph of double immunodiffusion plate containing TAS in the central well and the following 9 day embryonic extracts in the peripheral wells: brain (9B) serum (9Se) and red cell lysate (9RL).
Note precipitin line with 9 day serum and 9 day brain extract demonstrating a reaction of identity.
No precipitation occurred with 9 day red cell lysate.

Figure 11 B Photograph of dried and stained immunoelectrophoresis plate. 9 day embryonic serum (9Se) in the upper well and 9 day embryonic brain extract (9) in the lower well were reacted with TAS in the trough.
Note the single precipitin band with common electrophoretic mobility in each extract.

Figure 11 C Photograph of dried and stained immunoelectrophoresis plate. 9 day embryonic liver extract (9L) in the upper well and 9 day embryonic brain extract (9) in the lower well were reacted with TAS in the trough.
Note the single precipitin band with common electrophoretic mobility in each extract.

Figure 11 D Photograph of dried and stained immunoelectrophoresis plate. 9 day embryonic kidney (9K) in the upper well and 9 day embryonic brain extract (9) in the lower well were reacted with TAS in the trough.
Note the single precipitin band with common electrophoretic mobility in each extract.


II A

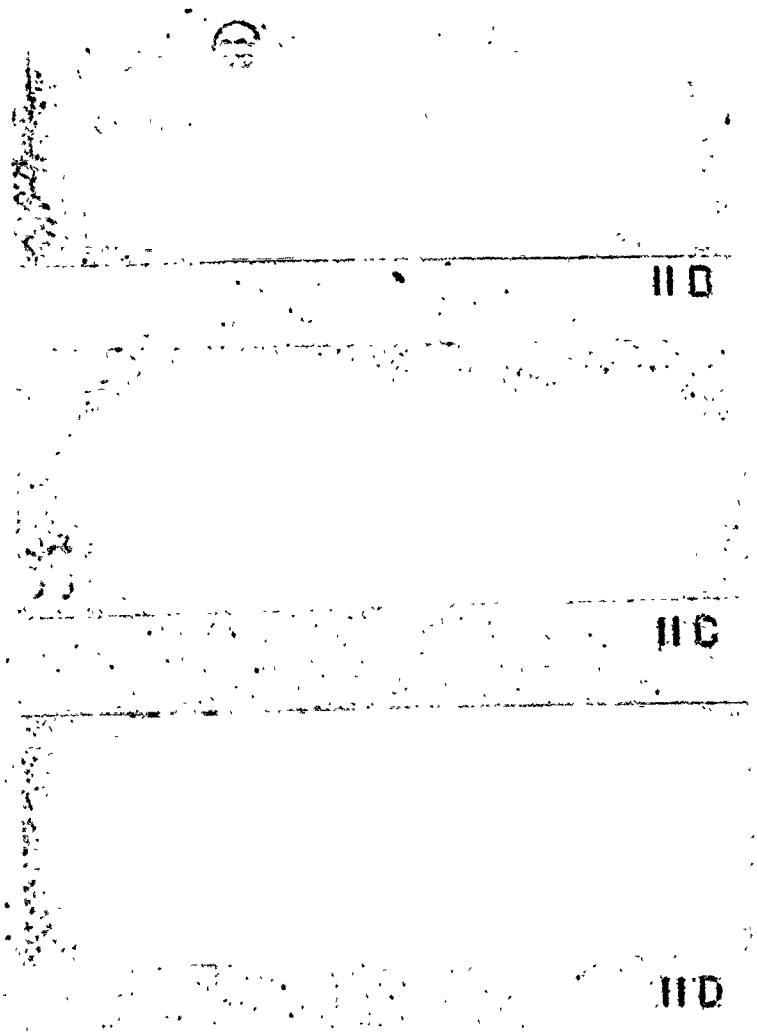
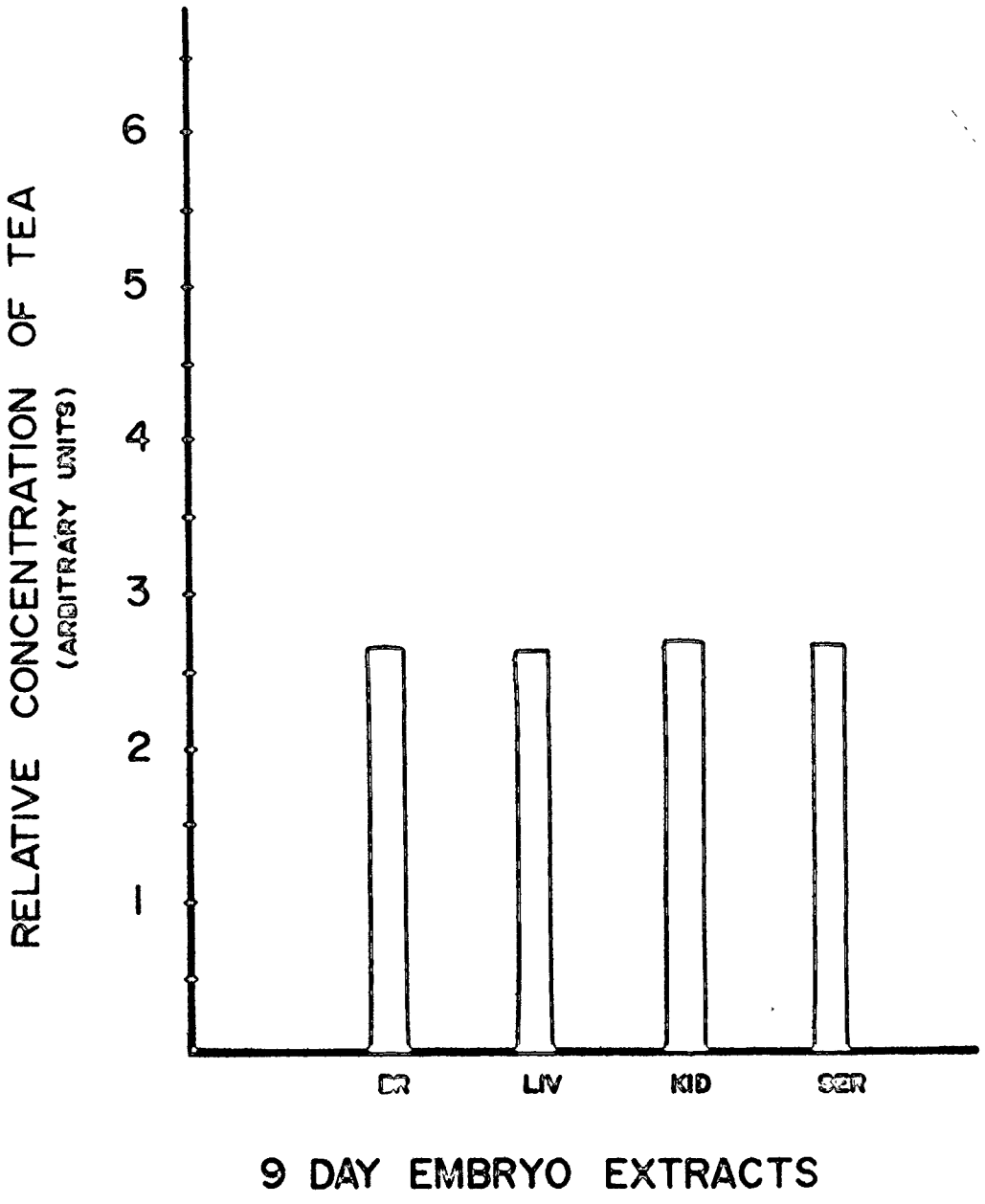

II D
II C
II D

Figure 12 Diagrammatic representation of rocket electrophoresis of 9 day embryonic extracts of brain (Br), liver (LIV), kidney (KID) and serum (SER) into TAS containing agarose gel. The relative concentration of TEA in each of these extracts is depicted.


