CHANGES IN THE ANTIGENIC PATTERN AND THE IDENTIFICATION OF BRAIN SPECIFIC ANTIGENS IN ZEBRAFISH DEVELOPMENT

by

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DEDICATION

To my wife, my parents and my aunt

and uncle, Mr. and Mrs. Sukhan.

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I wish to express sincere thanks to my supervisor, Dr. H.W. Laale, for having suggested this type of experimentation, and for his numerous constructive criticisms during all phases of this project. Thanks also to Dr. H.E. Welch, head of the Department of Zoology, for having made the facilities of the Department available to me.

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ABSTRACT

Developmental stages from eight cells to 28 days post hatching and nine adult organs and tissues of the zebrafish were analysed by immunoelectrophoresis with unabsorbed and absorbed rabbit antiserum to adult zebrafish brain.

From the earliest embryonic stage to the oldest post hatching stage tested, a total of thirty different antigens was detected. Six antigens were found to be consistently present in all stages. Of four other antigens present in the earliest stage analysed, one showed an irregular appearance at various stages and the other three exhibited alterations in electrophoretic mobility at different stages. Fourteen antigens appeared during development at various stages, two of these were transitory.

Three brain specific antigens were identified with absorbed sera. One of these was found in all developmental stages; a second, which contains a lipid moiety appeared shortly after hatching and the third was detected only in the adult brain.

INTRODUCTION

Ontogenetic development is characterised by a progressive specialization in macromolecular synthesis. In the adult most cells are fully differentiated. The differentiated state consists of a characteristic set of proteins which are determined by differential gene activity (Grobstein, 1966). One of the central problems of current embryological research is the precise mechanism of cellular differentiation. An investigation of the time of appearance and disappearance of organ and tissue specific products should help in the elucidation of the synthetic phenomena leading to the differentiated state.

Immunology provides a unique tool for the elucidation of antigenic patterns in ontogeny and phylogeny because macromolecules have an almost universal ability to induce the formation of specific antibodies.

Cooper (1946) and Tyler (1955) have reviewed much of the earlier work in immunoembryology. Since the mid-forties the literature in this field has grown steadily.

Clayton (1953) and Spar (1953) have shown that succeeding stages of developing amphibian embryos acquire an increasing antigenic complexity. This antigenic complexity according to Burke et al (1944), in the chick, becomes evident by the appearance of organ antigens subsequent to histological differentiation. Schectman (1948) and Ebert (1950) have since demonstrated that organ specific antigens in the chick may appear prior to histological differentiation.

Within the last twenty years emphasis has been placed on the elucidation of organ specific antigens in ontogeny.

The lens, because of the relative simplicity and stability of its antigenic structure, has been the subject of numerous studies. Ten Cate and Van Doorenmaalen (1950) reported the presence of adult lens antigens in the lens vesicles of 60 hour chick embryos. These results were confirmed by Langman (1959a). Langman (1959b) subsequently demonstrated a progressive increase in the number of lens antigens with embryonic development in the chick. Maisel and Langman (1961) demonstrated the temporal development of lens antigens by using biochemical fractionation techniques in conjunction with immunological methods. These authors also investigated the distribution of lens antigens in other vertebrate classes. Barabanov (1966a, 1966b, 1967) who experimented with mouse embryos, arrived at essentially the same conclusions as Langman (1959a, 1959b) and Maisel and Langman (1961). In Rana temporaria the organ specific lens antigens has been shown to appear with the formation of the lens placode (Kirzon, Averkina and Vyazov, 1969).

The antigenic structure of the embryonic chick brain has been the subject of a series of investigations. Schalekamp (1960) found that three brain specific antigens appeared in the ontogeny of the chick brain by using antisera to adult chick brain in immunoelectrophoresis. McCallion and Langman (1964), who tested embryonic saline

soluble brain extracts against antiserum to adult chick brain in immunodiffusion tests, found two series of antigens. One series, nonspecific for nervous tissue, appeared early in ontogeny; the other series, specific for nervous tissue, appeared later in development. Evidence from immunodiffusion tests for the appearance of neural specific transient antigens in ontogeny has been provided by McCallion and Trott (1964) and confirmed by immunoelectrophoresis (McCallion and Trott, 1965). A recent study on the developing brain of the hamster (La Velle and Van Alten, 1969) strongly suggests that the disappearance of some brain antigens may be correlated with myelination. Using antiserum to adult rat brain in immunoelectrophoresis, Sviridov and Polyakova (1969) identified five brain specific antigens in the postnatal ontogeny of the rat.

Ben-Or and Bell (1965) with the aid of antisera to seven day epidermis and thirteen day feather extract of the chick embryo found that tissue specific and stage specific antigens arose during the course of skin differentiation.

Changes in the antigenic constitution of the mammary glands of the mouse have been shown by immunodiffusion to occur during postnatal development (Shchekolodkin, 1967). Antisera were produced to mammary gland antigens of 1, 30, and 90 day-old neonatals and it was found that mammary gland tissues for 1 and 7 day-old mice contained antigens which were not observed in the mammary glands of older mice.

Specific microsomal antigens, identified in the adult chick kidney by immunodiffusion were found to appear before birth (Okada and Sato, 1963). Lahti and Saxen (1966) investigated the appearance and localization of a kidney specific antigen in the embryonic development of the mouse by means of immunodiffusion, immunoelectrophoresis and immunofluorescence. That tissue specificity is acquired during organogenesis in the human kidney has been illustrated by immunodiffusion and immunofluorescence with antisera produced against human foetal and postnatal kidneys (Linder, 1969).

By testing saline extracts of spleen from neonatal mice of different age in immunodiffusion experiments with unabsorbed and absorbed antisera to spleens from 1, 7, 30 and 90 day-old mice, Maiskii and Shchekolodkin (1967) found that the antigenic structure of the spleen changes in postnatal development.

Adult chick liver antigens were found, by immunoelectrophoresis, to appear progressively in embryonic and newly hatched chick liver (Croisille, 1960). The results were confirmed by an immunological study of the antigenic components of cell fractions during the ontogeny of the chick liver (Mutolo et al, 1965). A study of changes in the parenchymal antigens during the early postnatal growth period of the rat liver shows that seven of the ten antigens found in the adult parenchymal microsomes appeared after birth (Raftell and Perlmann, 1968).

Chick serum albumin, which is present in yolk, has been identified in the serum of the four day chick embryo and also in the microsomal and lysosomal fractions of the eight day chick embryo liver by immunodiffusion and immunoelectrophoresis (Zaccheo and Grossi, 1967). Immunoelectrophoretic analysis of the serum of fetal, neonatal and adult rats show that most serum proteins appear during embryonic development while the remainder are detected shortly after birth (Afanas'eva, 1966). Three embryonic specific globulins have been identified in human fetal serum by immunochemical and electrophoretic methods (Tatarinov and Afanas'eva, 1967; Tatarinov et al, 1967). Tatarinov and Afanas'eva (1965) found cross-reacting antigenic determinants on the embryonic specific alpha globulins of man and certain mammals (dog, cow, sheep and pig). Monjour and Mariage (1969) also found three embryo specific serum proteins in the fetal rat.

Thus far few immunoembryological studies have been reported on fishes. A comparative study of the antigenic structure of oocytes and developing embryos of the Black Sea garfish and the sevruga fish has been made (Apekin, 1964; 1965). Since, phylogenetically, fishes are the oldest vertebrates, useful correlations may be made by comparing the structural and functional molecules of fishes with those of other classes. Kaplan (1965) considered the functional relationships between lactate dehydrogenase isoenzymes from the various classes

of vertebrates and the possible evolutionary significance of these relationships.

The primary object of this study was to trace the ontogenetic development of the antigenic pattern of the brain of a teleost. This study may be considered to be part of a project the aim of which is to describe the ontogenetic and phylogenetic development of living organisms in molecular terms.

The zebrafish, Brachydanio rerio (Hamilton-Buchanan), was chosen as the material for study. This species can provide eggs throughout the year (Hisoaka and Battle, 1958). The eggs are non-adhesive and have a short incubation period, 96 hours at 26°C.

MATERIALS AND METHODS

I. Animal Stock

Maintenance of fish

Zebrafish stock were obtained through the Hudson Bay Company. They were maintained within the temperature range of 25-27°C in dechlorinated hatchery water. A photoperiod of 13 hours of light followed by 11 hours of darkness was used. A variety of preserved and live fish foods were used to secure optimal health conditions.

Dissection of organs and tissues

Prior to dissection adult zebrafish were placed in a waterbath at 4°C for 20-30 minutes. Organs and tissues were dissected with the aid of an International dissecting microscope; brain, liver, ovary, muscle, skin, spleen, testis and blood samples were removed and maintained at 0-4°C. All organs and tissues except blood were freed of all visible adhering tissue and washed in physiological saline at 0-4°C before storage at -20°C.

Preparation of adult zebrafish brain homogenate for inoculation of rabbits

Dissected brain tissue was freeze-dried with an automatically refrigerated Vir Tis freeze mobile. The freeze-dried brain tissue was weighed and suspended in borate buffer pH 7.8 (see Appendix I) to yield a final concentration of 40 mg per ml of mixture. The mixture was homogenized at 0-4°C, for six thirty second intervals with thirty

second rest intervals between, to avoid heating, with a Tri-R Stir R model K 43 homogenizer. The brain homogenate was preserved in 0.01% merthiolate and stored at -20°C in aliquots of 0.5 ml each.

Preparation of embryo extracts

Zebrafish eggs were obtained from laboratory stock and incubated at 26°C. The stages required were separated according to Hisoaka and Battle (1958). Eggs at specific stages were homogenized at 0-4°C for five minutes at a slow rate to reduce frothing. The homogenates were centrifuged at 3000 x g for 30 minutes and the supernatant stored in 50 microlitre quantities at -20°C.

Production of rabbit antisera to adult zebrafish brain

Four male New Zealand albino rabbits, obtained from the Canadian Breeding Laboratories, Quebec, were bled from the marginal ear vein to obtain preimmune sera. One week later each rabbit was injected with 20 mg of freeze-dried brain tissue in complete Freund's adjuvant (Difco Laboratories). The material for inoculation was prepared by thoroughly mixing 0.5 ml of brain homogenate containing 20 mg of freeze-dried brain tissue with 0.5 ml of complete Freund's adjuvant. 0.25 ml of the resulting mixture was injected subcutaneously into each of four widely separated sites along the back of the rabbit.

Four weeks subsequently the rabbits were given a second inoculation. A small quantity of blood was removed from each rabbit, and sera were tested in immunodiffusion tests for a suitable titer. When a suitable titer was obtained, 20-40 ml of blood were removed from the

marginal ear vein. The serum was separated from the blood and stored according to standard immunological procedure (Campbell et al, 1964; Kabat, 1961). Rabbits which did not produce a suitable titer were reinoculated four weeks after the second injection and the procedure repeated.

Antiserum to adult Rana pipiens brain was produced in the same manner as that described for antiserum to zebrafish brain.

Absorption of sera

To determine the minimum amount of freeze-dried mid-gastrula supernatant which must be used to absorb all cross-reacting antibodies from zebrafish brain antiserum, varying quantities of freeze-dried mid-gastrula supernatant were used to absorb separate aliquots of serum. The concentrations used were 10, 20, 50, 100, 150, and 200 mg per ml of antiserum. After addition of the freeze-dried supernatant to the antiserum the mixture was incubated for two hours at 37°C, kept at 4°C for two days, then centrifuged at 40,000 x g for 30 minutes. Supernatants were removed and used in immunoelectrophoretic tests on fresh mid-gastrula supernatants. The control consisted of the analysis of fresh mid-gastrula supernatant with unabsorbed brain antiserum. Slides were prepared for observation as described below (See page 1). The minimum amount of freeze-dried mid-gastrula supernatant required to fully absorb one ml of antiserum to zebrafish brain was 20 mg.

The quantities of material which were found to fully absorb zebrafish brain antiserum with freeze-dried 49 hour embryo, adult ovary and liver supernatants were 44 mg/ml, 20 mg/ml and 70 mg/ml respectively.

II. Experimental Techniques

A. Immunoelectrophoresis

An adaptation of Grabar's and Williams' immunoelectrophoretic analysis to microscope slides (Scheidegger, 1955) was employed.

The Plexiglass electrophoresis chamber was designed by me and made by the Science Technical Workshop of the University of Manitoba (Fig. 1). Each buffer compartment has a capacity of 1.5 liters and is divided into four sections by three baffles to reduce changes in the ionic constitution of the buffer in the vicinity of the gels. A close fitting cover was used to reduce evaporation. The distance between the buffer compartments is 11.4 cm and eight microscope slides can be accommodated.

Gel preparation and electrophoresis

Since preliminary tests showed that dust interfered with the clarity of precipitin bands all possible precautions were taken to reduce the incorporation of dust on microscope slides.

Microscope slides were thoroughly washed with detergent, rinsed in distilled water and dried with a circulating stream of air at 70°C. Dust free slides which were dipped in hot 0.2% agar and dried, were placed on a level surface and 1.7 ml of a hot 1% agarose solution in barbital acetate buffer (see Appendix II) of pH 8.6 and ionicity 0.05 were poured onto it. The solution distributed itself over the slide and was retained by surface tension at the edges. After the

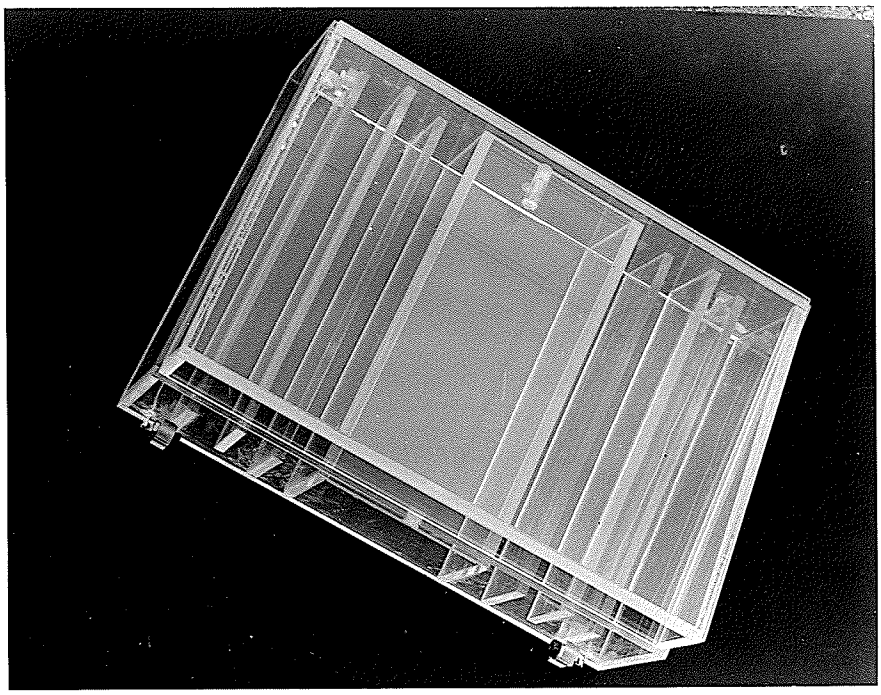


Figure 1. The Plexiglass electrophoresis chamber used.

Note: Slides are placed on the raised centre section at right angles to the buffer compartments.

layer had gelled the slide was placed in a Petri dish which had a close-fitting paraffin mold (for microscope slides) on the inner surface and a diagram of an immunoelectrophoretic template aligned with the paraffin mold and attached to the outer surface of the dish (Fig. 2). A ruler and a scalpel with two clean blades, the latter separated from each other by 1.5 mm were used to cut the longitudinal sides of the antiserum well. The antigen wells were made with a glass tube of diameter 1 mm. Strips of gel of the same width, thickness and constitution as that used for immunoelectrophoresis were used to make contact with electrode vessels. Samples to be electrophoresed were applied with capillary glass tubing.

Electrophoresis was carried out with a Gelman power supply at 8-10^{o}C. Barbital acetate buffer pH 8.6 and ionicity 0.05 was made and precooled to 4^{o}C before use. An electromotive force of 75 volts was applied for 60 minutes or 80 minutes. The current flow observed when eight microscope slides were used was 13.0-16.0 milliamperes. Following electrophoresis the narrow layer of gel was removed from the antiserum trough, approximately 70 microlitres of antiserum were added and the plates were subsequently incubated for 24 hours at 26^{o}C in a humid chamber.

Staining of gels and preparation of permanent mounts

Gels were washed in several changes of filtered 0.85% sodium chloride for 48 hours at room temperature to remove nonconjugated reactants present in the gel. Sodium chloride remaining in the gels

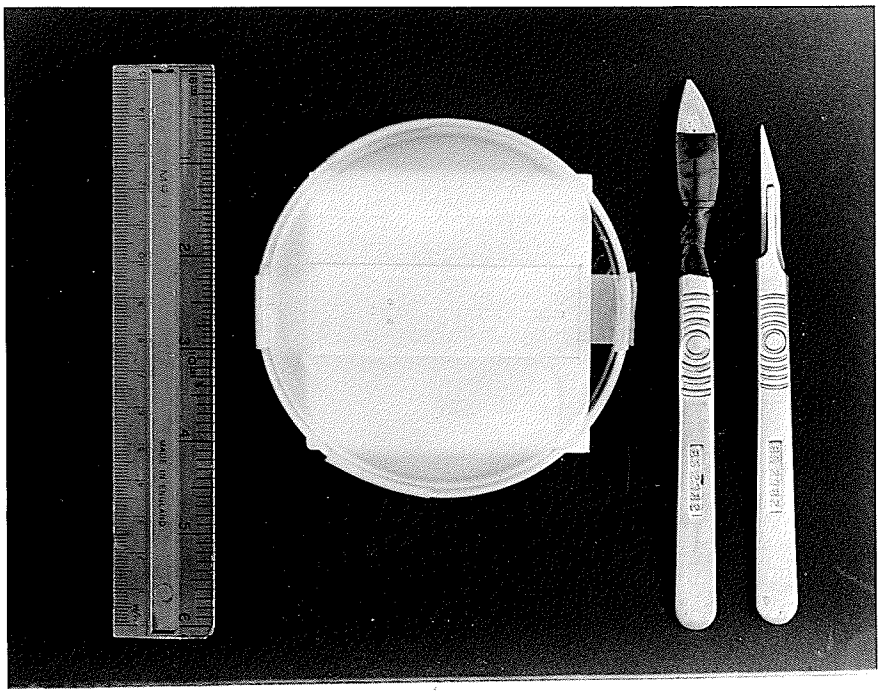


Figure 2. Instruments used to make troughs in gels.

was removed by immersing the gels in distilled water for one hour.

The gels were stained in amido black (Uriel, 1964) and light green SF (Brighton, 1967) and made into permanent mounts. Gels were dried at 37°C in a relatively dust free cabinet and stained in filtered amido black staining solution (see Appendix IIIA) for 30 minutes. Excess stain was removed by immersing the gels in filtered wash solution (see Appendix IIIB) for 30 minutes. Gels were then stained in light green SF (Appendix IV) for 20 minutes and destained for 30 minutes in 3% acetic acid. The gels were dehydrated in an ascending series of alcohol, cleared in xylene and mounted in De Pex brand mounting fluid.

Recording of results

In diffuse white light, stained precipitin bands appeared blue green. In orange filtered light containing wave lengths of 500 mu and greater, diffusion bands appeared clearer, thicker and red in colour. Precipitin bands were almost invisible when viewed in blue or green filtered light lacking wave lengths in the range 570-680 mu.

The absorption spectrum of dilute amido black staining solution as determined by a Unicam SP 800 A spectrophotometer shows that amido black absorbs light in the range 520-670 mu with maximum absorption at 620 mu. Precipitin bands stained in amido black appear blue in diffuse white light and red in incident white light.

Light green SF was found to absorb in the range 560-700 mu (maximum at 640 mu) and to transmit wave lengths outside of that

range. Precipitin bands stained in light green SF appear green in diffuse white light and red in incident white light. Hence it seems that both amido black and light green SF absorb orange light and emit red light.

Kodak infrared film sensitive in the range 670-870 mu was found to record bands adequately. An orange filter which absorbs all wave lengths shorter than 500 mu is employed because Kodak infrared film is sensitive to some of the wave lengths shorter than 500 mu. A precipitin band pattern (the normal brain pattern) is shown in Figure 3 as recorded in diffuse white light, Figure 3(a), and in direct incident white light, Figure 3(b), with Kodachrome II colour film; Figure 3(c) shows the same pattern as recorded in orange filtered direct incident light by Kodak infrared film.

In all cases line drawings were made of precipitin band patterns independently of the photographic record. The drawings were made by superimposing a microscope slide bearing a grid etched with a diamond pencil on the precipitin band pattern and recording the precipitin bands on drawing paper which was placed over a proportionate fascimile of the grid employed (Figure 4). Magnifying lenses which gave a total enlargement of approximately four times were used to facilitate the observation of precipitin bands. All slides and line drawings were examined by an independent observer to verify the accuracy of the drawings.

Figure 3. A precipitin band pattern as recorded by Kodakchrome II colour film (a) in diffuse white light (b) in direct incident white light; and (c) as recorded by Kodak infrared film in orange filtered direct incident light.