RELATIVE CONCENTRATION OF TEA
(ARBITRARY UNITS)
6
5
4
3
2
1
CR
LIV
KID
SGR
9 DAY EMBRYO EXTRACTS

Since TEA was common to several embryonic tissue extracts two possibilities were considered: that TEA was a protein common to each of the embryonic tissues tested or that TEA was a circulating component of the blood. Since several blood components have been shown to demonstrate transience in several species including the chick, studies were undertaken to determine if TEA was one of these.

When whole blood collected from 9 day chick embryos was tested against TAS by immunodiffusion a precipitin line resulted (not shown). Nine day embryonic blood was further separated into serum and red cell components. The embryonic red cell lysate diluted to several concentrations and 9 day serum were tested by immunodiffusion against TAS (Fig. 11A). A precipitin line, showing a reaction of identity with 9BE formed with the serum but not with the red cell lysate. Immunoelectrophoresis of 9 day embryonic serum against TAS demonstrated a precipitin arc corresponding to TEA in 9 day embryonic brain extract (Fig. 11B).

b) Quantitation in embryonic tissue extracts

Rocket immunoelectrophoresis of 9 day embryonic extracts of brain, liver and kidney and 9 day embryonic serum diluted to an equal total protein concentration was run in agarose containing TAS (Fig. 12). The results demonstrated that TEA was present in all extracts tested, at similar relative concentrations.

DISCUSSION

Classical experiments have furnished descriptions of the gross morphological changes occurring during embryogenesis. In recent years, scientific advances have added to this information by providing the means for studying the developmental process at the molecular level. One tool, the highly specific antigen-antibody reaction has been used to study the synthesis and localization of antigenic substances during the course of development. The appearance of antigens which react with antiserum against specific tissue antigens is considered to reflect the synthesis of these substances in the embryo. The present study utilized this technique to determine the ontogenic pattern and to identify some characteristics of a transient embryonic antigen (TEA) in the chick embryo. Identification of a particular antigen in a complex immunoelectrophoretic profile can be misleading and isolation and purification techniques are often difficult. Consequently, TEA was identified by preparing antiserum (TAS) specific for that antigen.

Studies by McCallion and Trott (1964, 1965) described a neural specific embryonic antigen in chick embryo extracts. Results from the present study were unable to confirm this report. Although TEA, a transient antigen, was exhibited in embryonic chick brain by antiserum prepared against 9 day embryonic neural tissue it was not neural specific. Contrary to their reports, embryonic tissues, namely 9

day liver, kidney and serum also demonstrated TEA immunoelectrophoretically (Fig. 11). In addition, while TEA migrated anodally in an electric field, the neural antigen displayed no mobility and precipitated around the antigen well (McCallion and Trott, 1965).

Comparison of the precipitin bands against 9 day embryonic brain extract using unabsorbed serum (AS) in the present study with those obtained by McCallion and Trott (1965) demonstrated very different immunoelectrophoretic profiles, suggesting differences in the quality of the antiserum used. Large individual variations in the abilities of rabbits to produce antibodies to a given tissue extract as well as the lack of uniformity of the antiserum under slightly different courses of immunization are well known problems (Harboe and Inglid, 1973). Although both studies used 9 day embryonic chick brain extracts for immunization, great differences in antibody response were demonstrated. This stresses the fact that unless identical antiserum is used, comparisons among studies are difficult.

Identification of TEA in embryonic tissues other than brain lead to the suggestion that it may be a common embryonic tissue antigen. However, because the chick embryos were not perfused prior to tissue collection and TEA was detected in the blood, specifically in the serum, it was thought to be a serum component isolated from the embryonic tissues during collection procedures. This is probable, since serum proteins are widely disseminated throughout the vascularized tissues of the body. In addition to the transience exhibited

by TEA, its electrophoretic anodal migration similar to that of an alpha globulin at pH 8.6 suggested that this embryonic serum component was an alpha-fetoprotein.

Alpha-fetoprotein (AFP) has been extensively studied and characterized in the human and in many mammalian species. Several studies of AFP in the chick embryo have demonstrated that it is not confined to mammalian development. TEA was compared with known chick AFP characteristics to determine homology between these two transient antigens.

Two chick embryo alpha (α) - globulin serum proteins (α-3 and α-4 globulin) were identifed immunoelectrophoretically by Weller and Bowdon (1974a). The α-4 globulin was embryo-specific. However α-3 globulin demonstrated partial cross-reactivity with adult antiserum. Review of the absorption procedures used in their study revealed that a minimal volume of hen serum (0.2ml/1.0ml antiserum) was added, and absorption was considered complete when no precipitate was seen macroscopically. In the present investigation it was found that much larger volumes (up to 0.5ml/1.0ml antiserum) of adult tissue extracts and serum were needed for absorption before no cross-reactivity could be detected by the more definitive test of immunodiffusion. Although two α-3 populations may be present, the cross-reactivity of α-3 globulin with both chick embryo antiserum and adult antiserum may also be the result of incomplete absorption techniques.

Weller and Bowdon (1974a, 1974b) performed further studies to

determine the developmental pattern of chick serum components. These involved comparisons of either the intensity of the stained precipitin line obtained by immunoelectrophoresis (Weller and Bowdon, 1974a) or the width of the stained disc bands obtained by polyacrylamide disc gel electrophoresis (Weller and Bowdon, 1974b) and must therefore be considered qualitative techniques. With these methods serum components including α-4 globulin showed a progressive increase up to the fifteenth day of incubation followed by a drastic decrease until none could be detected by one day post hatching. Densitometric scanning patterns obtained from the disc gels confirmed the general developmental pattern of α-4 globulin but the peak was detected at 12 days rather than 15 days incubation (Weller and Bowdon, 1974b).

Only one quantitative ontogenic study of purified chick AFP has been reported (Slade and Milne, 1978). Electroimmunoassay (rocket electrophoresis) experiments from seven days of incubation to one week post hatching demonstrated a linear increase in AFP from a concentration of 1.65 mg/ml to a peak value of 2.5 mg/ml on day 13; thereafter it declined rapidly to barely detectable levels at 5 days post hatching. Quantitative analyses were not undertaken before seven days of incubation.

In the present study immunoelectrophoresis was initially used to establish the general ontogenic pattern of TEA (Fig. 3 to Fig. 6). Further studies utilized the quantitative technique of rocket immunoelectrophoresis (Fig. 7). With this sensitive method, protein levels

as low as 0.1 to 1.0 μg/ml can be detected (Weeke, 1973a). While immunoelectrophoresis demonstrated the initial appearance of TEA at three days and its disappearance at 18 days of incubation, rocket immunoelectrophoresis detected low levels at two days of incubation and later in 18 and 19 day embryonic extracts. Precipitation of TEA could not be visualized in one day or twenty day embryonic extracts nor in adult brain extracts by either method.

The quantitative ontogenic profile of TEA obtained by rocket electrophoresis is very similar to the studies of AFP development. An initial spurt of TEA production early in development was followed by a progressive increase in concentration until twelve days of incubation. Thereafter TEA levels decreased rapidly until it could no longer be detected in 20 day embryos. The initial peak which was also described in the qualitative studies by Weller and Bowdon (1974a) may be due to heterogeneous although antigenically indistinguishable forms of TEA. This is known to be the case in the mouse where five distinct forms of AFP have been demonstrated during fetal development (Higgins, 1979). The initial peak may however, be due to rapid proliferation of AFP synthesizing cells early in development, followed by the activation of a regulatory mechanism such as the onset of endodermal differentiation. From seven days of incubation on, the quantitative results described in Figure 7 closely parallel those obtained from purified chick AFP using the same technique (Slade and Milne, 1978).