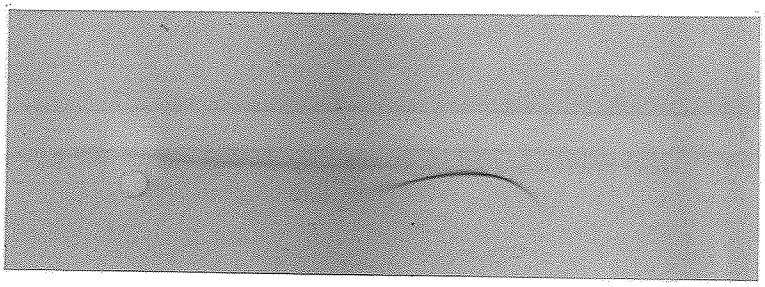


Figure 3(a)

Figure 3(b)

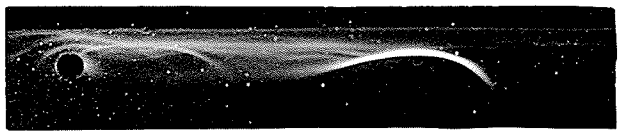


Figure 3(c)

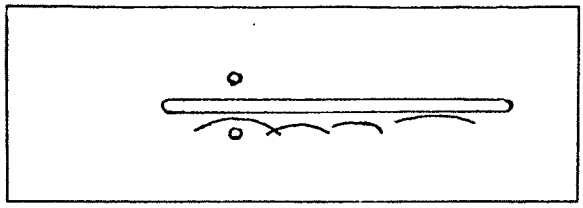
B. Immunodiffusion

Microscope slides with a thin layer of 1% agarose gel, containing the appropriate wells and troughs were prepared as previously described for immunoelectrophoresis. Antiserum and antigen preparations were introduced simultaneously and plates were incubated for 24 hours at room temperature. After incubation, plates were washed, stained, mounted and photographed as for immunoelectrophoresis.

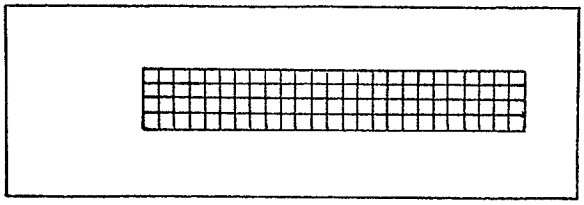
C. Sephadex column chromatography

Approximately 12 zebrafish brains were homogenized in 1 ml of barbital acetate buffer (Appendix II) and centrifuged at 40,000 x g for 30 minutes. The supernatant was chromatographed.

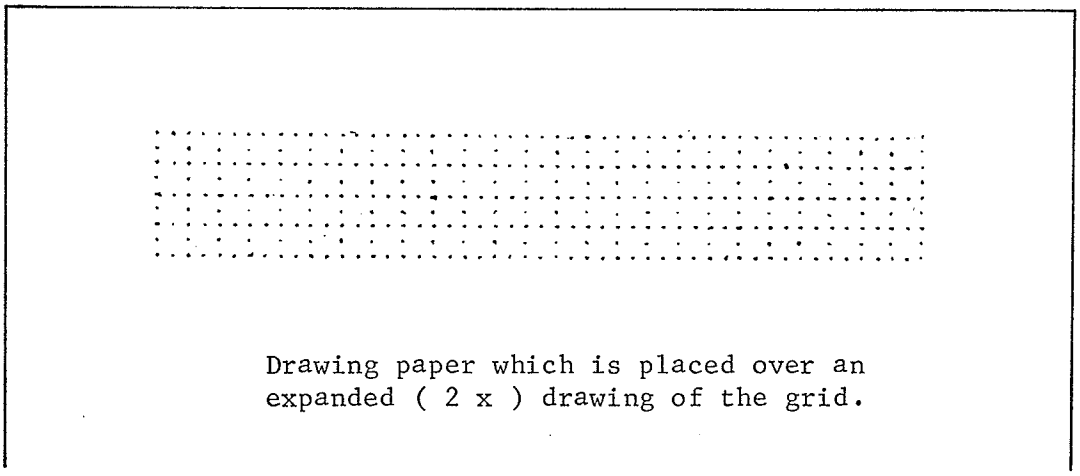
Superfine Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden) was swollen in barbital acetate buffer as suggested by Pharmacia Fine Chemicals (1966). The swollen Sephadex was poured as a thick slurry into a K 15/30 column (Pharmacia Fine Chemicals) to a height of 25 cm approximately. A disk of filter paper, 1.5 cm diameter was placed on the packed gel and approximately 1 ml of the adult zebrafish brain supernatant was carefully layered on the disk. After the sample had entered the gel the column was filled with buffer and attached to a buffer chamber which was designed to introduce buffer at a constant pressure. The column was maintained at $4^{o}C$ by a jacket of coolant at $0\text{-}4^{o}C$. Fractions were collected at 20 minute intervals by an automatic refrigerated Buchler fraction collector. The spectra of the samples at 260 mu and 280 mu were recorded. Samples were



Slide with the precipitin band pattern.



Slide bearing grid which is superimposed on the slide with the precipitin band pattern.



Precipitin bands are observed with reference to the squares of the grid on the microscope slide and are reproduced on the drawing paper with reference to the squares of the enlarged drawing of the grid.

Fig. 4. Method used to make line drawings.

pooled on the basis of their spectrum at 280 mu. Pools were dialysed against distilled water at 4°C with the aid of a magnetic stirrer. The dialysed pools were freeze-dried and resuspended in electrophoresis buffer in a concentration of 100 mg/ml. Immunoelectrophoresis was then conducted on the concentrated pools.

D. Lipid extraction

Lipids were extracted from zebrafish brain according to the method of Folch et al (1957). The lipid extract was freeze-dried and resuspended in electrophoresis buffer. This was used in immuno-electrophoresis and immunodiffusion.

III. Precipitin Band Classification

Precipitin bands were labelled in small letters starting at 'a' from antiserum trough to antigen well and from cathode to anode (see Figure 5).

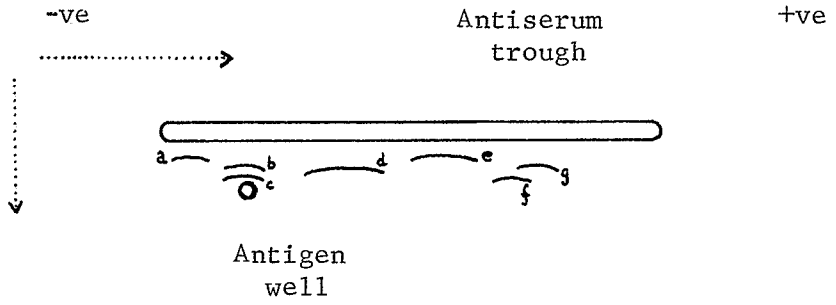


Figure 5. Primary method of band classification.

Use of subscripts in the identification of bands

Precipitin bands representing antigens which may be related antigenically and with respect to physical properties were named with the same letter bearing different numerical subscripts e.g. h, h_1, h_2, h_3 in embryonic and post hatching stages. In the case of one band giving rise to two separate bands the latter bands were named with the same letter but with different subscripts e.g. band 'f' changing to give bands 'f_1', and 'f_2' (see Figures 19 and 20). In absorption experiments precipitin bands have been identified which have electrophoretic mobilities and diffusion rates similar to bands which were known to be absorbed. Such previously undetected precipitin bands were identified with the identical letter of the known absorbed bands accompanied by a unique subscript e.g. bands 'f_3' and 'j_1' of Figure 21(b). A precipitin band which disappears simultaneously from several organs when the organs were tested with a given absorbed serum was given the same name in each of the different organ patterns e.g. band 'c_1' in Figures 26-34.

RESULTS

Immunoelectrophoretic analysis was performed on the supernatants of 10 embryonic stages, four post hatching stages and nine adult organs and tissues. The analytical agent was rabbit antisera against zebrafish brain. Controls consisted of the immunoelectrophoretic analysis of organs (tissues), embryos and post hatching stages with the aid of antiserum to zebrafish brain fully absorbed with freeze-dried brain supernatant and preimmune serum. The number of times that each supernatant was subjected to immunoelectrophoresis with the aid of unabsorbed and absorbed sera is shown in Table I.

I. Immunoelectrophoresis of Embryonic and Post Hatching Stages

A. Analysis with unabsorbed rabbit anti-zebrafish brain sera

Immunoelectrophoretic analysis of the eight cell stage resulted in the formation of a maximum of 10 precipitin bands (Figure 7). By comparing the results obtained with the eight cell stage with that of the ovary two bands, 'b', and '1', have been tentatively labelled in Figure 6. The band patterns of the eight cell stage and high blastula differ only in the presence of band 'k' in the high blastula (Figures 7 and 8).

Analysis of the mid-gastrula stage (Figure 9) showed that the relative position of band 'h' has changed slightly and bands 'g' and 'k' are not visible. Results obtained with the 10 hour embryo stage are almost the same as those of the mid-gastrula. The

Table I

The total number of times that specific embryos, post hatching stages and adult organs and tissues were subjected to immunoelectrophoresis with unabsorbed and absorbed rabbit antiserum to zebrafish brain. Controls consisted of the analysis of embryos, post hatching stages and adult organs and tissues with brain absorbed antiserum to zebrafish brain and preimmune serum.

Material Analysed	Number of times						
	Unabsorbed rabbit anti-zebrafish brain serum	Rabbit anti-zebrafish brain serum absorbed with					Preimmune serum
		Mid-gastrula	49 hour embryo	Ovary	Liver	Brain	
IA Embryonic Stages							
Eight cells	8	2	2	1	2		1
High Blastula	8				1		1
Mid-gastrula	16	4	1	1	2	1	1
10 hours	12				1		1
20 hours	6						1
27 hours	12	1	1	1	1		1
37 hours	14		1				1
49 hours	10	2	5	1	1	1	1
72 hours	9	1					1

continued

Table I continued

Material Analysed	Number of times						
	Unabsorbed rabbit anti-zebrafish brain serum	Rabbit anti-zebrafish brain serum absorbed with					Preimmune serum
		Mid-gastrula	49 hour embryo	Ovary	Liver	Brain	
96 hours	17		1	1	1		1
IB Post Hatching Stages							
1 day	6						1
2 days	6						1
7 days	7	2	3	1	1		1
28 days	3	2	2	1	1	1	1
II Adult organs (tissues)							
Brain	55	3	2	4	3	2	2
Blood	6	1	1	2	2	1	2
Heart	6	1	1	2	2		1
Liver	8	1	1	2	3	1	1
Muscle	6	1	1	2	2		1
Ovary	5	1	2	5	4	1	2
Skin	7	1	1	2	2		1
Spleen	5	1	1	2	2		1
Testis	6	1	1	2	2		1

shape of band 'h' now named band 'h_1' and its position relative to band 'j' has changed (see Figures 9 and 10). The pattern of the 20 hour embryo (Figure 11) shows an extension in band 'h_1', now designated band 'h_2' towards the anode. Band 'g' is apparent at the 20 hour embryonic stage. The 27 and 37 hour embryos yielded patterns identical with 20 hour embryos (Figures 11, 12 and 13).

Precipitin line 'm' appeared in the 49 hour embryonic pattern as a distinct band apparently joined to band 'j' (Figure 14). Band 'h_2', now named 'h_3', is closely aligned to band '1'. Analysis of the 72 hour embryo showed the detection of a new antigen 'n' with an electrophoretic mobility slightly less than antigen 'h_3' (Figure 15). Band 'g' is not evident in the 72 hour embryonic pattern.

Band 'n' was not apparent in the 96 hour embryonic pattern (Figure 16). However a new poorly defined band 'o' appears in the region of the anodic extremities of bands 'j' and 'm'. Analysis of 1, 2, 7 and 28 days post hatching larvae showed a progressive increase in the intensity and length of band 'o' in the direction of the anode (Figures 17-20).

A distinct hump formation can be seen in band 'f' at one day post hatching (Figure 17). At the seven-days post hatching stage the effect was greatest and band 'f' became divided into two parts, 'f_1', close to the antigen well and 'f_2' which showed a greater electrophoretic mobility (Figure 19). By 28 days post hatching the two bands were separate (Figure 20).

Band 'd' appears to exhibit a lower diffusion rate at 2, 7 and 28 days post hatching (Figure 18-20) than in the one day post hatching and earlier stages. Hence in the 2, 7 and 28 day post hatching stages band 'd_1' is identified and not 'd'.

At one day post hatching (Figure 17) a poorly defined band, 'p', appears. This band showed a greater electrophoretic mobility than 'e' but less than 'g' and a greater diffusion rate than either.

In the 2 day post hatching stage, band 'p' was more intense and a new band 'q', which had an electrophoretic mobility similar to 'e' and a diffusion rate greater than 'e' but less than 'p' appeared (Figure 18).

In the 7 day post hatching stage there was no significant change in the band pattern (Figure 19). However, in the 28 days post hatching stage two new precipitin bands were seen. Bands 'r' and 's' had very low electrophoretic mobilities but their diffusion rates were less than that of antigen 'b' but greater than 'c' (Figure 20).

B. Analysis with rabbit anti-zebrafish brain serum absorbed with freeze-dried mid-gastrula supernatant

Rabbit anti-zebrafish brain serum was absorbed with 30 mg. of freeze-dried mid-gastrula supernatant per ml of antiserum. No precipitin lines were observed when mid-gastrula supernatant was tested with the absorbed serum indicating that the absorption was complete. Precipitin bands observed with this absorbed serum against the developmental stages (except the mid-gastrula stage) and adult organs and tissues are therefore not common to the mid-gastrula stage.

No precipitation was observed with the eight cell stage. Analysis of the 27 hour embryo revealed some ill-defined precipitation on the anodic side of the origin. Figure 21(a) shows that at least four and probably five precipitin bands are discernible in the 49 hour embryo. The band with the lowest electrophoretic mobility occurred in the vicinity of bands 'b' and 'c' (compare with Figure 14). This precipitin band, named band 't' and not previously detected by inspection of the normal embryonic patterns is probably synthesized between 27 and 49 hours of development (at 26°C). The complex of at least two bands, and probably three, which have electrophoretic mobilities and diffusion rates similar to bands 'f', 'j', and 'm' of Figure 14 are named bands 'f_3', 'j_1' and 'm'. Since bands 'f' and 'j' were identified in the mid-gastrula absorbing tissue (Figure 9) they should not be found in 49 hour embryos analysed with mid-gastrula absorbed brain antiserum. Probably, new antigens, e.g. 'f_3' and 'j_1' have been formed between 27 and 49 hours of development which have similar physical properties to 'f' and 'j'. As shown in Table I band 'm' is apparently synthesized between 37 and 49 hours of development. Thus band 'm' of Figure 21(b) is most probably identical to band 'm' of Figure 14. The fifth band observed in Figure 21(b) has the physical characteristics of band 'h_3', of Figure 14 and therefore is named 'h_3'.

Immunoelectrophoresis of the 7 day post hatching stage (Figure 21(b)) shows that one band has become evident in addition to the five bands observed in the 49 embryo (compare with Figure 21(a)). This band is named band 'o' because it bears identical physical properties to band 'o' of Figure 19. At 7 days post hatching the 'f_3', 'j_1', 'm' band complex is extended and connected to band 'h_3'.

Analysis of the 28 day post hatching stage (Figure 21(c)) shows that the 'j_1', 'm', 'h_3' band complex completely overlaps with, or is modified to yield two bands 'u' and 'v' which have electrophoretic mobilities similar to each other as well as to the 'j_1', 'm', 'h_3' complex of Figure 21(b). At this stage band 'o' is slightly more extended than in the 7 day post hatching stage.

Analysis of the adult brain (Figure 21(d)) displayed a slight change in the relative positions of 'u' and 'v', a more intense and extended band 'o' and the appearance of an additional band. The latter has the highest electrophoretic mobility observed and a high diffusion rate. It is named band 'w'.

C. Analysis with rabbit anti-zebrafish brain serum absorbed with freeze-dried 49 hour embryo supernatant

Zebrafish brain antiserum absorbed twice with 22 mg of freeze-dried 49 hour embryo supernatant per ml was used in the analysis of eight cell, mid-gastrula, 27 hour, 49 hour and 96 hour embryonic stages and 7 and 28 day post hatching stages. All development stages up to the 7 day post hatching yielded negative results with this

absorbed serum. Hence any bands observed in later stages are not common to the 49 hour embryo stage. The 28 day post hatching stage (Figure 22(a)) showed the formation of two precipitin lines. By comparing Figure 22(a) with Figures 20 and 21(c) one line has been identified as band 'o'. From Figure 20 the second band seems to be band 'm'. However, since band 'm' was identified in the 49 hour embryo absorbing tissue and later stages and since the absorption was complete with 49 hour embryo extract, this band cannot be homologous with band 'm' of the 49 hour 72 and 96 hour embryos and the 7 day post hatching stage. It may represent a molecular species 'm_1' which has been synthesized between 7 and 28 days post hatching and has similar physical characters to band 'm'. This band, 'm_1' (Figure 22(a)) appears to coincide with the anodic extremities of band 'u' (Figure 21(c)) suggesting that band 'u' may actually represent more than one antigenic molecular species which possess similar physical properties.

Adult brain was analysed with brain antiserum which was absorbed only once with 20 mg of freeze-dried 49 hour embryo supernatant. As Figure 22(c) shows this absorption was not complete; hence all of the bands observed in Figure 22(b) are not necessarily absent from the 49 hour embryo. Comparing Figures 21(c) and 22(a) it is evident that the antigenic molecules responsible for bands 't', 'd_1', 'f_3' and 'v' which are not present in the mid-gastrula are synthesized by 49 hours. The suggestion that antigen 'd_1' is synthesized by 49 hours of embryonic development apparently conflicts with its detection with unabsorbed brain antiserum only in the 2, 7 and 28 day post hatching stages. However, it is possible that antigen'd_1' was present in the

49 hour embryo in quantities detectable by immunoelectrophoresis. The occurrence of bands 't', 'd_1' and 'f_3' (identified by comparison of Figure 22(b) with Figure 21(d)) in the analysis of the adult brain with brain antiserum partially absorbed with freeze-dried 49 hour embryo supernatant, is most probably due to the incomplete nature of the absorption rather than to differences in the antigenic constitution of the 28 day post hatching stage and the adult brain. As observed in Figure 21 band 'w' as seen here (Figure 22(b)) is characterized by its high electrophoretic mobility and appears only in the adult brain.

D. Analysis with rabbit anti-zebrafish brain serum absorbed with freeze-dried ovary supernatant.

Rabbit antiserum to zebrafish brain was absorbed with 20 mg of freeze-dried ovary supernatant per ml of antiserum. This absorption was complete since immunoelectrophoresis of ovary showed no precipitin line formation. The antigens detected with this absorbed serum are therefore not present in the ovary.

The five embryonic stages tested, eight cells, mid-gastrula, 27 hour, 49 hour and 96 hour yielded no precipitin bands. Only one band was observed in the 7 day post hatching stage (Figure 23(a)). It is identified as 'f_1' by comparison with Figure 19. Five bands were obtained when the 28 day post hatching stage was analysed (Figure 23(b)). Bands 'f_1' and 'o' were identified from Figure 20. Figure 21(c) aided in the identification of bands 'u' and 'v'. Since the fifth band which has an electrophoretic mobility of approximately

zero was not found in the 7 day post hatching stage but only in the 28 day post hatching stage it may be homologous with 'r' or 's'. This band is labelled 'r_1'.

Analysis of the adult brain yields five bands (Figure 23(c)). Comparison of the latter with Figure 23(b) and 21(d) allows for the identification of bands 'w', 'v', 'o' and 'u'. The fifth band, which has an electrophoretic mobility of almost zero, is probably identical with the band 'r_1' in Figure 23(b).

E. Analysis with rabbit anti-zebrafish brain serum absorbed with freeze-dried liver supernatant

Zebrafish brain antiserum was absorbed successively with 30 mg per ml, 20 mg per ml, and 20 mg per ml (total 70 mg per ml), of freeze-dried zebrafish liver supernatant. This absorption was shown to be complete. Therefore antigens detected with this absorbed serum do not share antigenic determinants with saline soluble liver molecules.

The ovary did not yield any bands with this serum. However, the eight cell, mid-gastrula, 27 hour, 49 hour and 96 hour embryos and the 7 day post hatching stage all yielded one band (see Figure 24 including legend). This precipitin line was identified as band 'e' by comparing the results obtained at each stage with the corresponding normal pattern (Figures 7, 9, 12, 14, 16, and 17). The 28 day post hatching stage yielded two bands, 'e' and 'o' (Figure 24(d)). Band 'o' was identified by comparing Figures 20 and 24(d).

Analysis of the brain showed the development of five bands (Figure 24(e)). Bands 'e' and 'o' were identified by a comparison of Figure 24(e) and 24(d). Band 'w' was identified by comparing Figure 24(e) with Figures 21(d), 22(b) and 23(c). The band labelled 'v_1' in Figure 24(e) has a similar electrophoretic mobility and diffusion rate to band 'v' of Figures 21(e), 21(d), 23(b) and 23(c). However, band 'v_1' is probably not homologous with band 'v' because the latter is present in both the 28 day post hatching stage and the adult brain whereas 'v_1' is present in the adult brain but not in the 28 day post hatching stage. The fifth band, characterized by a low electrophoretic mobility, was labelled band 'x' because it was not present in the 28 day post hatching stage but only in the adult brain. All of the antigens with very low electrophoretic mobilities mentioned before ('a', 'b', 'c', 'd', 'd_1', 'r', 's', 't', and 'r_1') have been identified in the antigenic patterns of embryos and post hatching stages obtained with unabsorbed brain antiserum; and mid-gastrula-, 49 hour embryo- and adult ovary- absorbed sera.

Fresh ovary supernatant did not yield any bands with the zebrafish brain antiserum which was absorbed with a total of 70 mg of freeze-dried liver supernatant per ml of antiserum (Figure 24). Thus it was concluded that the molecules of the ovary did not possess any antigenic determinants which were not already present on liver molecules.

This further suggested that ovary-absorbed sera should also detect antigen 'e' in embryos, post hatching stages and the adult brain. However, band 'e' was not observed in any of these cases. Since freeze-dried supernatants were being used for absorptions and frozen and thawed supernatants (fresh) for immunoelectrophoretic analysis it seemed fruitful to test for a difference in the antigenic patterns of freeze-dried and fresh ovary supernatants. Figure 25 shows the results obtained in the analysis of the two ovary supernatants (fresh and freeze-dried), and some early embryonic stages with zebrafish brain antiserum absorbed twice with 20 mg per ml (total 40 mg per ml) of freeze-dried liver supernatant. From Figure 25(b) band 'e' is present in the freeze-dried ovary supernatant whereas Figure 25(a) shows the presence of two other precipitin bands with the fresh ovary supernatant. The precipitin band with the lower electrophoretic mobility in Figure 25(a) has been called band 'e_1' because this band may represent an antigen which when freeze-dried yields the antigen 'e'. The occurrence of antigen 'e' in freeze-dried ovary supernatant explains why ovary-absorbed serum did not detect band 'e' in the embryonic and post hatching stages and the adult brain. The second band in Figure 25(a) was not named and may represent a molecular species which when freeze-dried is so changed that it is no longer recognized by its specific antibody (or antibodies). Hence it is not evident in Figure 25(b). The absence of precipitin lines in the analysis of fresh ovary supernatant with brain antiserum absorbed with 70 mg of freeze-dried liver supernatant per ml, and the formation of two precipitin lines when fresh

ovary supernatant is analysed with brain antiserum absorbed with 40 mg of freeze-dried liver supernatant per ml presents a conflict which is not readily resolved.