Reports disagree on the timing of the disappearance of AFP from chick serum. Some studies have reported that this event occurs just prior to hatching (Weller and Bowden, 1974a) while others have indicated that low AFP levels remain up to one week post-hatching (Slade and Milne, 1979). In the present study TEA could not be demonstrated after 19 days of incubation. While these discrepencies may be due to differing sensitivities in the techniques used, in all studies in the chick these transient serum antigens could not be detected in the adult. The differing rates of AFP disappearance reported may also be due to the use of different strains of chicken in the various studies, similar to the differential expression of the transient red blood cell membrane antigen (CFA) among avian species reported by Dietart and Sanders (1978).

Similar ontogenic patterns have been established for the rate of TEA production in the present study with that of AFP. However, it must be kept in mind that the TEA levels reported were obtained from chick embryo extracts. No significant differences in TEA levels in tissue extracts or serum in 9 day embryos were found (Fig. 12). However, during later stages of development when TEA levels have diminished, the absolute concentration of TEA in the brain may not reflect that in the serum. Thus, although the circulating levels in the serum may be high enough, the concentration of TEA in brain extracts during later stages of incubation may not have been sufficient for detection by the techniques used.

Studies in mammalian systems have also reported variable rates of AFP disappearance from the circulation. This rate appears to be dependent on the degree of maturity of the new-born individual (Zizovsky et al, 1970). Sensitive radioimmunoassay techniques have also demonstrated low levels of circulating AFP in normal human adult serum (Ruoslahti and Seppala, 1972b). Regardless of the variability of these findings the level of AFP synthesis after birth is negligible compared to the maximum concentrations achieved in the embryo. While the AFP peak in development suggests its primary functional role is in the embryo, the detection at low levels in the human adult may be significant when considering the mechanism of regulation of AFP synthesis during both embryogenesis and oncogenesis.

Several other characteristics determined for TEA were also found to be comparable to alpha-fetoprotein. Chick AFP isolated from 4 to 8 day embryonic extracts demonstrated a molecular weight of 69,000 daltons by sodium dodecylsulphate (SDS) gel electrophoresis and 70,000 daltons by gel filtration (Lindgren et al, 1974). Similar AFP molecular weights have also been determined in the mammalian species studied (Ruoslahti and Pinko, 1975). Reports of human AFP molecular weight vary from 65,000 (Ruoslahti and Seppala, 1972a) to 76,000 (Masopust et al, 1970) daltons; it is generally considered to be 70,000 daltons (Ruoslahti and Pihko, 1975). The molecular weight of TEA was determined by gel filtration to be 73,000 daltons (Fig. 9), comparable to that of AFP in all species studies. The TEA profile determined by fused rocket immunoelectrophoresis demonstrated

that TEA was eluted over several fractions (Fig. 9A). It was found that the application of concentrated samples of 9 day embryonic brain extract (40.0 mg/ml total protein) were needed for TEA identification after fractionation. This may have caused column overloading resulting in the elution noted. On the other hand heterogeneous TEA populations may be represented. A purified sample of TEA was not prepared and since SDS was found to affect the binding site so that TEA could not be identified immunologically by the specific antiserum (TAS), verification of TEA molecular weight by SDS polyacrylamide gel electrophoresis could not be done.

The isoelectric point (pI) of purified AFP in the chick embryo has not yet been reported, but the pI of human AFP determined by isoelectric focusing on polyacrylamide gels was found to be at pH 5.08 (Masopust et al, 1970). The pI of TEA, using similar methods was at pH 4.8 (Fig. 10). When the samples were eluted from the focused gel and run against TAS by fused rocket electrophoresis, TEA was eluted from two 2.0 mm fractions. The pI of TEA was considered to be the average pH of these two fractions. However, the major part of the focused band, represented by the higher peak in the fused rocket profile (Fig. 10B), was eluted from the fraction of higher pH. Thus the pI of TEA may be slightly higher than calculated.