All antigens identified in the developmental stages with the absorbed sera are summarized in Table II.

II. Immunoelectrophoretic Analysis of Adult Organs and Tissues with Unabsorbed and Absorbed Rabbit Anti-zebrafish Brain Serum

Analysis of the organs and tissues of the adult zebrafish with unabsorbed brain antiserum resulted in characteristic band patterns (Figures 26-34). The number of bands observed in each pattern in different organs and tissues is variable, yet a similarity in basic pattern often persists. Seven bands each were observed in the muscle and heart patterns, nine bands each in the ovary, blood and testis patterns, eleven bands in the liver, eight in the skin and six in the spleen.

The relationship of various organ (or tissue) band patterns to one another is not obvious. That some degree of overlap in precipitin band pattern occurs in some organs is understandable when one realizes that these organs often have basically the same tissue components. This is seen where electrophoretic mobilities and diffusion rates are identical; though such physical identity in molecular behaviour need not necessarily represent a common unique antigenic molecule. The disappearance of a precipitin band in two or more different organs (tissues) when tested with the same absorbed serum may not necessarily

Table II

Antigens detected in developmental stages with brain antisera fully absorbed with freeze-dried supernatants of mid-gastrula, 49 hour embryo, adult ovary and adult liver.

Developmental stages	Absorbing tissue	Antigens detected											
		d_1	e	f_1	f_3	h_3	j_1	m	m_1	o	r_1	t	u	v
Eight cell	Mid-gastrula													
	49 hour embryo													
	Ovary													
	Liver		+											
Mid-gastrula	49 hour embryo													
	Ovary													
	Liver		+											
27 hours	Mid-gastrula													
	49 hour embryo													
	Ovary													
	Liver		+											
49 hours	Mid-gastrula				+	+	+	+				+		
	Ovary													
	Liver		+											
96 hours	49 hour embryo													
	Ovary													
	Liver		+											

continued

Table II continued

Developmental stages	Absorbing tissue	Antigens detected												
		d_1	e	f_1	f_3	h_3	j_1	m	m_1	o	r_1	t	u	v
7 days post hatching	Mid-gastrula				+	+	+	+		+		+		
	49 hour embryo													
	Ovary			+										
	Liver		+											
28 days post hatching	Mid-gastrula	+			+					+		+	+	+
	49 hour embryo								+	+				
	Ovary			+						+	+		+	+
	Liver		+							+				

indicate the presence of the same antigenic molecule in those organs (tissues). Since an antigenic molecule which possesses several different antigenic determinants may induce the formation of several different specific antibodies, and since two otherwise different antigenic molecules may share a common antigenic determinant, it is possible that a given type of antibody molecule may react with two relatively different antigenic molecules.

In short, in considering the precipitin band patterns of different organs (tissues), precipitin lines will not be labelled as being identical on the basis of similar electrophoretic mobilities and diffusion rates alone. Instead, additional evidence such as the persistence or disappearance of a band with a given absorbed serum will determine the labelling of bands. In addition, the application of the same label to bands in different organs (tissues) merely indicates the probability of those bands being identical.

The mid-gastrula-absorbed, ovary-absorbed and liver-absorbed rabbit anti-zebrafish brain sera used to analyse the adult zebrafish organs and tissues (Figures 35, 37 and 38 respectively) were all completely absorbed; i.e. no precipitin bands were formed when each absorbed serum was tested with fresh supernatant of the corresponding absorbing tissue (organ, embryonic or post hatching stage). However, the 49 hour embryo-absorbed brain antiserum was not a complete absorption (Figure 36(k)). Thus the results obtained with the 49 hour embryo-absorbed serum can only be of limited value. All antigens of organs and tissues which were named in immunoelectrophoretic

patterns obtained with unabsorbed and absorbed sera have been summarized in Tables III and IV respectively.

Immunoelectrophoretic analysis of zebrafish organs and tissues with brain antiserum absorbed with 30 mg of freeze-dried mid-gastrula supernatant per ml of antiserum showed the simultaneous disappearance of a band with low electrophoretic mobility and a relatively high diffusion rate in seven different organs (skin, spleen, heart, ovary, testis, liver and brain). This band was labelled 'c_1' (See Figures 26-34) because of its similar electrophoretic mobility and diffusion rate to band 'c' of the embryonic and post hatching stages (See Figures 7-20). A second band, identified by a high diffusion rate and a high electrophoretic mobility, though variable in different organs (tissues) has also been found to disappear in all organs and tissues with mid-gastrula absorbed serum. This band has been named 'l_1' because of its similar physical characteristics to band 'l' of embryonic and post hatching stages (Figures 7-20). Since 'l_1' represents antigenic molecules which display differing electrophoretic mobilities (Figures 26-34) these antigens are probably similar in parts of their molecular structure but are not of the same molecular size and structure.

A third antigen characterized by a high diffusion rate and a high electrophoretic mobility was not detectable with mid-gastrula-absorbed brain antiserum in brain, liver, ovary, spleen and testis though it was observed when these organs were tested with unabsorbed brain antiserum. This precipitin band is named 'h_4' because of its

Table III

Antigens named in the immunoelectrophoretic patterns of adult organs and tissues and the total number observed when the adult organs and tissues were analysed with unabsorbed brain antiserum.

Organs and tissues	Antigens named			Total number of antigens
	c_1	h_4	1_1	
Blood			+	9
Heart	+		+	7
Liver	+	+	+	11
Muscle			+	7
Ovary	+	+	+	9
Skin	+		+	8
Spleen	+	+	+	6
Testis	+	+	+	9
Brain	+	+	+	18

similar physical characteristics to band 'h_3'. Figure 21(a) shows that antibodies to antigen 'h_3' are not absorbed by freeze-dried mid-gastrula supernatant. Thus antigens 'h_3' and 'h_4' are not identical.

The analysis of blood with mid-gastrula- (Figure 35(a)), ovary- (Figure 37(a)), liver- (Figure 38(a)) and brain-absorbed brain antiserum, unabsorbed brain antiserum (Figure 30) and pre-immune serum resulted in the formation of a precipitate in the vicinity of the antigen well which is most probably of a non-specific nature. When blood was analysed with mid-gastrula-absorbed brain antiserum two precipitin bands were observed in addition to the suggested non-specific band (Figure 35(a)). Analysis with 49 hour embryo-absorbed brain antiserum yield at least three bands (Figure 36(a)). As indicated above, since this lot of absorbed serum was not completely absorbed with freeze-dried 49 hour embryo supernatant the results obtained will not be considered. A precipitate similar in appearance to the suggested non-specific precipitate is observed when blood is electrophoresed, washed, stained and mounted.

When heart was analysed with mid-gastrula (Figure 35(b)) and 49 hour embryo (Figure 36(b)) absorbed brain antisera an antigenic molecule with relatively low electrophoretic mobility was detected in both cases. This band referred to as band 'e_2' has the same physical characteristics as band 'e' but it is not identical with the latter because the principal character of band 'e' is its detection by

Table IV

Antigens identified in adult organ (tissue) supernatants analysed with brain antiserum absorbed with freeze-dried supernatants of mid-gastrula, 49 hour embryo, adult ovary and adult liver.

Organs and tissues	Absorbing material	Antigens identified																
		a_1	d_1	e	e_1	e_2	f_3	h_3	l_2	o	r_1	t	u	u_1	v	v_1	v_2	x	w
Heart	Mid-gastrula					+													
	49 hour embryo					+													
Liver	Ovary	+															+		
Muscle	Mid-gastrula							+	+										
	Ovary													+					
Ovary (fresh)	Mid-gastrula				+														
	49 hour embryo				+														
Ovary (freeze-dried)	Liver			+															
Brain	Mid-gastrula		+				+			+		+	+		+				+
	49 hour embryo		+				+			+		+	+						+
	Ovary									+	+		+		+				+
	Liver			+						+						+		+	+

N.B. (a) All precipitin bands formed were not named in all patterns obtained (see Figures 35-38). Only those patterns which contained bands that were named have been included.

(b) The 49 hour embryo absorbed serum was not completely absorbed.

liver absorbed brain antiserum (Figures 24 and 25) and it does not meet this criterion (Figure 38). The heart does not show the formation of any precipitin lines when tested with ovary and liver-absorbed brain antisera.

Analysis of the liver with ovary-absorbed brain antisera (Figure 37(b)) yielded three precipitin bands. A fourth area of precipitation is suspected to be non-specific. The band representing the antigen with the greater electrophoretic mobility was similar to band 'v' (See Figures 21(c) and 21(d)). Thus it was named band 'v_2'. Since the same ovary-absorbed serum also showed the formation of band 'v' in both the 28 day post hatching stage and the adult brain (Figures 23(b) and 23(c)) 'v_2' is probably homologous with band 'v'. When liver supernatant was tested with mid-gastrula and 49 hour embryo-absorbed brain antisera six and five precipitin bands were observed respectively. Hence the adult liver possesses at least six antigens which are not found in the mid-gastrula embryo.

When muscle was analysed with mid-gastrula-absorbed serum at least four bands were formed (Figure 35(d)). One of these has a high electrophoretic mobility and was named 'h_3'. This band did not develop when muscle was tested against 49 hour embryo-absorbed brain antiserum from which antibodies to antigen 'h_3' have been removed (Figure 36(d)). Similarly antigen 'h_3' was not detected by ovary and liver-absorbed brain antisera suggesting the presence of antigenic determinants of the 'h_3' antigen in these organs.

However it should be emphasized that antigen 'h_3' was not observed when liver and ovary were analysed with mid-gastrula-absorbed brain antiserum.

Muscle supernatant formed one band with ovary-absorbed brain antiserum which has an electrophoretic mobility similar to band 'u' (compare Figures 37(c) and 37(e)). Thus this band was named 'u_1'. Band 'u_1' and 'u' may represent closely related if not identical antigenic molecules because they both occurred in the presence of ovary-absorbed sera and were both absent in the presence of 49 hour embryo and liver-absorbed sera.

Analysis of fresh ovary supernatant with mid-gastrula-absorbed brain antiserum (Figure 35(e)) and 49 hour embryo-absorbed brain antiserum (Figure 36(e)) yielded two precipitin lines each. As mentioned in section I E above, fresh ovary supernatant appears to possess an antigen 'e_1' which is not found in the liver or in the various embryonic stages (Figures 24 and 25). Thus one of two bands in each case may be 'e_1'.

Six bands were formed when skin was analysed with mid-gastrula-absorbed brain antiserum (Figure 35(f)). At least four of these bands were apparent with 49 hour-absorbed brain antiserum. No precipitin bands were observed when skin was tested with ovary-absorbed (Figure 37 legend), or liver-absorbed (Figure 38 legend) brain antisera. Thus the antigens of the skin (Figure 26) which may be detected by unabsorbed brain antiserum are all held in common with both ovary (Figure 31) and the liver (Figure 33).

Spleen supernatant formed three bands with mid-gastrula absorbed brain antiserum (Figure 35(g)), one band with 49 hour embryo-absorbed serum (Figure 36(g)) and no bands with ovary (Figure 37 legend) and liver (Figure 38 legend) absorbed brain antisera. Hence, like the skin, the antigens of the spleen which may be detected with zebrafish brain antiserum are all held in common with both the ovary and the liver. One of these antigens is probably synthesized after 49 hours of development.

The testis formed two bands with the mid-gastrula-absorbed brain antiserum (Figure 35(h)), three bands with the 49 hour-absorbed brain antiserum (Figure 36(h)), probably a weak band with the ovary-absorbed brain antiserum (Figure 37(d)) and no bands with the liver-absorbed brain antiserum (Figure 38). Therefore the testis probably possesses an antigen which is not found in the ovary whereas the liver contains all antigens found in the testis.

Analysis of the brain with the four absorbed sera was described above when the analysis of the embryonic and post hatching stages and the adult brain with the aid of absorbed sera was considered. The results of these analyses are summarized in Table IV.

III. Analysis of Antigen 'o'

Band 'o' is a distinct brain specific precipitin line. In one series of immunoelectrophoretic tests band 'o' of the brain pattern exhibited a peculiar hump formation (Figure 39). This suggested that each molecule of antigen 'o' may consist of two basic parts with

different electrophoretic mobilities. Presumably, under certain conditions the bonds of a certain percentage of these molecules which hold these two fractions together may be broken resulting in the formation of a hump in the band. From this observation it seemed pertinent to investigate whether antigen 'o' was a protein-prosthetic molecular combination.

A preliminary division of a highly complex mixture of macromolecules in the zebrafish brain supernatant into a number of less complex mixtures was effected with Sephadex G-100. In Figure 40(a) band 'o' is observed in the immunoelectrophoretic patterns of pool I primarily. Several other antigens are also present in pool I. Pool II (Figure 40(b)) contains antigen 'w', and probably four other antigens including antigen 'o'.

Because of the common occurrence of lipoproteins in the brain it seemed pertinent to make a lipid extraction of adult brain supernatant and to determine whether antigen 'o' contained a lipid moiety. Thus a lipid extract, made according to the Folch method, was used in immunodiffusion and immunoelectrophoretic tests. In immunoelectrophoresis the strongest precipitin line has an electrophoretic mobility of zero (Figure 40(c)) and in immunodiffusion tests the strongest precipitin line of the lipid extract shows the reaction of partial identity with the strong antigen of the brain and the common antigen of pools I and II (Figure 41).

IV.A. Cross-reactions between rabbit anti-zebrafish brain serum and supernatants from the embryonic stages of Catastomus commersoni and the adult brains of various species of fish.

Saline soluble preparations from the early gastrula, optic vesicle, yolk constriction and hatching stages of the white sucker, Catastomus commersoni, showed the formation of weak precipitin bands with zebrafish brain antiserum in immunodiffusion tests (Figure 42). However the pigmentation stage (post hatching) and the adult sucker brain exhibited stronger cross-reactions (Figure 42).

Figure 43 also shows varying cross-reactions between brain antigens of the walleye, brook trout and pike and zebrafish brain antiserum.

B. Test for cross-reacting antigens in the brains of Rana pipiens and the zebrafish.

Adult zebrafish brain and zebrafish embryo supernatants were analysed in immunoelectrophoresis with antiserum to Rana pipiens adult brain, but there was no precipitin band formation. The opposite is also true, i.e. R. pipiens adult brain and R. pipiens embryo supernatants when tested against zebrafish brain antiserum yielded negative results. Therefore, brains of the zebrafish and the leopard frog do not appear to share any common antigenic determinants with respect to those brain macromolecules which are soluble under the conditions employed.

FIGURES 6 - 43

Note: The time of electrophoresis in Figures 6-40 was 60 minutes except where otherwise noted.

Figure 6. Precipitin band pattern obtained on immunoelectrophoretic analysis of ovary with antiserum to adult zebrafish brain.

Figure 7. Precipitin band pattern obtained on immunoelectrophoretic analysis of the eight cell stage with antiserum to adult zebrafish brain.

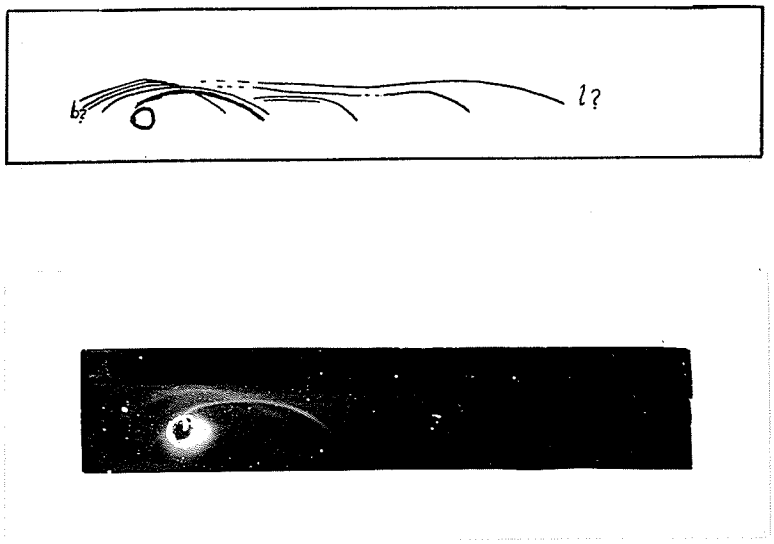


Figure 6.

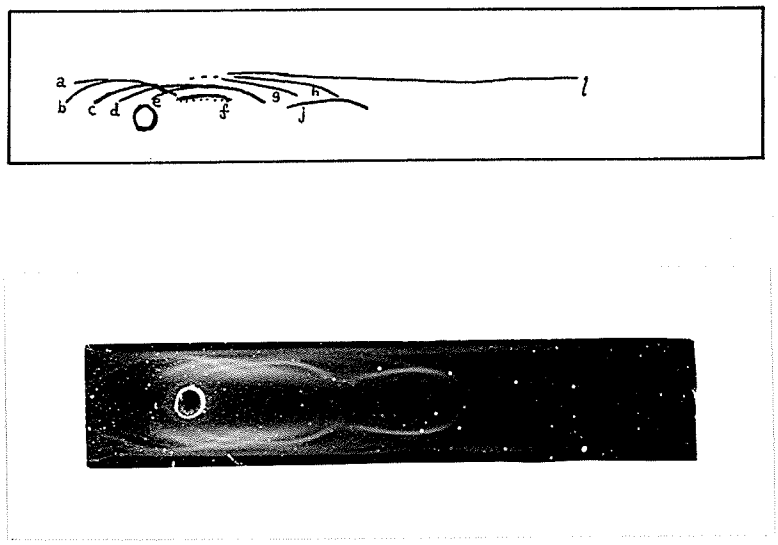
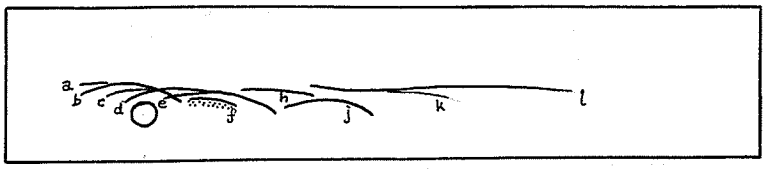


Figure 7.

Figure 8. Precipitin band pattern obtained on immunoelectrophoretic analysis of the high blastula with antiserum to adult zebrafish brain.

Figure 9. Precipitin band pattern obtained on immunoelectrophoretic analysis of the mid-gastrula with antiserum to adult zebrafish brain.



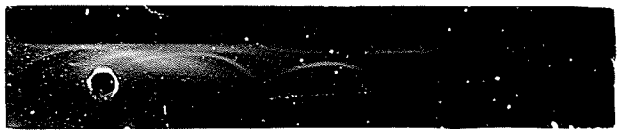
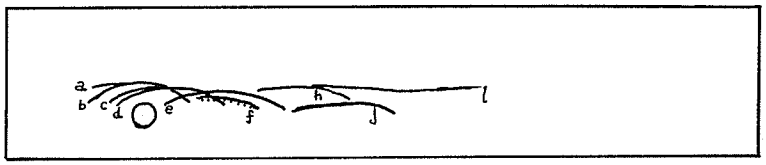


Figure 8.



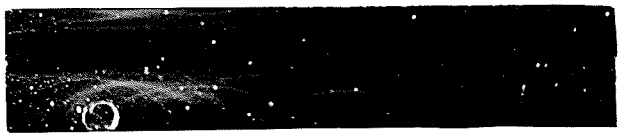


Figure 9.

Figure 10. Precipitin band pattern obtained on immunoelectrophoretic analysis of the 10 hour embryonic stage with antiserum to adult zebrafish brain.

Figure 11. Precipitin band pattern obtained on immunoelectrophoretic analysis of the 20 hour embryonic stage with antiserum to adult zebrafish brain.

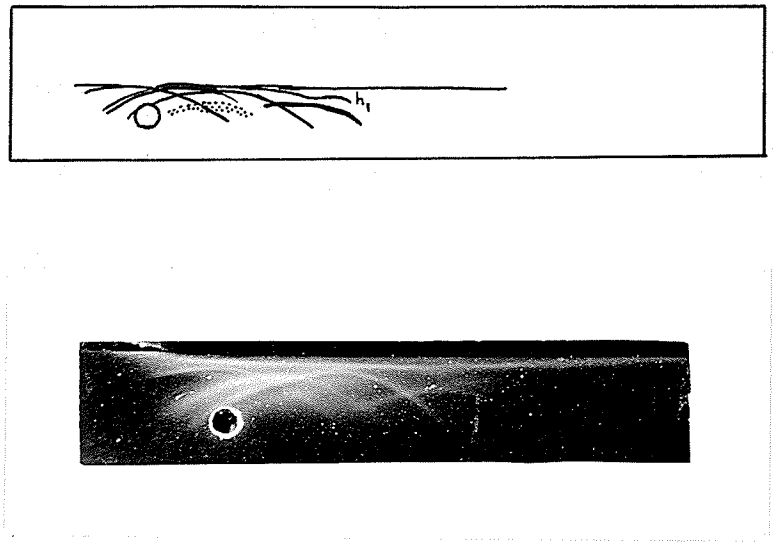


Figure 10.

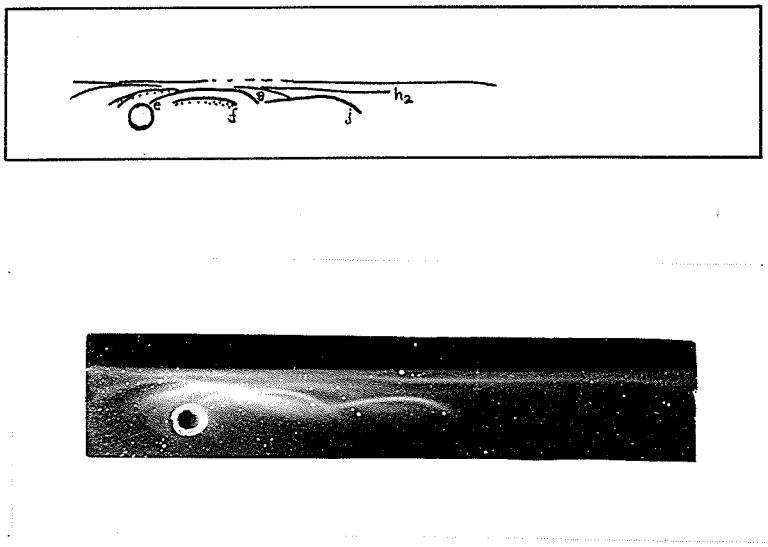


Figure 11.

Figure 12. Precipitin band pattern obtained on immunoelectrophoretic analysis of the 27 hour embryonic stage with antiserum to adult zebrafish brain.

Figure 13. Precipitin band pattern obtained on immunoelectrophoretic analysis of the 37 hour embryonic stage with antiserum to adult zebrafish brain.

Figure 14. Precipitin band pattern obtained on immunoelectrophoretic analysis of the 49 hour embryonic stage with antiserum to adult zebrafish brain.

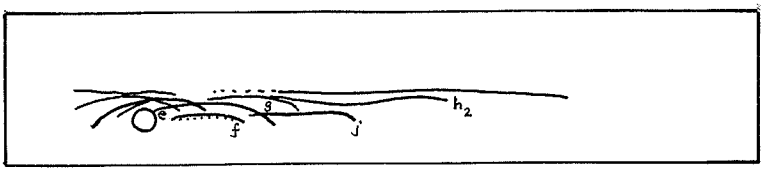


Figure 12.

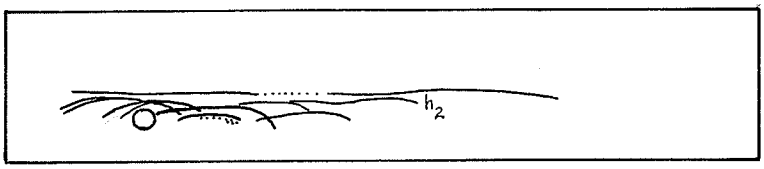
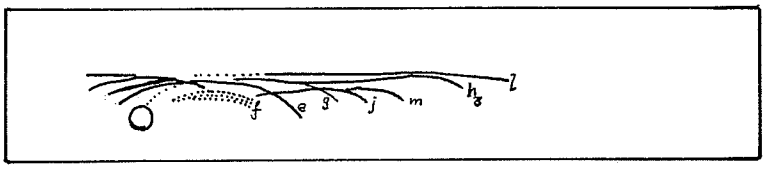


Figure 13.



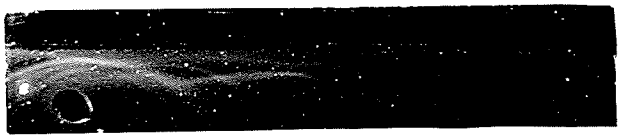


Figure 14.

Figure 15. Precipitin band pattern obtained on immunoelectrophoretic analysis of the 72 hour embryonic stage with antiserum to adult zebrafish brain.

Figure 16. Precipitin band pattern obtained on immunoelectrophoretic analysis of the 96 hour embryonic stage with antiserum to adult zebrafish brain.

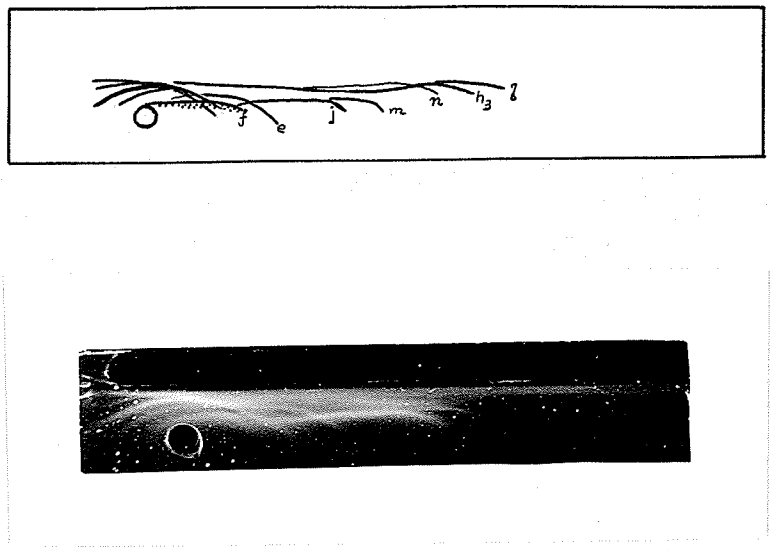


Figure 15.

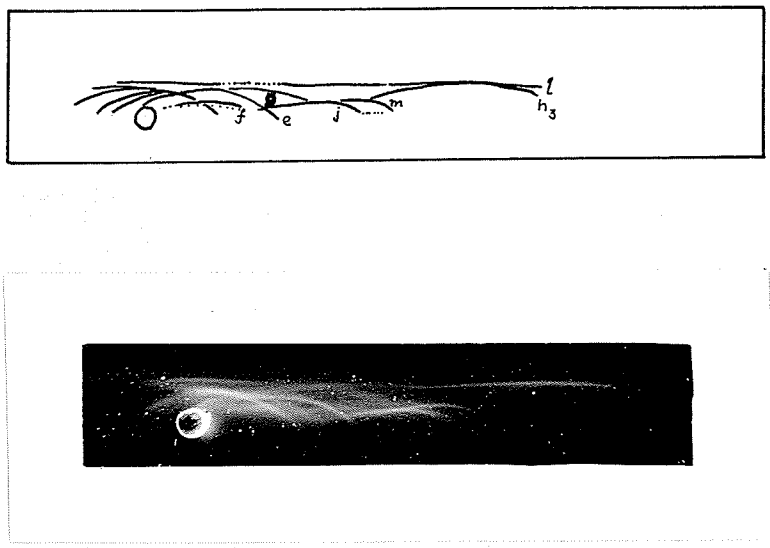


Figure 16.

Figure 17. Precipitin band pattern obtained on immunoelectrophoretic analysis of the 1 day post hatching stage with antiserum to adult zebrafish brain.

Note: Electrophoresis was conducted for 80 minutes.

Figure 18. Precipitin band pattern obtained on immunoelectrophoretic analysis of the 2 days post hatching stage with antiserum to adult zebrafish brain.

Note: Electrophoresis was conducted for 80 minutes.

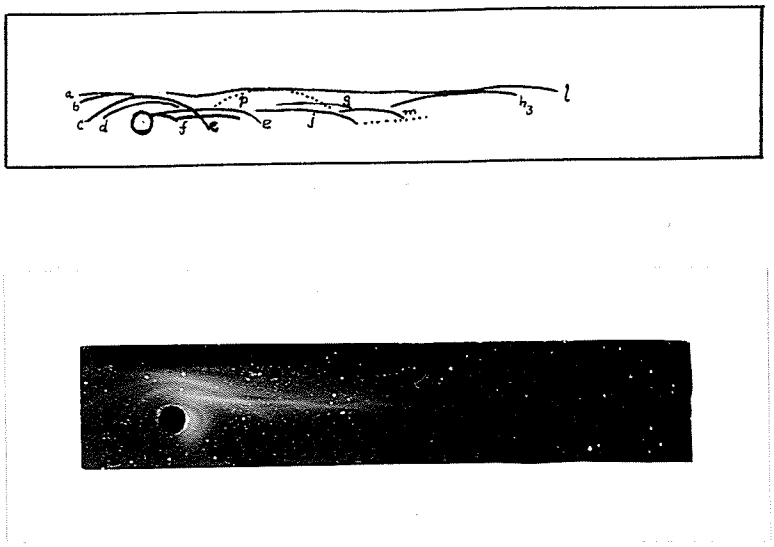


Figure 17.

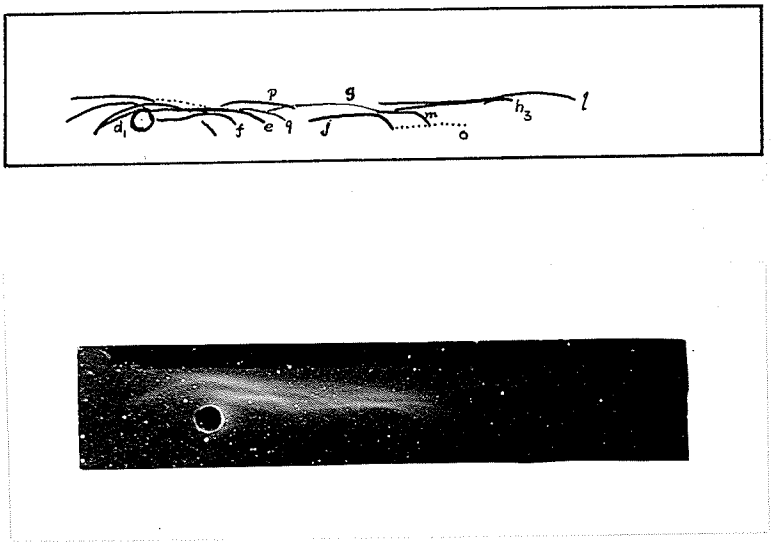


Figure 18.

Figure 19. Precipitin band pattern obtained on immunoelectrophoretic analysis of the 7 days post hatching stage with antiserum to adult zebrafish brain.

Note: Electrophoresis was conducted for 80 minutes.

Figure 20. Precipitin band pattern obtained on immunoelectrophoretic analysis of the 28 days post hatching stage with antiserum to adult zebrafish brain.

Note: Electrophoresis was conducted for 80 minutes.

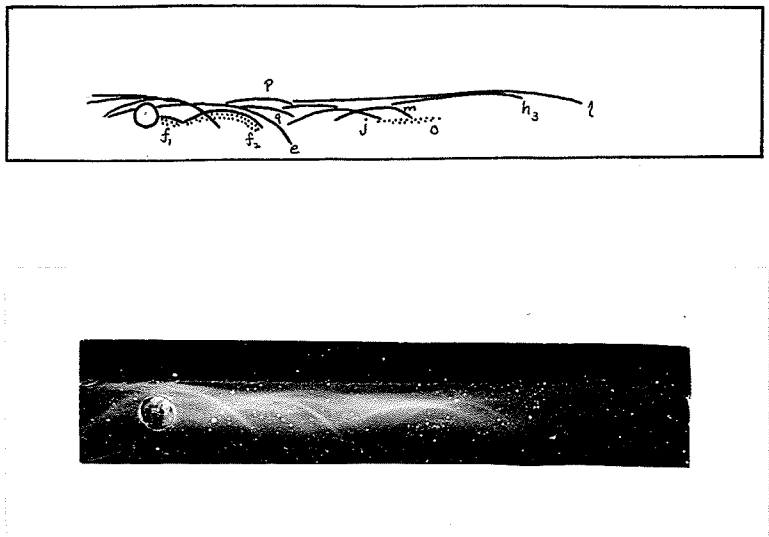


Figure 19.

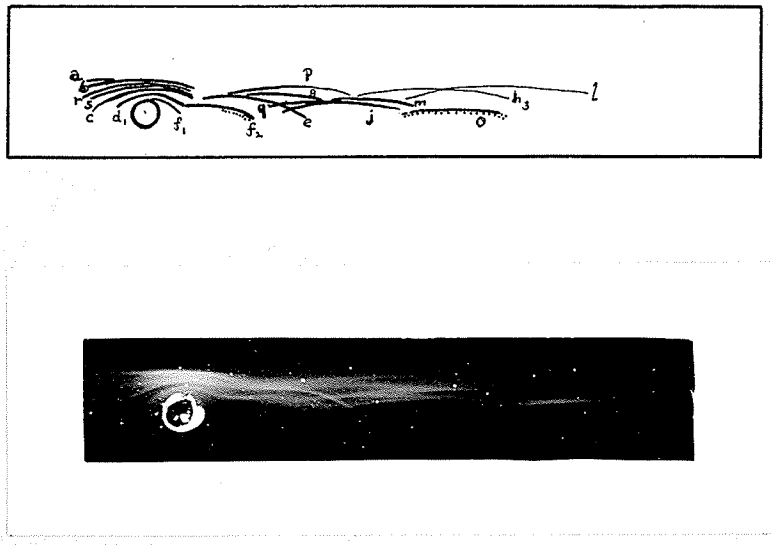


Figure 20.

Figure 21. Precipitin band patterns obtained on immunoelectrophoretic analysis of embryonic and post hatching stages and the adult brain with antiserum to adult zebrafish brain absorbed with 30 mg per ml of freeze dried mid-gastrula supernatant.

Note: The eight cell stage and the mid-gastrula showed no precipitin band formation.

Supernatants of the 7 and 28 days post hatching stages and the adult brain were electrophoresed for 80 minutes.

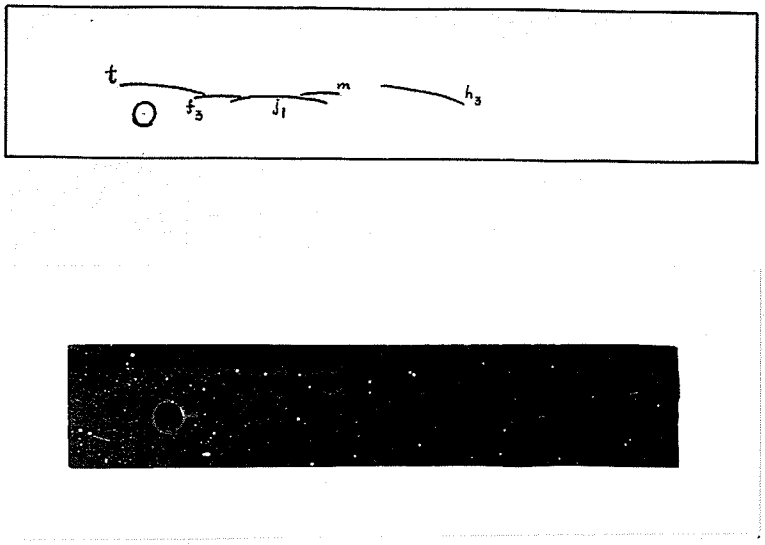


Figure 21(a). 49 hour embryo.

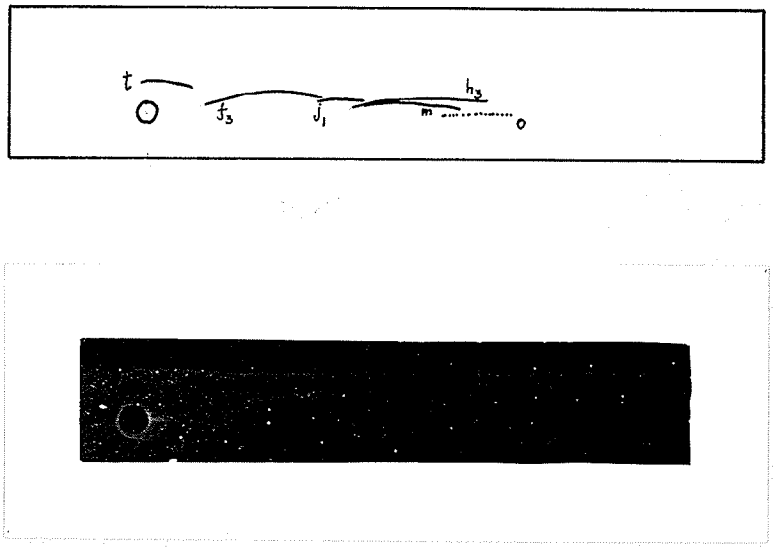
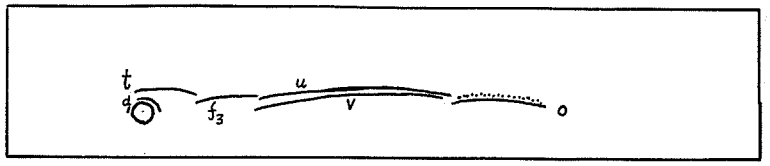


Figure 21(b). 7 days post hatching stage.



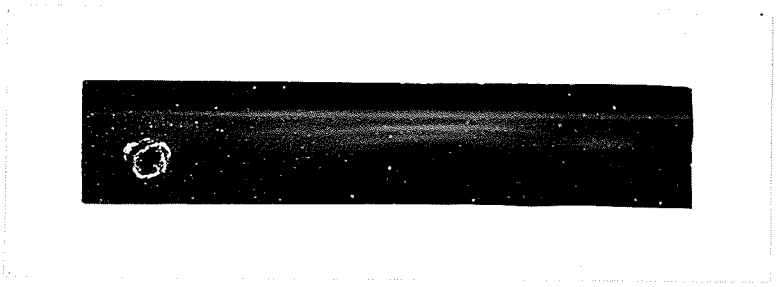


Figure 21(c). 28 days post hatching stage.

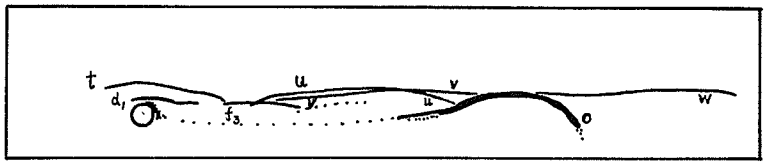


Figure 21(d). Adult brain.

Figure 22. Precipitin band patterns obtained on immunoelectrophoretic analysis of embryonic and post hatching stages and the adult brain with antiserum to adult zebrafish brain absorbed with freeze dried 49 hour embryo supernatant. Note: Two lots of absorbed sera were used. All developmental stages were analysed with antiserum absorbed twice with 22 mg of freeze dried supernatant per ml (44 mg per ml total). The adult brain supernatant was analysed with antiserum absorbed with 20 mg of freeze dried supernatant per ml. The eight cell, mid-gastrula, 27 hour, 49 hour and 96 hour embryos and the 7 days post hatching stage did not yield any precipitin bands. Supernatants of the 7 and 28 days post hatching stages and the adult brain were electrophoresed for 80 minutes.

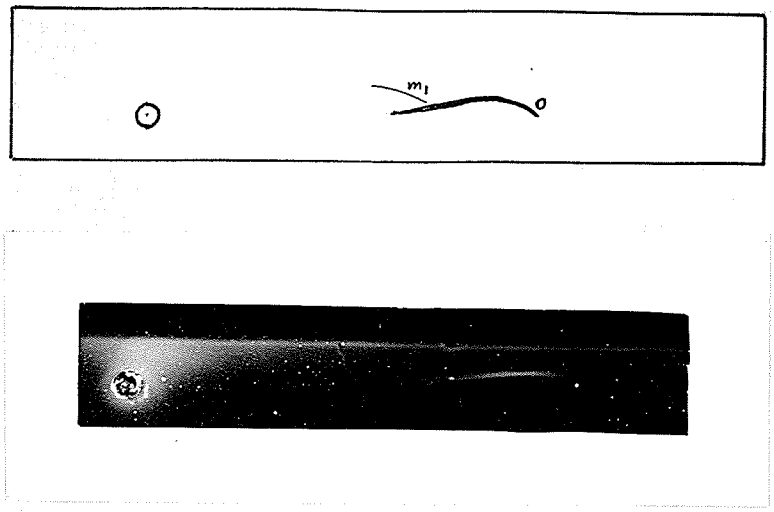


Figure 22(a). 28 days post hatching stage.

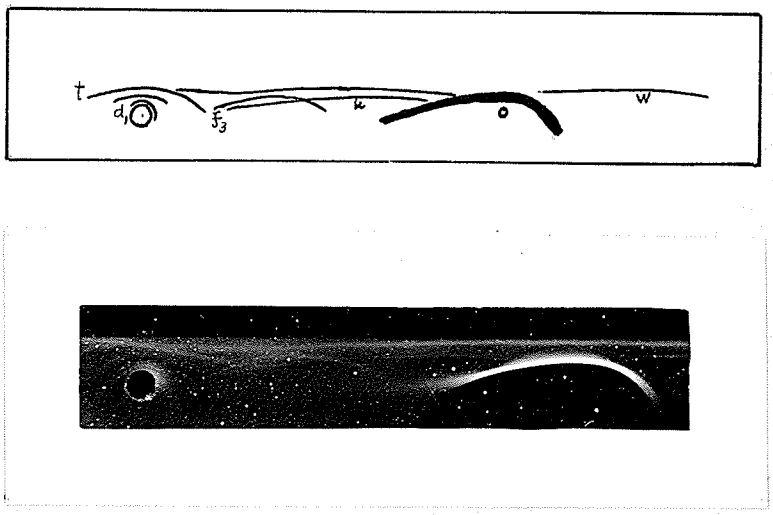


Figure 22(b). Adult brain.

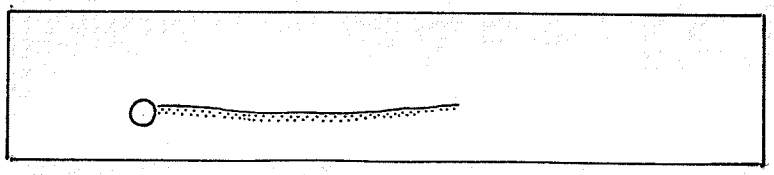


Figure 22(c). 49 hour embryo against antiserum absorbed with 20 mg per ml.

Figure 23. Precipitin band patterns obtained on the immuno-electrophoretic analysis of embryonic and post hatching stages and the adult brain with antiserum to adult zebrafish brain absorbed with 20 mg per ml of freeze dried ovary supernatant.

Note: The eight cell, mid-gastrula, 27 hour, 49 hour and 96 hour embryos did not yield any precipitin bands. Supernatants of the 7 and 28 days post hatching stages and the adult brain were electrophoresed for 80 minutes.