In all species studied including the human (Ruoslahti and Seppala, 1972a) and the chick (Weller and Bowden, 1974b; Ruoslahti and Pihko, 1975) alpha-fetoprotein has been identified as a

glycoprotein. Similarly studies of AFP amino acid sequences in several mammals, including man (Ruoslahti and Enguall, 1978) have demonstrated that although they are not identical molecules there is a certain degree of sequence homology among animals. These characteristics were not determined in the present study and further investigations are necessary before these AFP features can be compared with TEA.

In the present investigation however, the nature of the antigenic site of TEA was studied (Fig. 8). Pronase is a general proteolytic enzyme preparation while trypsin and chymotrypsin are more specific; trypsin hydrolyses the peptide linkages involving the carboxyl groups of arginine and lysine, while chymotrypsin digests peptide bonds containing carboxyl groups of aromatic amino acids. Since the reactivity of TEA was only inhibited by pronase, the antigenic site, although proteinaceous in character was not sensitive to trypsin or chymotrypsin hydrolysis. Further investigations of the possible carbohydrate or lipid contributions are needed before the chemical nature of this site is fully understood. Comparable experiments have not yet been reported for chick or mammalian AFP, thus, comparison of the antigenic sites of these transient antigens is not feasible at this time.

A comparison of the known features of TEA and AFP demonstrated that these two transient embryonic antigens have common ontogenic and chemical characteristics. Radioisotope incorporation studies have

shown that embryonic brain does not secrete serum proteins (Weller, 1976). However, immunofluorescence experiments (Slade and Milne, 1977) of AFP in the brain ventricles and neural canal in five day chick embryos demonstrated intense fluorescence, indicating that the fluid within these cavities was rich with AFP. This provides further evidence for the identity of TEA with chick AFP.

The ultimate test to determine if TEA was in fact a chick alpha-fetoprotein would be to cross-react either TEA with anti-AFP serum or AFP with antiserum specific for TEA. However, since mammalian and chick AFP systems do not cross-react (Lindgren et al, 1974) and purified chick AFP was not available, this could not be done.

In mammals, the yolk sac and subsequently the liver are the principle sites of AFP synthesis (Gitlin et al, 1972). In the chick, initial studies concluded that only the yolk sac was actively involved in AFP synthesis (Gitlin and Kitzes, 1967). However, recent experiments using very sensitive immunoautoradiography techniques have demonstrated that while the yolk sac is the primary site of AFP synthesis, small amounts are also produced by the liver throughout development (Slade and Milne, 1977). In the present study no significant difference in TEA level was found in the liver compared to other 9 day embryo tissue extracts (Fig. 12). If TEA is also synthesized by the embryonic chick liver the level is too low at 9 days incubation to be detected by the techniques used.

The presence of AFP as a major component of embryonic serum suggests a role of primary significance in development. In addition, rather than being confined to a short temporal existence, AFP is found throughout most of the gestational period of all the animal models studied. Even in animals where the yolk sac becomes atretic early in gestation levels are still maintained by AFP synthesis in the fetal liver.

Although the function of AFP is as yet unknown a number of its inherent properties have resulted in several theories. The finding that AFP was capable of binding to estrogen in the rat (Uriel et al, 1972) led to the speculation that AFP was involved in the hormonal control of development. More specifically, it was suggested that AFP might keep estrogens from affecting estrogen sensitive targets by reducing unbound circulating hormone plasma concentrations. However, since this property is not universal, neither chick (Slade and Milne, 1977) nor human (Ruoslahti, 1979) AFP binds estrogen, it is unlikely to be of functional significance. Other experiments have demonstrated that mammalian AFP displays immunosuppressive properties in vitro (Murgita and Tomasi, 1975). These observations prompted the suggestion that AFP might be important in preventing the fetus from immunological attack. An immunosuppressive role in the feto-maternal relationship whereby the curtailment of AFP synthesis at birth would result in immunological rejection of the placenta, initiating labor, has also been considered (Gitlin & Gitlin, 1975). Although this may

be the case in mammalian species, a different AFP function must be considered in non-placental animals.