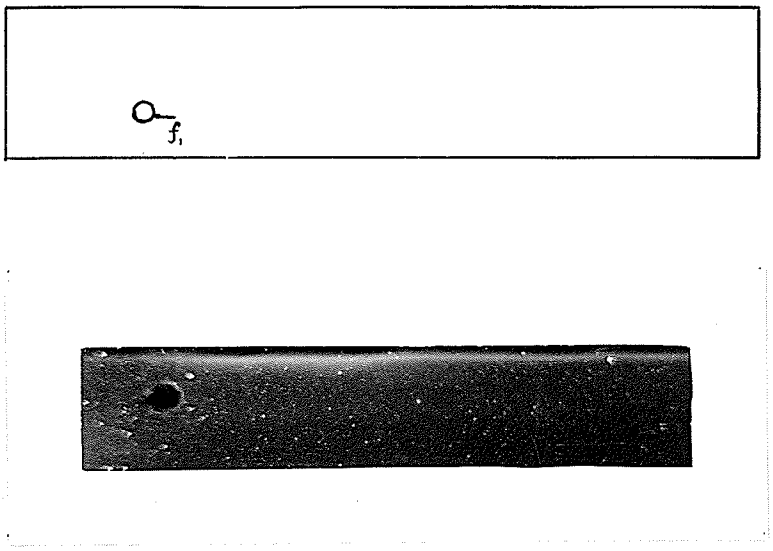


Figure 23(a). 7 days post hatching stage.

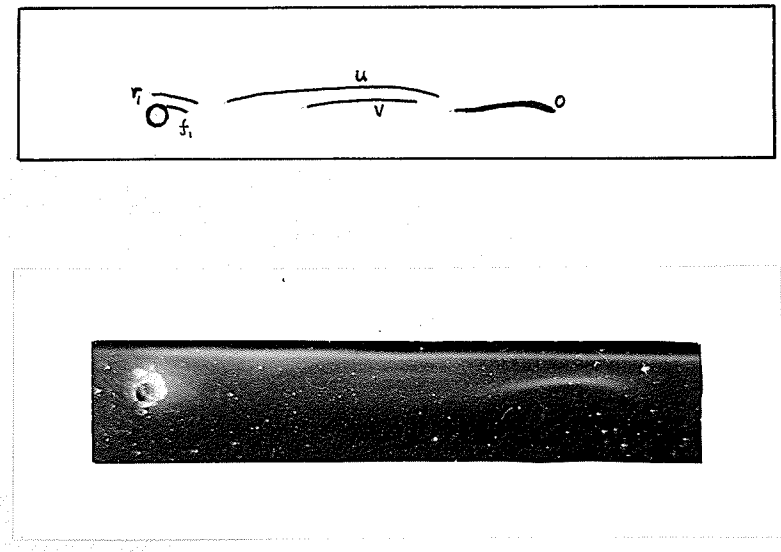
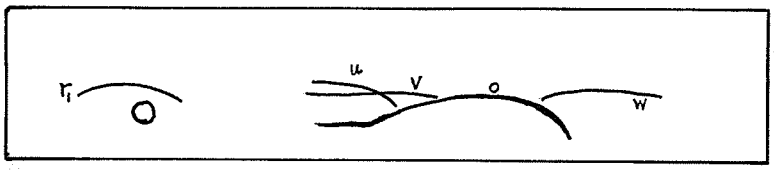


Figure 23(b). 28 days post hatching stage.



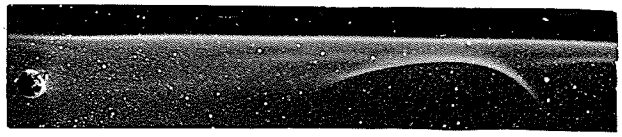


Figure 23(c). Adult brain.

Figure 24. Precipitin band patterns obtained on the immuno-electrophoretic analysis of embryonic and post hatching stages and the adult brain with antiserum to adult zebrafish brain absorbed successively with 30 mg per ml, 20 mg per ml and 20 mg per ml (70 mg per ml total) of freeze dried liver supernatant. Note: The 49 hour and 96 hour embryos and the 7 days post hatching stage yielded the same precipitin band pattern as did the 27 hour embryo. Supernatants of the 7 and 28 days post hatching stages and the adult brain were electrophoresed for 80 minutes.

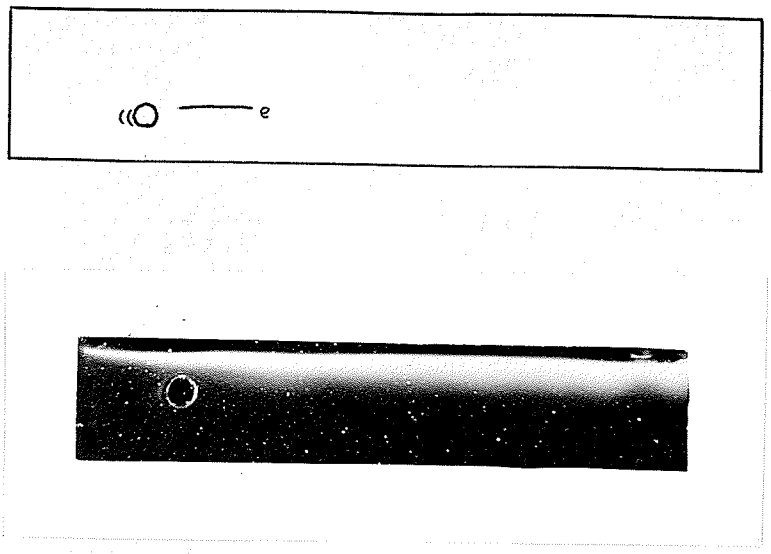


Figure 24(a). Eight cell stage.

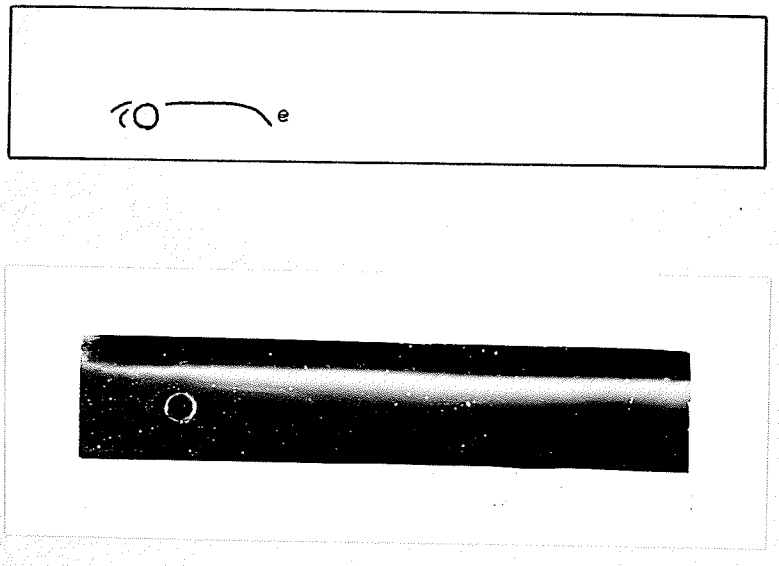


Figure 24(b). Mid-gastrula.

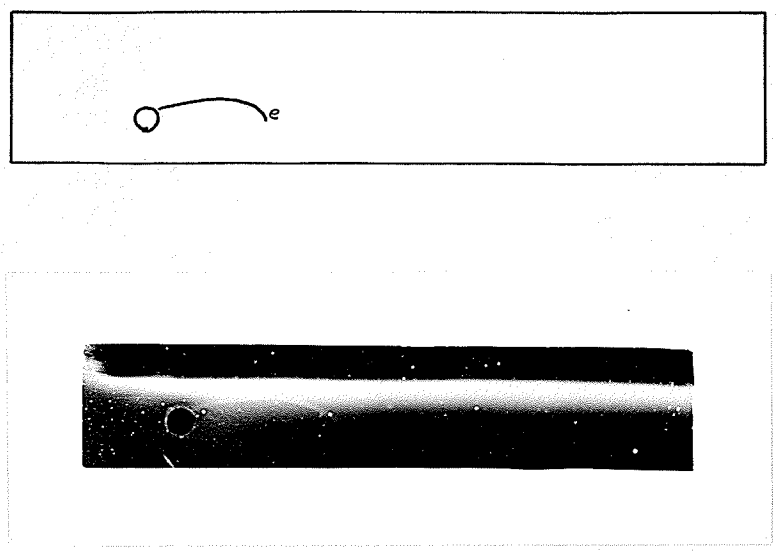


Figure 24(c). 27 hour embryo.

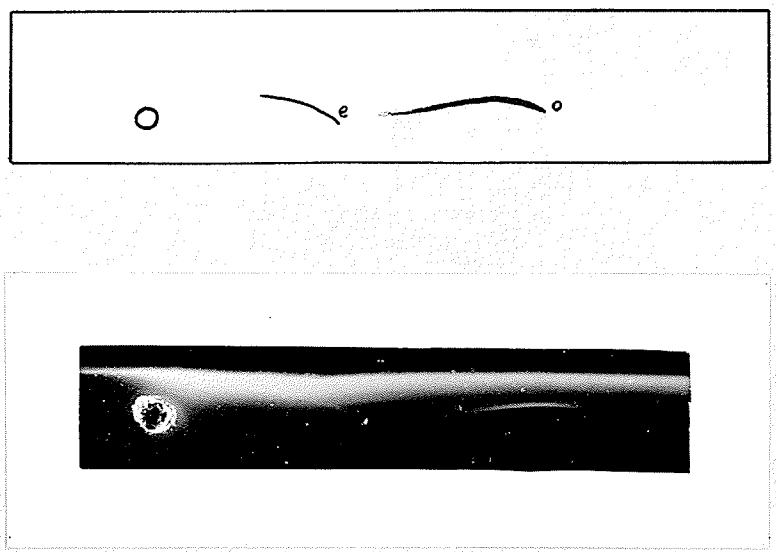
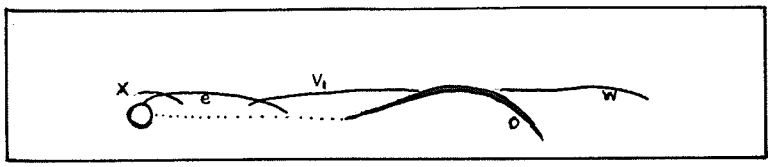


Figure 24(d). 28 days post hatching stage.



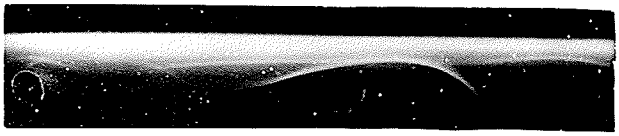


Figure 24(e). Adult brain.

Figure 25. Precipitin band patterns obtained on the immuno-electrophoretic analysis of fresh ovary, freeze dried ovary, eight cell, high blastula, 4 hour, mid-gastrula and 10 hour embryos with antiserum to adult zebrafish brain absorbed twice with 20 mg per ml of freeze dried liver supernatant (40 mg per ml total).

Note: The high blastula, 4 hours and mid-gastrula embryos yielded the same precipitin band pattern as the eight cell stage.

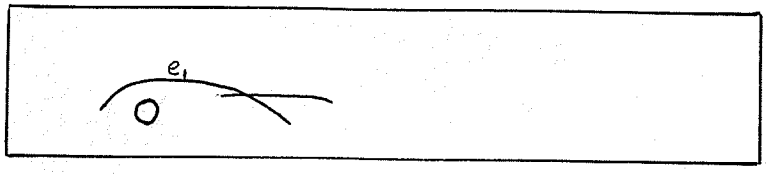


Figure 25(a). Fresh ovary.

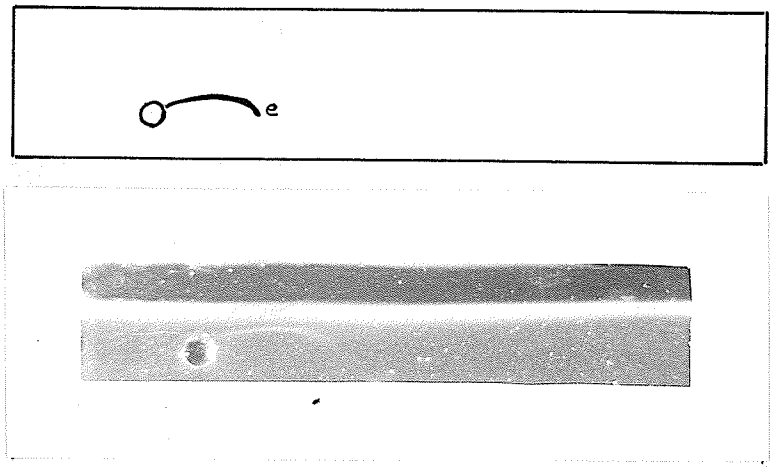


Figure 25(b). Freeze dried ovary.

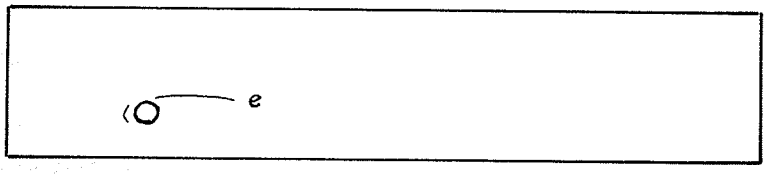


Figure 25(c). Eight cell stage.

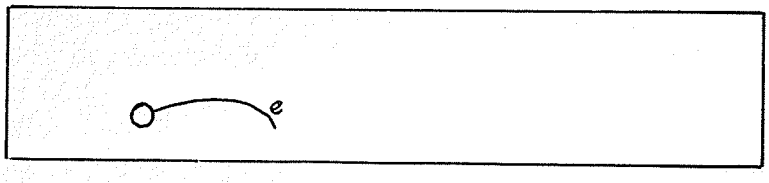


Figure 25(d). 10 hour embryo.

Figure 26. Precipitin band pattern obtained on immunoelectrophoretic analysis of adult skin with antiserum to adult zebrafish brain.

Figure 27. Precipitin band pattern obtained on immunoelectrophoretic analysis of adult spleen with antiserum to adult zebrafish brain.

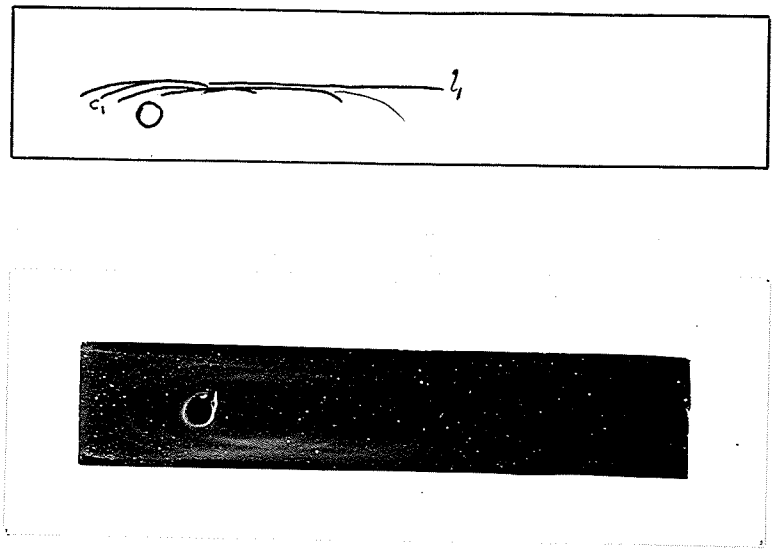


Figure 26.

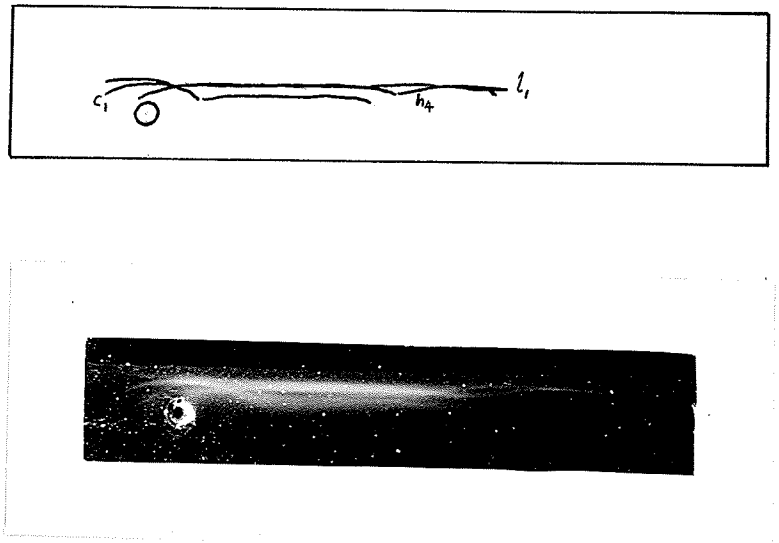


Figure 27.

Figure 28. Precipitin band pattern obtained on immunoelectrophoretic analysis of adult muscle with antiserum to adult zebrafish brain.

Figure 29. Precipitin band pattern obtained on immunoelectrophoretic analysis of adult heart with antiserum to adult zebrafish brain.

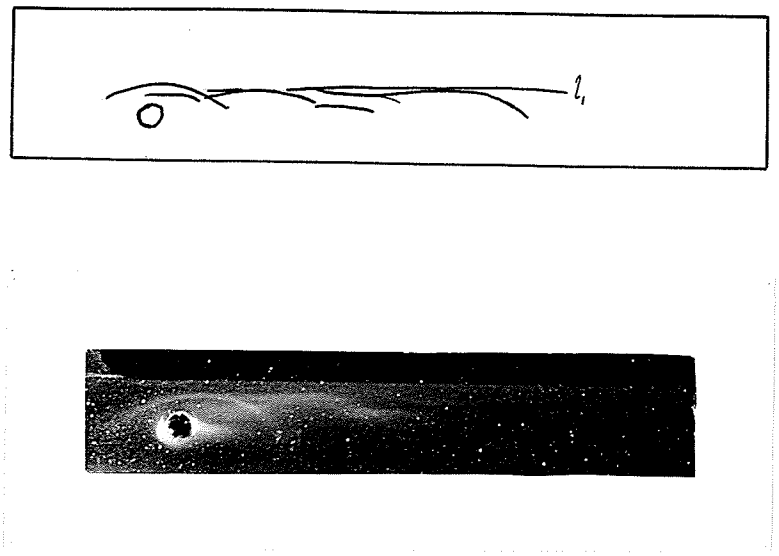


Figure 28.

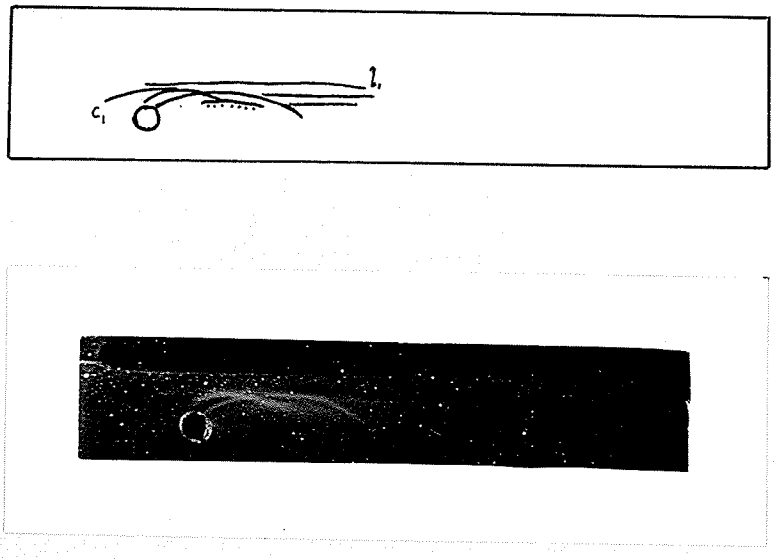


Figure 29.

Figure 30. Precipitin band pattern obtained on immunoelectrophoretic analysis of adult blood with antiserum to adult zebrafish brain.

Figure 31. Precipitin band pattern obtained on immunoelectrophoretic analysis of adult ovary with antiserum to adult zebrafish brain.

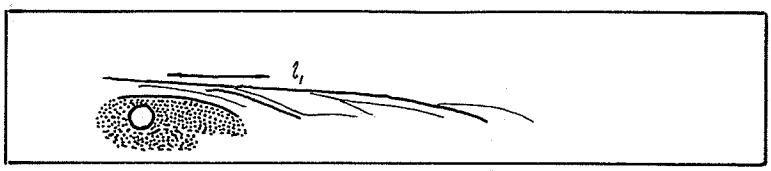


Figure 30.

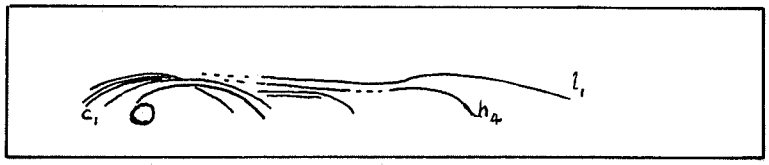


Figure 31. Same as Figure 6.

Figure 32. Precipitin band pattern obtained on immunoelectrophoretic analysis of adult testis with antiserum to adult zebrafish brain.

Figure 33. Precipitin band pattern obtained on immunoelectrophoretic analysis of adult liver with antiserum to adult zebrafish brain.