Alternatively, since alpha-fetoprotein and albumin have been found to share similar structural properties such as molecular weight (Peters, 1975) and amino acid sequencing (Ruoslahti and Terry, 1976) it has been suggested that AFP may be a fetal counterpart of albumin (Abelev, 1971). The major physiological roles of albumin are: maintenance of the osmotic pressure of the blood, transport of fatty acids, and sequestering and transport of bilirubin (Peters, 1975). Similar binding properties have recently been demonstrated for AFP (Ruoslahti, 1979) suggesting that these two serum proteins may have similar functions as general carriers in the plasma and perhaps as osmotic pressure regulators. Further investigations are necessary since AFP probably has specialized characteristics offering an advantage over albumin in the embryo.

The complex differentiation process involves the sequentially programmed activation and repression of many gene sequences. Although activation may be a result of temporally controlled cellular interactions several processes may be involved in the suppression of gene sequences coded for transient antigens. In the chick the ontogenic pattern of AFP appears to be related to the contribution of the yolk sac during development. The proliferation of yolk sac AFP-producing cells early in development may result in the initial AFP surge while the subsequent decrease in circulating levels may simply reflect the progressive atrophy of the yolk sac in later stages of development.

However, in those animals in which the liver is also a major contributor to AFP synthesis other mechanisms must be considered. Immunofluorescence studies in the mouse (Abelev, 1979) showed that in the liver of fetuses where the trabecular structure had not yet been established, all hepatocytes were equally active in AFP synthesis. However, following the establishment of the definitive cords in the post natal liver, a gradient of AFP intensity was formed, with brightly fluorescing cells located around the central veins. With time, the cell number and fluorescence diminished. This prompted the hypothesis that establishment of the definitive hepatic cords leads to the repression of AFP synthesis.

Results obtained from immunofluorescence studies of regenerating mouse liver following CCl_4 poisoning also support this concept (Abelev, 1979; Engelhardt et al, 1979). Within 24 hours after treatment AFP began to be synthesized. However the AFP positive cells demonstrated a highly specialized localization in a narrow zone immediately bordering the necrotic or damaged area. This suggests that impairment of intercellular contacts in the definitive liver cord releases the suppression of AFP synthesis established during hepatocyte development.

The detection of low levels of AFP in the serum of normal human adults and its increased synthesis in liver damage and carcinoma have demonstrated that inactivation, by whatever mechanism, is neither total nor irreversible. Immunofluorescence studies by Engelhart et al, (1979) have demonstrated AFP localization in morphologically differ-

entiated hepatocytes following CCl_4 poisoning. This suggests that these cells have at least genetically "dedifferentiated" by reactivating AFP genes. However, another possibility must be considered. Genes coded for AFP may be inactivated in differentiated cells but the presence in adult liver of small numbers of undifferentiated cells capable of AFP synthesis may result in the low circulating levels detected. In addition, in cases of hepatic damage or carcinoma the rapid proliferation of these undifferentiated cells rather than the reactivation of specific AFP gene sequences in differentiated hepatic cells may cause the increased circulating AFP levels. It must be kept in mind that the natural distribution of secreted antigens does not necessarily coincide with sites of synthesis. Thus, further studies including immunomorphological electron microscopy are necessary to elucidate the relationship of AFP synthesis with hepatocyte surface structure and degree of differentiation.

In summary a transient antigen in the chick embryo has been identified and characterized. Although antigen extracts and antibody production were derived from embryonic neural tissue TEA was not a neural specific antigen. Its electrophoretic mobility, ontogenic appearance, molecular weight and isoelectric point suggest that TEA is a chick alpha fetoprotein. The presence of a chick fetal serum protein homologous to mammalian AFP suggests that the chick embryo with its inherent advantages is a valuable system for further investigations. Because many characteristics of AFP have been established

it is a useful model for studying the role of transient embryonic antigens in development and differentiation.

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