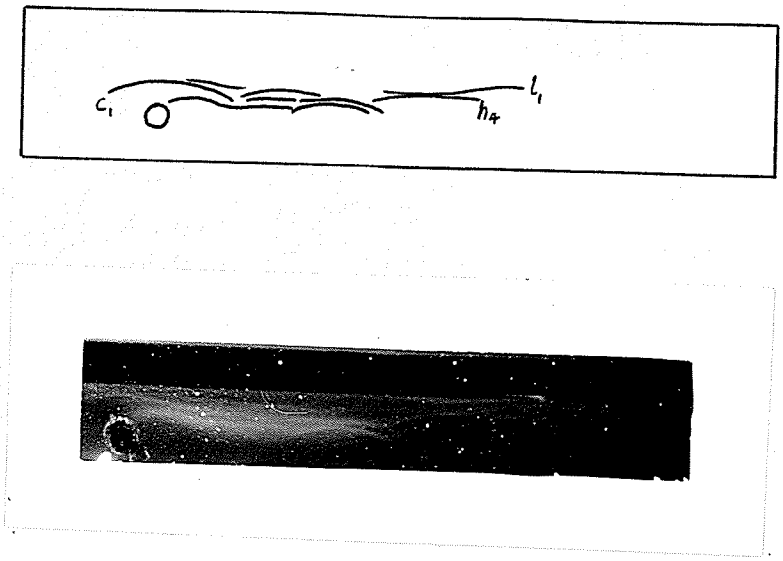


Figure 32.

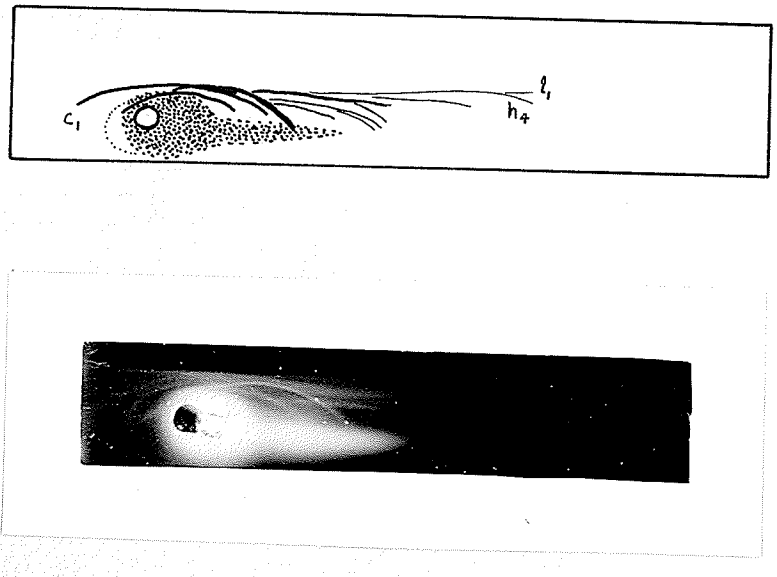
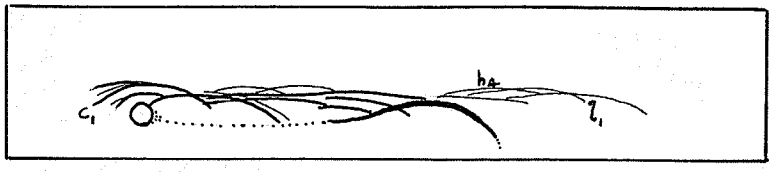


Figure 33.

Figure 34. Precipitin band pattern obtained on immunoelectrophoretic analysis of adult brain with antiserum to adult zebra-fish brain.



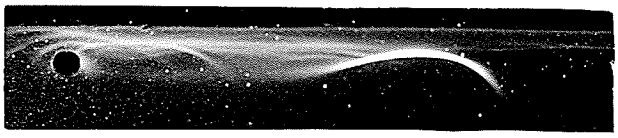


Figure 34.

Figure 35. Precipitin band patterns obtained on immunoelectrophoretic analysis of adult organs and tissues with antiserum to adult zebrafish brain absorbed with 30 mg per ml of freeze dried mid-gastrula supernatant.

Note: The brain and liver supernatants were electrophoresed for 80 minutes.

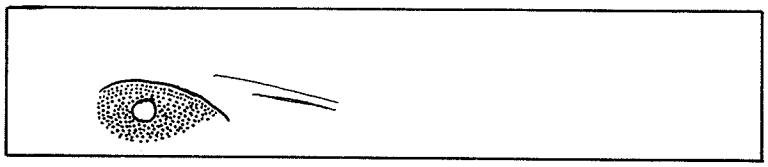


Figure 35(a). Blood.

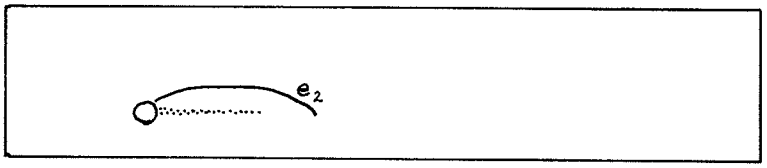


Figure 35(b). Heart.

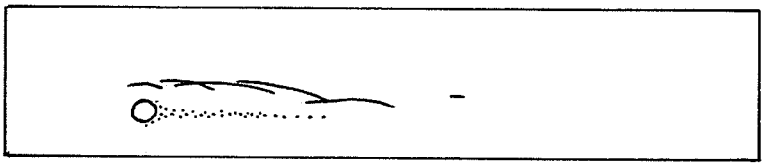


Figure 35(c). Liver.

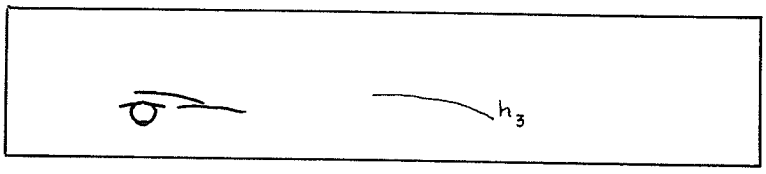


Figure 35(d). Muscle.

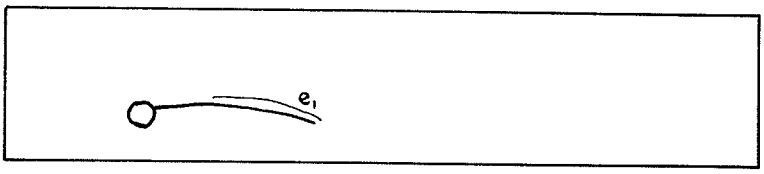


Figure 35(e). Ovary.

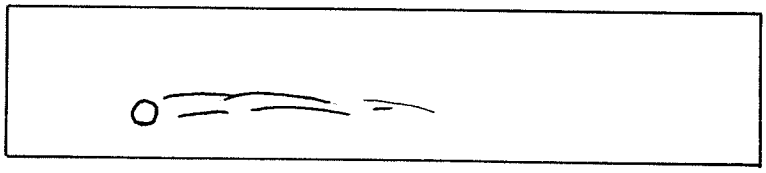


Figure 35(f). Skin.

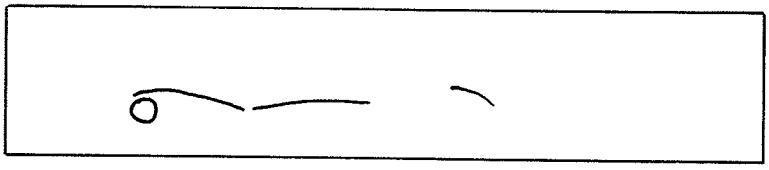


Figure 35(g). Spleen.

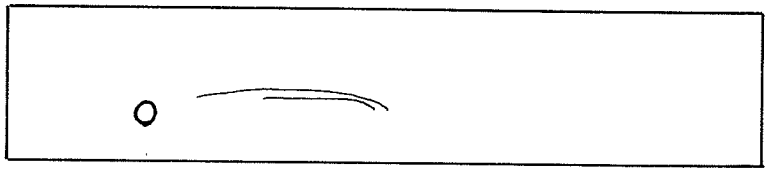


Figure 35(h). Testis.

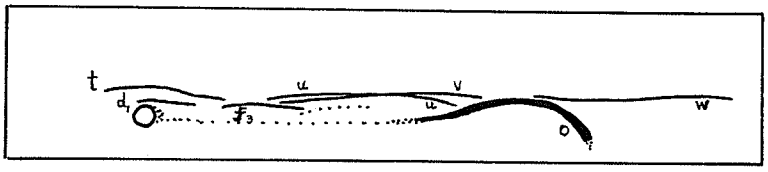


Figure 35(i). Brain.
Same as Figure 21(d).

Figure 36. Precipitin band patterns obtained on immunoelectrophoretic analysis of adult organs and tissues with antiserum to adult zebrafish brain absorbed with 20 mg per ml of freeze dried 49 hour embryo supernatant.

Note: This absorption was not complete. The brain, liver and ovary supernatants were electrophoresed for 80 minutes.

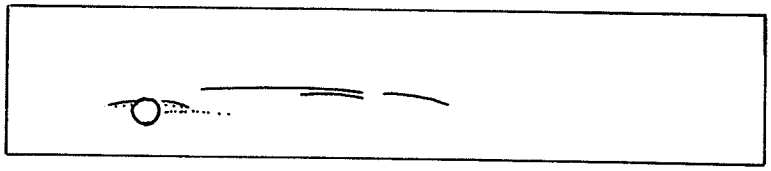


Figure 36(a). Blood.

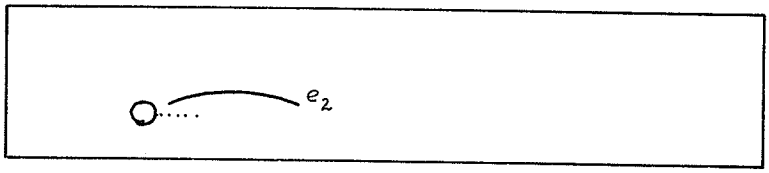


Figure 36(b). Heart.

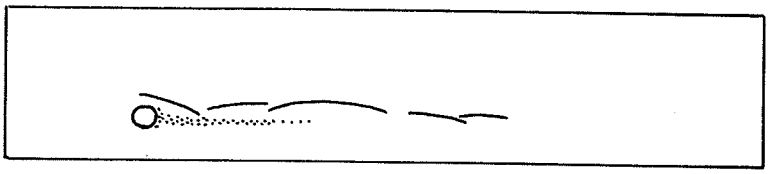


Figure 36(c). Liver.

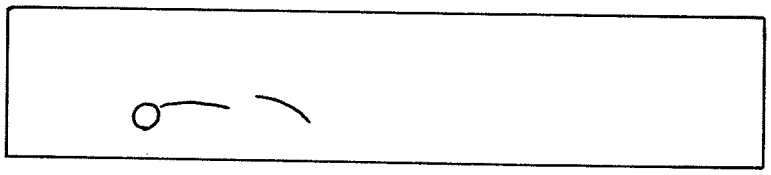


Figure 36(d). Muscle.

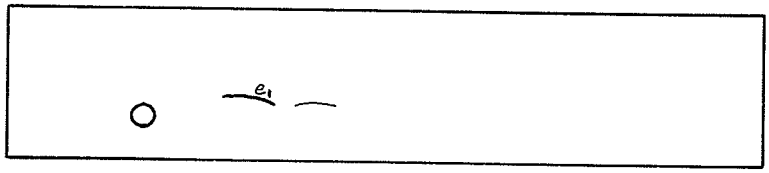


Figure 36(e). Ovary.

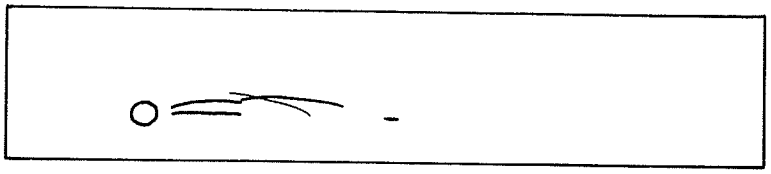


Figure 36(f). Skin.

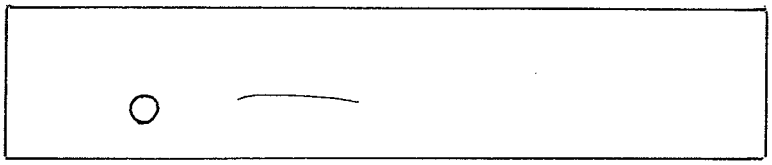


Figure 36(g). Spleen.

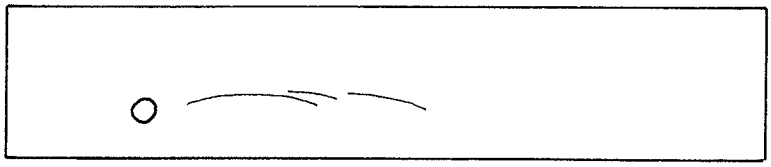


Figure 36(h). Testis.

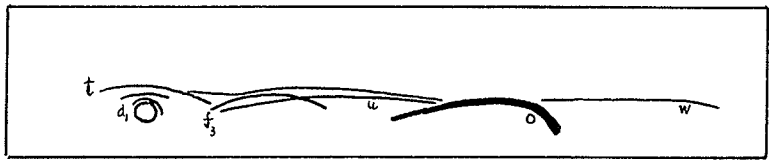


Figure 36(i). Brain.
Same as Figure 22(b).

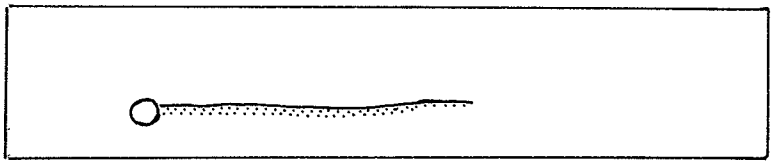


Figure 36(j). 49 hour embryo.
Same as Figure 22(c).

Figure 37. Precipitin band patterns obtained on immunoelectrophoretic analysis of adult organs and tissues with antiserum to adult zebrafish brain absorbed with 20 mg per ml of freeze dried ovary supernatant.

Note: The heart, ovary, skin and spleen supernatants did not yield any precipitin bands.

The brain and liver supernatants were electrophoresed for 80 minutes.

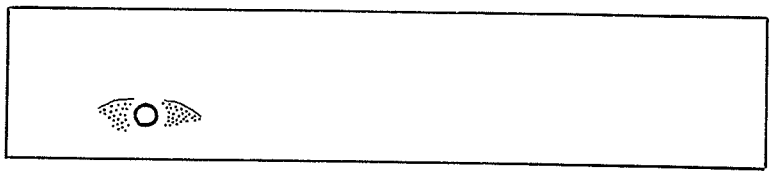


Figure 37(a). Blood.

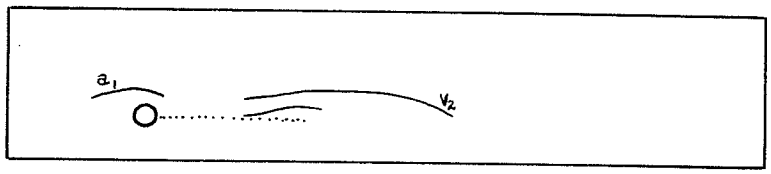


Figure 37(b). Liver.

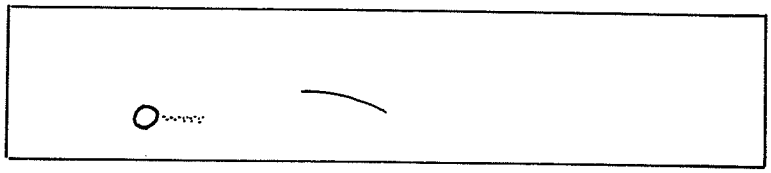


Figure 37(c). Muscle.

Figure 37(d). Testis.

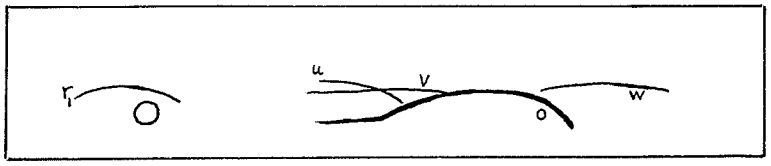


Figure 37(e). Brain.
Same as Figure 23(c).

Figure 38. Precipitin band patterns obtained on immunoelectrophoretic analysis of adult organs and tissues with antiserum to adult zebrafish brain absorbed successively with 30 mg per ml, 20 mg per ml and 20 mg per ml (70 mg per ml total) of freeze dried liver supernatant.

Note: Heart, muscle, ovary, skin, spleen and testis showed no precipitin band formation.

The brain and liver supernatants were electrophoresed for 80 minutes.

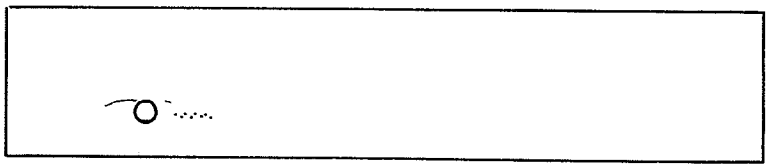


Figure 38(a). Blood.

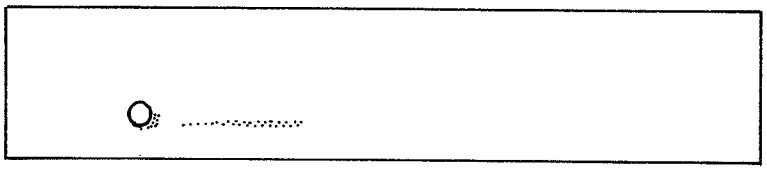


Figure 38(b). Liver.

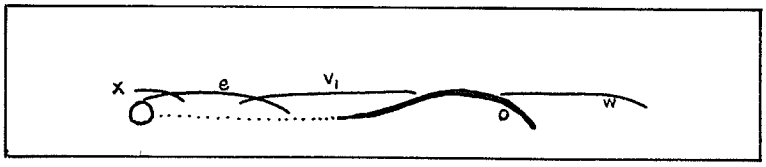


Figure 38(c). Brain.
Same as Figure 24(e).

Figure 39. Peculiar hump formation in preciptin band 'o' observed after the immunoelectrophoretic analysis of one preparation of adult zebrafish brain supernatant with antiserum to adult zebrafish brain.

Figure 40. Precipitin band patterns obtained on immunoelectrophoretic analysis of Sephadex G-100 Pool I, Sephadex G-100 Pool II and the Folch lipid extract with antiserum to adult zebrafish brain.
Note: Comparing Figures 40(a) and 40(b), a partial separation of precipitin bands 'o' and 'w' are observable.

Figure 39.

Figure 40(a). Sephadex G-100 pool I.

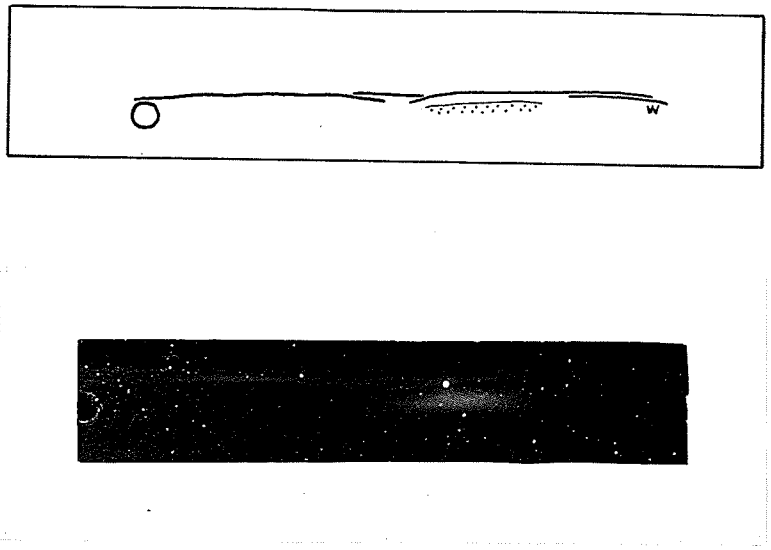


Figure 40(b). Sephadex G-100 pool II.

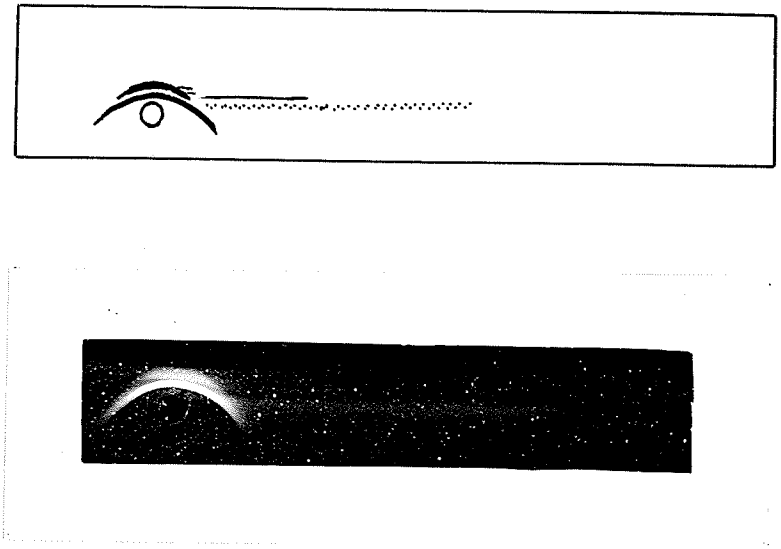


Figure 40(c). The Folch lipid extract.

Figure 41. Precipitin band patterns obtained on immunodiffusion of adult zebrafish brain, the Folch lipid extract and Sephadex G-100 Pools I and II with antiserum to adult zebrafish brain.

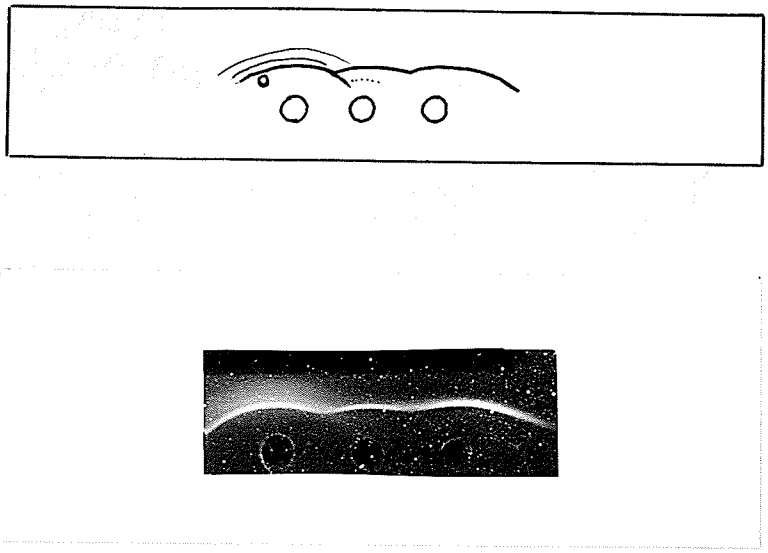


Figure 41(a).

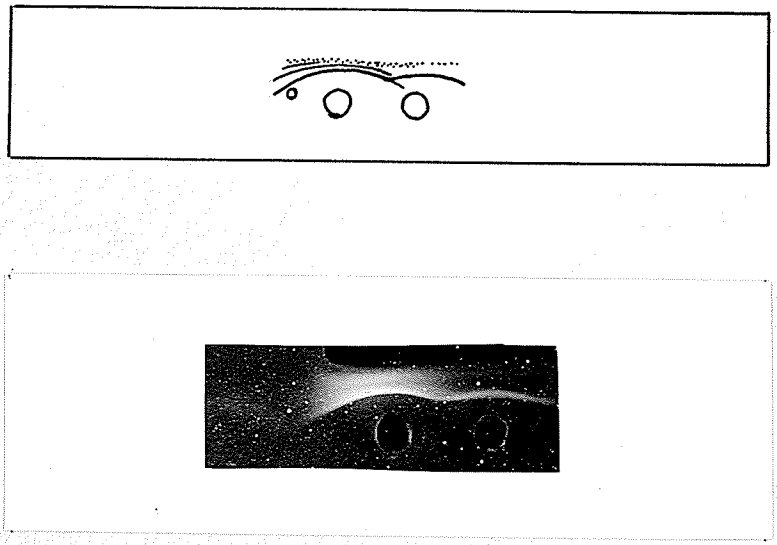


Figure 41(b).

Figure 42. Precipitin band patterns obtained when developmental stages of the white sucker, Catastomus commersoni, were analysed with antiserum to adult zebrafish brain in immunodiffusion.

Figure 43. Precipitin band patterns obtained when brain supernatants of three heterospecific teleosts were analysed with antiserum to adult zebrafish brain in immunodiffusion.

Pigmen-
tation

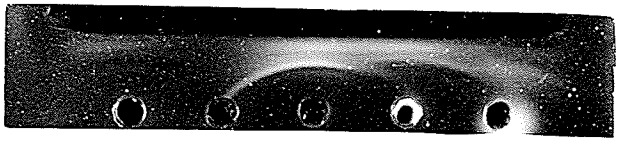
Yolk
Constric-
tion

Early
Gastrula

Hatch-
ing

Optic
Vesicle

Figure 42



Walleye

Zebra-
fish

(Sucker
Pigmentation)

Trout

Sucker

Figure 43

DISCUSSION

The mechanism of gene control in ontogeny is one of the central problems facing developmental biologists at present. The largely unexplained methods of gene activation and repression in eucaryotic cells, preclude a direct attack on this problem. Proteins are the products of genes and the relationship between proteins and the genetic material have been well investigated. Differentiation is associated with changes in the population of protein molecules in the differentiating cells (Davidson, 1968). Proteins have a varied structure and function which make them particularly amenable to experimental analysis. Thus studies pertaining to the identification, organ and species specificity, time of appearance and disappearance, location and kinetics of synthesis of proteins in embryonic development are relevant to an understanding of gene control in ontogeny.

Studies on lactate dehydrogenase isozymic patterns in mouse ontogeny strongly suggest that the synthesis of lactate dehydrogenase isozymes is epigenetically controlled (Markert and Moller, 1959; Markert and Ursprung, 1962; Cahn et al, 1962; Markert, 1963). Similarly the results of experimental studies on the pancreas have led to the proposal of a multiphasic type of regulation in the differentiation of the rat pancreas (Reboud, Pasero and Desnuelle, 1964; Reboud et al, 1966; Pascale, Aurameas and Uriel, 1966; Rutter et al, 1968).

Models such as those proposed above by Markert (1963) and Rutter et al (1968) are useful in suggesting future avenues of research. Presumably when enough experimental data on the antigenic structure of the embryonic and adult brain have accumulated a meaningful model of genetic control of the synthesis of brain antigens in development may be postulated.

Among the techniques used to investigate antigens in the course of development two of the most potent and universally applicable for the qualitative study of mixtures of antigens are immunodiffusion (Ouchterlony, 1948) and immunoelectrophoresis (Grabar and Burtin, 1953). Other methods used: fluorescent antibody technique, autoradiography and complement fixation are useful for studying the localization, time of synthesis and rate of accumulation of antigens respectively, but not for identification. Although some electrophoretic methods yield excellent separations, immunoelectrophoresis is superior for qualitative analyses because it allows for the identification of antigens on the basis of diffusion rates and antigenic specificity in addition to electrophoretic mobility. However, one limitation of immunological methods is that a given molecular species must be antigenic in the animal producing the antiserum before it can be detected.

In the present investigation soluble antigens in successive developmental stages and adult organs (tissues) were studied by

immunodiffusion and immunoelectrophoresis and an attempt was made to characterize brain specific antigens.

Both unabsorbed and absorbed sera were employed. The absorbed sera were used to further characterize the antigens observed with unabsorbed serum, and the absorbing materials were chosen in accordance with certain considerations as follows:-

(1) Absorption should help to clarify the relationship between apparently related antigenic molecules of different stages and thus demonstrate possible stage specificity. Thus mid-gastrula embryo and 49 hour embryo extracts were used as absorbing materials to distinguish between the 'h', 'h_1', 'h_2', 'h_3' precipitin band complex formed between fertilization and the 49 hour embryonic stage. These absorbed sera were also used to establish the stage specificity of bands 'k' and 'm' which occur at the high blastula and 49 hour embryonic stages respectively. (2) The use of absorbed sera should aid in distinguishing between embryonic and maternal antigenic products. Since eggs are produced in the ovary it is necessary to test for differences in antigenicity between the ovary and embryos. During the development of the sea bass, Serranus atrarius, the yolk is incorporated into the liver (Wilson, 1889). This is also true of the frog (Huettner, 1941). Therefore it seemed appropriate to absorb separate aliquots of brain antiserum with freeze-dried liver and ovary supernatants and to use the absorbed sera to analyse embryonic and post hatching stages. (3) Absorbed sera should assist

in the identification of organ specific antigens. Observations of the immunoelectrophoretic patterns of the zebrafish organs and tissues against unabsorbed brain antiserum (Figures 26-34) show that both liver and ovary cross react considerably with unabsorbed brain antiserum. Hence brain antiserum absorbed separately with freeze-dried liver and ovary supernatants might aid in the detection of brain specific antigens.

Problems encountered in the absorption of brain antiserum and the use of absorbed sera are:-

(1) Absorbing materials showed differential absorbing capacities. Thus it was found that quantities of absorbing material which allowed for complete absorptions in the case of freeze-dried mid-gastrula (20 mg per ml) and adult ovary (20 mg per ml) supernatants caused incomplete absorptions with freeze-dried 49 hour embryo (20 mg per ml) and adult liver (20 mg per ml) supernatants. This necessitated the use of large quantities of freeze-dried 49 hour embryo (44 mg per ml) and adult liver (70 mg per ml) supernatants to effect complete absorptions. (2) Absorbed antisera occasionally exhibited a non-specific precipitation area in and around the antiserum troughs. This was observed when the 49 hour embryo- and adult liver-absorbed antisera were used. In an absorbed serum, in addition to the usual serum components are soluble non-antigenic molecules of the absorbing tissue soluble antigen-antibody complexes and antigenic molecules whose homologous antibodies have been absorbed. It is

possible that the non-specific precipitation observed is due to the presence of these contaminants. (3) The presence of these contaminants may lead to variations in the degree of sensitivity observed between the absorbed and unabsorbed brain antisera and also between different absorbed brain antisera. For example, with unabsorbed brain antiserum precipitin band 'o' is barely detectable in the 96 hour embryo and the one day post hatching stage and is readily seen in the 2 day, 7 day, and 28 day post hatching stages. Brain antiserum absorbed with freeze-dried mid-gastrula supernatant (20 mg per ml) showed the presence of band 'o' in the 7 day post hatching stage. However, brain antiserum absorbed with the same quantity of freeze-dried adult ovary supernatant (20 mg per ml) or larger quantities of freeze-dried 49 hour embryo supernatant (44 mg per ml) and freeze-dried adult liver supernatant (70 mg per ml) do not show the presence of band 'o' in the 7 day post hatching stage but only in the 28 day post hatching stage (Figures 22-24). To attribute this variation in results to non-specific precipitation in and around the troughs containing absorbed sera might be valid for the 49 hour embryo- and adult liver-absorbed sera but not for the adult ovary-absorbed serum. In the latter case there was little non-specific precipitation in and around the antiserum troughs. Here, the reduced sensitivity of this absorbed serum might be due to other factors. For example, it is known that the combination of an antigenic determinant with its homologous antibody involves the formation of non-covalent bonds

(Pressman and Grossberg, 1968). Therefore it is possible that the high concentration of macromolecules in the vicinity of an antiserum well which contains an absorbed serum might tend to reduce the number of antigen-antibody precipitates which are formed in the immediate vicinity. This would reduce the ability of the absorbed serum to detect an antigen.

There is little change in the antigenic pattern in the early phases of embryonic development, namely, cleavage, blastula, gastrula and neurula. This at first appears to be at variance with the accepted picture of antigen synthesis, and increasing antigenic complexity demonstrated in the sea urchin (Westin, Perlmann and Perlmann, 1967); and with the changes in the antigenic structure observed in frog embryos during gastrulation (Spar, 1953). Upon consideration of the analytical reagent used this conflict disappears. Both Spar (1953) and Westin, Perlmann and Perlmann (1967) prepared antisera against different embryonic stages of the species under investigation. In the present study the analytical reagent was antiserum to adult zebrafish brain. Hence a lack of change in the antigenic pattern in embryos before and after gastrulation does not imply a lack of antigen change in the zebrafish gastrula. Instead it suggests that there is no change in the antigenic structure of the blastula, gastrula and neurula with respect to the adult brain.

Cross reaction between the ovary and eight cell to 10 hour embryo and zebrafish brain antiserum suggests that the macromolecules

of the adult brain, adult ovary and eight-cell to 10 hour embryonic stages, though most probably not identical, do share a number of common antigenic determinants. It is known that the brain and the yolk of the ovary and embryonic stages both have a high lipoprotein content. Whether the latter accounts for the cross reactivity in any way is still to be verified.

In assessing the results obtained by analysing supernatants of embryos, post hatching stages and the adult brain with unabsorbed brain antiserum and brain antiserum absorbed with freeze-dried mid-gastrula, 49 hour embryo, adult ovary and adult liver supernatants, the following picture emerges. (1) Precipitin bands observed in embryos, post hatching stages and the adult brain with unabsorbed brain antiserum are:- 'a', 'b', 'c', 'd', 'd_1', 'e', 'f', 'f_1', 'f_2', 'g', 'h', 'h_1', 'h_2', 'h_3', 'j', 'k', 'l', 'm', 'n', 'o', 'p', 'q', 'r', 's', and 'w' (Table V). Precipitin bands 'a', 'b', 'c', 'j' and 'l' were identified in all embryos and post hatching stages, showed no apparent change in development, and their homologous antibodies were seemingly absorbed by each of the absorbing tissues employed. Hence these antigen ('a', 'b', 'c', 'j' and 'l') have no distinguishing features other than those already mentioned and will not be further considered. Antigen 'e' was also identified in all embryos and post hatching stages; however, its homologous antibodies were not absorbed by freeze-dried liver supernatant. Thus antigen 'e' has been partially characterized with liver-absorbed brain antiserum and will be subsequently discussed. (2) Data obtained

some precipitin bands through the use of unabsorbed brain antiserum were supported by experiments conducted with absorbed sera. These bands are 'm', 'h_3', 'd_1', 'f_1', 'e', 'o' and 'w'. (3) The detection of some precipitin bands, namely 'k', 'h_1', 'h_2', 'n', 'p', 'q', and 's' with unabsorbed brain antiserum was not supported by observations made with absorbed sera. In the case of the weak bands, 'k', 'h_1', 'h_2' and 'n' it is possible that the relative insensitivity of the absorbed sera may have made detection difficult. In all cases it is possible that molecules of the absorbing tissue contain antigenic determinants in common with these antigens. (4) Some antigens, 'f_3', 'j_1', 'm_1', 't', 'u', 'v', 'v_1', 'x' and 'r_1' were detected only with absorbed sera, their presence being not supported by observations made with unabsorbed serum. These precipitin bands were not observed with unabsorbed brain antiserum probably because of the overlap of these bands with other precipitin bands.

In the following, precipitin bands will be considered according to their interrelatedness.

Band 'h' is identified in the eight cell, high blastula and mid-gastrula stages; 'h_1' in the 10 hour stage; 'h_2' in the 20, 27 and 37 hour stages; and 'h_3' in the 49 hour stage. When the eight cell, mid-gastrula and 27 hour stages were analysed with brain antiserum absorbed with mid-gastrula supernatant no bands were observed. This implies that antigens 'h' and 'h_2' may possess identical antigenic

Table V

Antigens detected in developmental stages with unabsorbed rabbit antiserum to zebrafish brain.

Developmental stage	Antigen																						
	a	b	c	d	d_1	e	f	f_1	f_2	g	h	h_1	h_2	h_3	j	k	l	m	n	o	p	q	r	s
Eight cells	+	+	+	+		+	+			+	+				+		+							
High blastula	+	+	+	+		+	+			+	+				+	+	+							
Mid-gastrula	+	+	+	+		+	+				+				+		+							
10 hours	+	+	+	+		+	+					+			+		+							
20 hours	+	+	+	+		+	+			+			+		+		+							
27 hours	+	+	+	+		+	+			+			+		+		+							
37 hours	+	+	+	+		+	+			+			+		+		+							
49 hours	+	+	+	+		+	+			+				+	+		+	+						
72 hours	+	+	+	+		+	+							+	+		+	+	+					
96 hours	+	+	+	+		+	+			+				+	+		+	+		?				
1 day post hatching	+	+	+	+		+	+			+				+	+		+	+		?	+			
2 days post hatching	+	+	+		+	+	+			+				+	+		+	+		+	+	+		
7 days post hatching	+	+	+		+	+		+	+	+				+	+		+	+		+	+	+		
28 days post hatching	+	+	+		+	+		+	+	+				+	+		+	+		+	+	+	+	+

determinants although antigen 'h_2' has a greater negative charge than 'h' which accounts for its greater electrophoretic mobility. Since the 10 hour stage was not tested with the mid-gastrula-absorbed brain antiserum it is not known whether antigen 'h_1' has the same antigenic determinant as antigens 'h' and 'h_2'. From the analysis of the 49 hour embryo supernatant with mid-gastrula-absorbed brain antiserum (Figure 21(a)) it is evident that at least some antibodies to antigen 'h_3' are not absorbed. The inference is that antigen 'h_3' is not identical with antigens 'h' and 'h_2'. Since antigen 'h_3' was not detected with ovary- and liver-absorbed brain antisera it probably occurs in both of these organs. It is not known whether antigen 'h_3' occurs in the brain because if it does, precipitin band 'h_3' would occupy the same position as the anodic extremity of band 'u' or band 'v_1'.

Precipitin band 'm' develops between 37 and 49 hours of development. Antigen 'm' is probably a molecular type which reaches detectable quantities at the 49 hour stage (Figure 14). The detection of antigen 'm' in the 49 hour embryo with mid-gastrula absorbed brain antiserum (Figure 21(a)) and its absence in the eight cell stage, mid-gastrula and 27 hour embryo when tested with the same serum proves that antigen 'm' is synthesized before 49 hours of development and after 27 hours. Antigen 'm' was also detected in the 7 day post hatching stage (Figure 21(b)). Band 'm' was not clearly visible in the 28 day post hatching stage probably because it was masked by bands 'u' and 'v' which have similar physical characteristics

(Figure 21(c)). When embryonic and post hatching stages were analysed with 49 hour embryo-absorbed brain antiserum a precipitin band which had similar physical characteristics to band 'm' was identified as band 'm_1' in the 28 day post hatching stage. Antigen 'm_1' apparently reaches detectable levels between 7 and 28 days post hatching since 'm_1' was not identified in the former but only in the latter. Band 'm_1' was not observed when the 28 day post hatching stage was tested with ovary- or liver-absorbed brain antisera. Hence antigen 'm' is probably found in these organs.

Precipitin band 'd' which has stable physical characteristics prior to 1 day post hatching shows a change at 2 days post hatching. Band 'd', now 'd_1' (Figure 18-20) is seen in closer association with the antigen well. This suggests that this molecular species probably has an increased molecular weight and (or) is present in lower concentrations relative to other antigens (Crowle, 1961). When the 28 day post hatching stage was analysed with mid-gastrula-absorbed brain antiserum band 'd_1' was identified. This band was not observed when the 7 day post hatching stage and the 49 hour, 27 hour, mid-gastrula and 8 cell embryonic stages were analysed with mid-gastrula absorbed brain antiserum. Since antigen 'd' was observed to change to 'd_1' between one and two days post hatching, it was expected that antigen 'd_1' would be detected at 7 days post hatching with mid-gastrula-absorbed serum. A reduced sensitivity of the absorbed serum and a relatively low concentration of antigen 'd_1' at this stage may explain why antigen 'd_1' was not detected. Antigen

'd_1' was not observed with any of the other three absorbed sera. This suggests that antigen 'd_1' might be a constituent of the 49 hour embryo, the adult ovary and the adult liver. Antigen 'd_1' was also identified in the adult brain (Figure 21(d)) with mid-gastrula-absorbed brain antiserum.

Precipitin band 'f' also displays certain changes after hatching. At the first and second day post hatching stages band 'f' appears as a single precipitin band with a low diffusion rate and low electrophoretic mobility. At 7 days post hatching (Figure 19) antigen 'f' divides into 'f_1' which has a lower diffusion rate and lower electrophoretic mobility than antigen 'f', and 'f_2' with a higher diffusion rate and higher electrophoretic mobility than antigen 'f'. The separation of bands 'f_1' and 'f_2' is greater at 28 days post hatching. The interpretation and significance of these observations is not clear. Around 1 day post hatching it is possible that antigen 'f' becomes bound to a macromolecule which yields a complex having a lower electrophoretic mobility than the original antigen 'f'. This hybrid molecule at the 7 day post hatching stage is split into two fractions. In addition to 'f_2', part of the original antigen 'f' remains with the macromolecule thereby forming a detectable antigen 'f_1'. Band 'f_1' was identified in the 7 day post hatching stage with ovary-absorbed brain antiserum, (Figure 23(a)). Antigen 'f_1' is not found in the brain supernatant and is probably not a native brain antigen, although the possibility should not be excluded that antigen 'f_1' is a brain antigen the antigenic determinants of which are masked

as development proceeds. La Velle and Van Alten, (1969) suggest that some brain antigens are probably masked in hamster development. The presence of antigen 'f_1' as detected with the absorbed sera agrees with the observations made on the normal immunoelectrophoretic patterns.

Antigen 'e' was detected in all embryos, post hatching stages and the adult brain with unabsorbed and adult liver-absorbed brain antiserum. Liver-absorbed brain antiserum showed that it was absent in fresh ovary supernatant, present in increasing concentrations in the eight cell, mid-gastrula and 27 hour embryos and apparently uniform concentrations in the 27 and 49 hour embryos, in the 7 and 28 day post hatching stages and the adult brain (Figures 24 and 25). The detection of antigen 'e' in freeze-dried ovary supernatant but not in the fresh ovary supernatant suggests that antigen 'e' may be present in an inactivated form in the unfertilized egg; possibly, activation occurs shortly after fertilization. The apparent increasing concentration of antigen 'e' from the 8 cell to the 27 hour embryo might imply a slow rate of activation.

Precipitin band 'o' is barely detectable with unabsorbed brain antiserum in the 96 hour embryo. It becomes well defined during the post hatching stages, the intensity of band 'o' increasing progressively through the 1, 2, 7 and 28 day post hatching stages to the adult brain. Band 'o' is not observed when the 8 cell, mid-gastrula, 27 hour and 49 hour embryo supernatants are tested against mid-gastrula-absorbed brain antiserum but is seen in the 7 and 28 day post hatching stages and the adult brain tested against mid-gastrula-absorbed brain antiserum

(Figure 21). That antigen 'o' appears subsequent to the 49 hour embryo is supported by the detection of this antigen in the 28 day post hatching supernatant by 49 hour embryo-absorbed brain antiserum (Figure 22(a)). Antigen 'o' is detected in the 28 day post hatching stage and adult brain with both ovary- and liver-absorbed brain antisera (Figures 23(c) and 23(d), 24(d) and 24(e)).

Band 'w' was not observed in any of the developing stages tested. It was detected in the adult brain only with unabsorbed and all absorbed sera. Apparently, antigen 'w' reaches detectable concentrations only after 28 days post hatching.

Band 'k' represents a molecular species which probably makes a transient appearance in development. Antigen 'k' is observed only in the high blastula. Presumably this molecule is catabolized, becomes removed from solution through the formation of an insoluble complex with another molecule or is undetectable because of the masking of its antigenic determinants. La Velle and Van Alten (1969) suggested the latter possibility to explain the detection of an antigen found in the adult hamster brain.

Antigen 'g' is probably an example of a molecular type which exhibits fluctuating levels in the course of embryonic development. Band 'g' is observed in the 8 cell stage and the high blastula, is not detected in the mid-gastrula and the 10 hour stage but is seen in all other stages analysed except the 72 hour embryonic stage.

Band 'n' which appears in the 72 hour embryo and disappears before 96 hours of embryonic development, as determined with unabsorbed

brain antiserum, probably represents an antigen which is synthesized between 49 and 72 hours of embryonic development. Analysis of the developmental stages with the absorbed sera does not support these findings probably for reasons similar to that given for antigen 'k'.

Precipitin band 'p' was observed in the normal patterns of the 1, 2, 7 and 28 day post hatching stages, (Figure 17-20). Antigen 'q' was detected in the 2, 7 and 28 day post hatching stages (Figures 18 and 19) with unabsorbed brain antiserum. However bands 'p' and 'q' were not detected with the absorbed sera suggesting that they have antigenic determinants which are present on molecules of the absorbing tissues.

Precipitin bands 'r' and 's' (Figure 20) were first identified at 28 days post hatching with unabsorbed brain antiserum. These bands were not observed when the 28 day post hatching stage was analysed with mid-gastrula- and 49 hour embryo-absorbed sera. Thus it seems that the antigenic determinants of antigens 'r' and 's' are present on molecules of freeze-dried mid-gastrula and 49 hour embryo supernatants. When the 28 day post hatching stage and adult brain supernatants were tested with ovary-absorbed brain antisera a precipitin band 'r_1' was observed which had similar physical characteristics to the bands 'r' and 's' of the normal pattern of the 28 days post hatching stage. Since this precipitin band may be synonymous with either band 'r' or band 's' it was named band 'r_1'. Analysis of the adult brain supernatant with liver-absorbed brain antiserum yielded a precipitin band having similar physical properties to bands 'r' and

's'. Since this band was not found in the 28 day post hatching stage it was assumed not to be identical with band 'r' or 's'; thus it was named 'x'. However in view of the relative insensitivity of the liver-absorbed brain antiserum (70 mg per ml) compared to the ovary-absorbed brain antiserum (20 mg per ml) it is possible that antigens 'r_1' and 'x' are the same and that both 'r_1' and 'x' are identical with either 'r' or 's'.

Antigen 'j_1' was detected in the 49 hour embryo and the 7 day post hatching stage by the mid-gastrula-absorbed brain antisera (Figures 21(a) and 21(b)). This antigen is not observed in the 28 day post hatching stage when tested with mid-gastrula-absorbed brain antiserum (Figure 21(c)). Since two new precipitin bands 'u' and 'v' with similar physical characteristics to antigen 'j_1' are present in the 28 day post hatching stage it is possible that band 'j_1' is obscured by one of these two precipitin bands. It is also possible that antigen 'j' is present in undetectable concentrations at 28 days post hatching as a result of reduced synthetic activity.

Precipitin bands 'f_3' and 't' were observed in 49 hour embryo, 7 and 28 day post hatching stages and the adult brain analysed with mid-gastrula-absorbed brain antiserum (Figure 21). These bands were not found in the 8 cell, mid-gastrula and 27 hour embryos when tested with mid-gastrula-absorbed brain antiserum. Also, bands 'f_3' and 't' were not detected with 49 hour embryo, adult ovary- and adult liver-absorbed brain antisera. Hence antigen 'f_3' and 't' appear to

be macromolecules which are synthesized between 27 hours and 49 hours of embryonic development and are found in the adult ovary, adult liver and adult brain. Antigen 'f_3' was not detected by unabsorbed brain antiserum presumably because it has the same physical characteristics as antigen 'f'. Similarly antigen 't' is not detected by unabsorbed brain antiserum probably because it has physical parameters similar to antigens 'c' and 'd'.

Bands 'u' and 'v' were observed when the 28 day post hatching stage and adult brain supernatants were analysed with mid-gastrula- and ovary-absorbed brain antisera. When the 7 day post hatching stage was tested with mid-gastrula-absorbed brain antiserum three of the precipitin bands observed were bands 'j_1', 'h_3' and 'm'. These bands were not apparent when the 28 day post hatching stage was tested with mid-gastrula-absorbed brain antiserum; instead bands 'u' and 'v' with similar physical parameters to bands 'j_1', 'h_3' and 'm' were observed. This suggested that antigens 'u' and 'v' may be related to antigens 'j_1', 'h_3' and 'm'. However antigens 'j_1', 'h_3' and 'm' were not observed when embryos and post hatching stages were tested with ovary-absorbed brain antiserum; whereas analysis of the 28 day post hatching stage with ovary-absorbed brain antiserum still showed bands 'u' and 'v'. The inference here is that antigens 'u' and 'v' bear no relation to antigens 'j_1', 'h_3' and 'm'. The lack of detection of bands 'u' and 'v' in the normal pattern of the 28 days post hatching stage is probably due to the overlap of these bands with other precipitin bands e.g. 'p', 'j', 'm' and 'h_3' which have similar

physical properties.

Antigen 'v_1' was identified only in the adult brain by liver absorbed brain antiserum. Antigen 'v_1' is assumed to be different from antigen 'v' of the 28 day post hatching stage since 'v_1' is detected only in the adult brain. However because of the relative insensitivity of the liver-absorbed serum it is possible that antigen 'v_1' is present in the 28 days post hatching stage and is synonymous with antigen 'v'.

Although, in early embryogeny morphological changes of the central nervous system are due primarily to differential mitotic activity and the migration of cells (Kallen, 1964), it is expected that some changes in the morphology of the developing brain, due to the appearance of differentiated cell types, may be associated with changes in the immunochemical structure. Consequently it might be useful to consider the morphological development of the teleost brain. By 10 hours of development, the nervous system of the zebrafish is solid and the anterior tip of the brain anlagen expands laterally (Hisoaka and Firlit, 1960). At 14 hours of development the third ventricle is formed in the diencephalon and simultaneously lateral outpouchings of the diencephalon form the optic vesicles. The otic placode appears at this stage as well. The rest of the nervous system is still solid. By 24 hours of development the optic vesicles are transformed into optic cups and lens placodes are formed. By 27-37 hours of development the diencephalon is rhomboid and encloses a large third ventricle. The walls of the mesencephalon and metencephalon have thickened and the myelencephalon encloses the fourth ventricle. At this stage the

optic cups are partly differentiated and lenses are present. The infundibulum is formed as an evagination of the floor of the diencephalon. Also olfactory placodes become evident at this stage. At 49 hours of development the rhombencephalon is elongated and by 72 hours the neurones in the central nervous system have become centrally located. After 96 hours of development the neurones in the diencephalon are centrally located and resemble a T-shaped mass. In the myelencephalon the neurones are found mainly in the dorsal half. The optic cups are further differentiated at this stage. Development of the nervous system continues after hatching. Weis (1968) has described the normal morphology and development of the spinal cord and ganglia of the zebrafish. She showed that the spinal cord does not become fully functional until at least two weeks after hatching and subsequently (2 to 4 weeks after hatching) the spinal ganglia appear. This late maturation of the spinal cord might suggest a similar late maturation of the brain.

Wilson (1889) has shown that the sea bass, Serranus atrarius has a shorter period of embryonic development (75 hours at 15.6^{o}C) than that described for the zebrafish. As in the zebrafish, the gross morphology of the central nervous system is evident at hatching; however the central nervous system of the sea bass is probably even less differentiated at this stage than that of the zebrafish (at hatching).

From the evidence given, it appears that the central nervous system of at least two species of teleosts, both of which display

relatively major gross morphological changes over short periods of embryonic development, is relatively undifferentiated on the cellular level at hatching.

Some synthetic activity may be correlated with the great morphological changes which occur between 27 and 37 hours of embryonic development. No precipitin bands were detected in the 27 hour embryo with mid-gastrula absorbed brain antiserum (Figure 21(a)), but five bands were found in the 49 hour embryo supernatant. If differentiation occurs in the immediate post hatching period one would expect a detection of new antigens during this period. The experimental results agree with this expectation to a considerable degree. Antigen 'o' probably appears at hatching; antigens 'p', 'q','d_1', 'f_1', 'f_2', 'r', 's', 'w', 'u', 'r_1', 'v', 'v_1' and 'x' appear after hatching.

It would be very useful if more precise correlations between specific antigens and individual morphological structures or cell types in the brain could be made. In the present investigation because of the techniques employed this correlation could not be made. The detection of an antigen by immunoelectrophoresis depends on the formation of a visible precipitin line between the antigen and its homologous antibodies. This only occurs above a minimum concentration of antigen. Therefore it is possible that an antigen is present in subminimal amounts for some time before it reaches detectable proportions. Investigations on the development of antigens in the chick lens have led to contradictory results in two different series of experiments. Langman (1959a), by exposing chick embryos to

antiserum against adult chick lens, demonstrated that the first lens antigen(s) appears before the first morphological change; that is, that macromolecular synthesis precedes morphological change. However, Langman (1959b), in immunodiffusion experiments demonstrated the exact opposite, that is, that the morphological event precedes the synthetic phase. Since antigen detection by immunodiffusion and immunoelectrophoresis depends on antigen concentration, primarily, it is readily conceivable that the times at which antigen detection and the observation of associated morphological changes occur, may bear little relation if any to each other.

The brain is such a complex organ that it is probable that several morphological changes in a given period of development may be accompanied by a number of changes in a precipitin bands pattern. In such an eventuality it is not possible to correlate a specific precipitin band with a particular morphological change.

A method which has been employed to study antigen localization with increasing frequency in recent years is the fluorescent antibody technique. The method is ideal for determining the cells in which a specific antigen occur if a specific antiserum to that antigen is available.

The analysis of the adult zebrafish organs with the absorbed sera (Results Section II and Figures 35 to 38) agree with the known picture of development, that is, mid-gastrula-absorbed brain antiserum forms a larger number of precipitin bands with all organs

than does either the ovary- or liver-absorbed brain antiserum.

Considering the results obtained with the ovary- and liver-absorbed brain antisera (Figures 37 and 38) it can be seen from the physical characteristics of the bands that antigens 'o' and 'w' are found only in the brain. Since the ovary-absorbed serum shows precipitin lines with blood, liver, muscle, and testis supernatants it is possible that any of the other three antigens detected in the brain by this serum, 'r_1', 'u' and 'v' are also present in one or more of the other organs or tissues which reacted with this serum. Since none of the other organs (or tissues) except brain showed specific antigen-antibody reactions with the liver-absorbed serum this serum is probably a brain specific serum. Antigen 'e' which was previously identified as an antigen which appears shortly after fertilization and is found in all embryos, post hatching stages and the adult brain, was not found in any of the other adult organ supernatants. Hence antigen 'e' is brain specific. Antigens 'x' and 'v_1' which were detected only in the adult brain by liver absorbed brain antiserum are probably brain specific as well. Unlike antigens 'o' and 'w' which are readily identified by their physical characteristics and antigen 'e' which was identified by its detection in all developmental stages with liver absorbed brain antiserum, antigens 'x' and 'v_1' have been identified only in this one case. Consequently it is premature to make any conclusions regarding antigens 'x' and 'v_1'. It is possible that anigens 'x' and 'v_1' like antigen 'w' are synthesized after

28 days post hatching, or are present in undetectable quantities. If antigen 'x' and 'v_1' are brain specific then the question arises: does the ovary-absorbed brain antiserum show the presence of these antigens in the brain? The ovary-absorbed brain antiserum forms two precipitin bands 'r_1' and 'v' (in addition to antigen 'u', 'o' and 'w') with the adult brain supernatants. These have similar physical characteristics to antigens 'x' and 'v_1' respectively. But antigens 'r_1' and 'v' were detected in the 28 day post hatching stage whereas antigens 'x' and 'v_1' were detected only in the adult brain. It is possible that antigens 'x' and 'v_1' did form precipitin lines with the ovary-absorbed serum, but were obscured by bands 'r_1' and 'v' respectively. It is conceivable that antigens 'x' and 'v_1' are identical with 'r_1' and 'v' respectively.

Hence the evidence suggests that there are three brain specific antigens, 'e', 'o' and 'w'. There may be two other brain specific antigens as well. As detected in immunoelectrophoresis, antigen 'e' appears in early cleavage, antigen 'o' at hatching or shortly after and antigen 'w' sometime after four weeks post hatching.

The separation of zebrafish brain supernatant into less complex mixtures of antigens by Sephadex G-100 suggests that separation on Sephadex G-100, G-150 or G-200 may be useful as preliminary steps in the preparation of pure samples of various zebrafish brain antigens. It might be noted that the chromatography of zebrafish brain supernatant conducted on Sephadex G-100 partially separated two brain specific antigens 'o' and 'w'. This fact may be useful if the preparation of

an antiserum reactive with one brain specific antigen is required. The separation of soluble rat, rabbit, beef and monkey brain proteins has been achieved with DEAE cellulose chromatography followed by starch gel electrophoresis (Moore and McGregor, 1965). Also soluble human brain proteins have been separated by DEAE cellulose chromatography and acrylamide gel electrophoresis (Bogoch et al, 1964).

The reaction of partial identity between one component of the lipid extract with the strongest precipitin line in the brain pattern (Figure 41(a)), precipitin line 'o', in immunodiffusion suggests that antigen 'o' has a lipid moiety. The negligible electrophoretic mobility of the component of antigen 'o' which is found in the Folch lipid extract suggests that the non lipid component of antigen 'o' carries a net negative charge at pH 8.6.

A number of studies have been reported on the brain specific antigens of various species. Schalekamp (1960) found three brain specific antigens in the chick brain with antiserum to adult chick brain in immunoelectrophoresis. One of these antigens appeared on the third day of embryonic development, the second on the tenth day and the third on the fifteenth day of embryonic development. McCallion and Langman (1964), who conducted an immunodiffusion study using anti-adult chick brain sera, also found three brain specific antigens in chick ontogeny. These antigens appeared on the fifth, eighth, and twelfth days of embryonic development. Subsequently McCallion and Trott (1965) using antiserum to 9 day chick embryo brain in immunoelectrophoresis detected three adult brain specific antigens by the sixth day of embryonic development and a transient embryonic neural

specific antigen by the eighth day of embryonic development. Since McCallion and Langman (1964) detected a brain specific antigen on the twelfth day of embryonic development, a minimum of five brain specific antigens have been identified in the chick one of which is a transient embryonic antigen. Friedman and Wenger (1965) using the complement fixation test to measure the accumulation of brain specific proteins identified a minimum of two brain specific antigens. One of these antigens appeared around two days of embryonic development and the second appeared at five and one half days of embryonic development. The greater sensitivity of the complement fixation test compared to precipitin in gel methods precludes a ready comparison between this study and those mentioned above.

Kosinski and Grabar (1967) reported the presence of five brain specific antigens in the adult rat brain. Sviridov and Polyakova (1969) who investigated the immunochemical composition of the rat brain in post-natal ontogeny verified the findings of Kosinski and Grabar (1967). They found that all five brain specific antigens appeared during post-natal development. The first brain specific antigen in rat development was observed in the five day old neonatal, the second at seven days, the third and fourth at 15 to 48 days old and the fifth in the adult brain.

It is worthy of note that neither in the chick nor the rat is there any evidence thus far of a brain specific antigen which appears as early in development as antigen 'e' of the zebrafish. Antigens 'o', 'w', 'v' and 'x' of the zebrafish brain appear sometime after the

morphological development of the brain as do the brain specific antigens of the chick and the rat which have been detected by immuno-electrophoresis.

Seven brain specific antigens have been identified in bovine brain (Hatcher and MacPherson, 1969). Two of these antigens are species specific as well. The other five are held in common with other mammalian brains. The two organ and species specific antigens were only detected with serum which was obtained after prolonged immunization. From this it is conceivable that additional antigenic molecules may be identified in the zebrafish brain if rabbits are immunized for a longer period.

Moore (1965) has described an acidic brain specific protein, S-100, which was originally separated from beef brain. This protein has been found in brains from species of all vertebrate classes by starch gel electrophoresis. The electrophoretic mobility of this protein in all species was high although there were small differences between species. Moore (1969) reported that the S-100 protein appeared at twelve to fifteen days in the post-natal development of the rat, and reached adult levels in approximately two months. It is not known whether this protein is identical with one of the brain specific antigens identified in the rat identified by Kosinski and Grabar (1967) and Sviridov and Polyakova (1969).

Friedman and Wenger (1970) who produced antiserum to beef S-100 protein by inoculation of rabbits with S-100 protein complexed with methylated bovine serum albumin emulsified with Freund's

incomplete adjuvant, detected an antigen in four to six day old chick embryo by the micro-complement fixation test which cross reacted with antiserum to bovine S-100 protein. Adult levels are attained in the 14 to 16 days embryo. The method used to produce antiserum to the S-100 protein suggests that this protein is not highly immunogenic when it is not complexed with another protein such as methylated bovine serum albumin. If this is true then it is unlikely that antibodies are produced to the S-100 protein when whole brain homogenates or supernatants constitute the material of innoculation as in studies of Kosinski and Grabar (1967) and Sviridov and Polyakova (1969). Hence the S-100 protein is probably not one of the brain specific antigens identified in the developmental stages of the zebrafish and the adult zebrafish brain, although its presence in the later developmental stages and the adult zebrafish brain is almost certain.

The first brain specific antigen in chick development has been detected by complement fixation at approximately two days of embryonic development (Friedman and Wenger, 1965). Other brain specific antigens appear in later embryonic stages. In rat development brain specific antigens have been detected at five days post-natal and later stages (Sviridov and Polyakova, 1969). In zebrafish development, however, antigen 'e' appears well before the development of the nervous system. The other brain specific antigens in the zebrafish were detected after the establishment of the central nervous system. With respect to the functioning of a hypothetical brain operon at least one

brain specific antigen is synthesized or made available during cleavage in the zebrafish. In most other species antigens appear at different periods in ontogeny after the gross morphology of the nervous system has been established. Hence macromolecular synthesis in the brain is probably controlled by a multiphasic type of regulation. This further suggests the existence of at least one polyoperon to control brain differentiation.

Apart from identifying some brain specific antigens and providing data on their times of appearance as detected in immunoelectrophoresis, this study may be used as a basis for future research. One of the objects of contemporary embryologists is to describe proteins with respect to times of appearance and disappearance, location, kinetics of synthesis and the qualities present at various stages of development. Therefore having identified at least three brain specific antigens (probably proteins) it is logical to describe these antigens as mentioned above.

Another problem worth investigating is the possible function of antigen 'e'. Since this brain specific antigen is present before the appearance of the central nervous system it is conceivable that it functions in an inductive capacity. The separation of a pure biologically active sample of antigen 'e' and the implantation of a piece of agar gel soaked in an appropriate solution of antigen 'e' into zebrafish blastulae and gastrulae may be illuminating. It might be noted that the continued presence of antigen 'e' suggests that if this molecule functions as an inductor then induction is not its only function;

in addition, it may act as a neural maintenance factor. If antigen 'e' is the primary inductor and if this antigen is present in the unfertilized egg in an inactive form which possesses slightly different physical and chemical properties when compared to the active form then antigen 'e' is coded for by the maternal genome.

The accumulation of data on the brain specific antigens of different species and the familiar picture of relatedness between macromolecules from widely separated species (Jukes, 1966; Dayhoff, 1969) leads one to speculate on the existence of relationships between brain specific antigens of different species of vertebrates and the functional significance of the differences between related molecules.

SUMMARY

1. Rabbit antiserum was produced to adult zebrafish brain by subcutaneous inoculation of a mixture of whole zebrafish brain homogenate with complete Freund's adjuvant. Separate aliquots of antiserum were absorbed with freeze-dried mid-gastrula, 49 hour embryo, adult ovary and adult liver supernatants.

2. Antigen preparations of the eight cell, high blastula, mid-gastrula, 10, 20, 27, 37, 49, 72, and 96 hour embryos and 1, 2, 7 and 28 day post hatching stages were analysed with unabsorbed brain antiserum. Antigen preparations of the eight cell,mid-gastrula, 27, 49 and 96 hour embryos and 7 and 28 day post hatching stages were tested with the absorbed sera.

3. Six antigens designated by the letters 'a', 'b', 'c', 'e', 'j' and '1' occur throughout development, from cleavage to 28 days post hatching without any observable variation.

4. Three antigens, 'h', 'd', and 'f' show changes during development. Antigen 'h' appears to acquire a greater negative charge progressively between mid-gastrula and 49 hours of embryonic development. Antigen 'd' probably becomes associated with another molecule at 1 to 2 days post hatching. Antigen 'f' apparently splits to yield antigens 'f_1', and 'f_2' between 2 and 7 days post hatching.

5. Antigen 'g' was detected in all developmental stages except the 10 hour, 20 hour and 72 hour embryos.

6. Antigens 'm', 'o', 'p', 'q', 'r' and 's' appeared progressively between 37 hours of embryonic development and 28 days post hatching. Antigens 'f_3', 'j', 't', 'm_1', 'r_1', 'w', and 'v' which were detected between 27 hours of embryonic development and 28 days post hatching with the absorbed sera only, also appeared progressively.

7. Two antigens are transient in development. Antigen 'k' was detected only in the high blastula and antigen 'n' only at 72 hours of embryonic development.

8. Adult blood, brain, heart, liver, muscle, ovary, skin, spleen and testis were analysed with unabsorbed and absorbed sera. Three antigens, 'e', 'o' and 'w' are shown to be brain specific. Antigen 'o' probably has a lipid moiety. Antigen 'w' was identified in the adult brain when the latter was analysed with all absorbed sera. Two other antigens, 'x' and 'v_1' which were detected only in the adult brain with liver absorbed brain antiserum, may be brain specific as well.

APPENDIX I

Boric acid - borax buffer, pH 7.8*

800 ml of 0.2M boric acid (12.4 gm per liter) per liter of buffer

200 ml of 0.05M sodium tetraborate.10 H_2O (19.05 gm per liter) per liter of buffer

* Williams and Chase, 1968.

APPENDIX II

Barbital-acetate buffer, ionicity 0.05, pH 8.6*

Sodium Barbital	5.4 gm
Sodium Acetate.3 H_2O	4.3 gm
0.1N hydrochloric acid	58.2 ml
Distilled water to give a total of 1 liter	

* Crowle, 1961.

APPENDIX III

A. Amido black staining solution.*

Amido black	1 gm
Acetic acid 1M	450 ml
Sodium acetate 0.1M	450 ml
Glycerol	100 ml

B. Wash Solution*

2% acetic acid containing 10 to 15% glycerol

* Uriel, 1964.

APPENDIX IV

Light green SF staining solution*

Light green SF	0.5 gm
Distilled water	70 ml
Ethyl alcohol	25 ml
Glacial acetic acid	5 ml

* Brighton, 1967.

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