AN IMMUNOELECTROPHORETIC STUDY OF THE EARLY EMBRYONIC STAGES OF THE ZEBRAFISH

by

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DEDICATION

To my parents

Mr. and Mrs. Sutong Law.

ACKNOWLEDGEMENT

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. R.Z. Hawirko of the Department of Microbiology, University of Manitoba, for providing certain facilities.

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ABSTRACT

The Ovary and six developmental stages of the zebrafish, Brachydanio rerio, namely: the Cleavage, High Blastula, Mid-gastrula, Embryonic-shield (11-12 hour), Optic Cup and Hatching stages were analysed by immunodiffusion and immunoelectrophoresis with unabsorbed and absorbed rabbit antisera to six developmental stages.

A total of thirty two to thirty four antigens was detected in all the stages tested. Twenty three antigens were detected in the Ovary, twenty four in the Cleavage stage, twenty six in the High Blastula and Mid-gastrula stages, twenty five in the Embryonic-shield and Optic Cup stages and twenty two in the Hatching stage. Altogether, twenty seven antigens were detected in the prehatching-stages. Of these, fourteen were common to the Hatching stage.

One antigen was unique to the Ovary stage, four new antigens appeared at the Cleavage stage, one transient antigen was detected in the High Blastula and Mid-gastrula stages and seven antigens first made their appearance at the Hatching stage.

Only one prehatching-stage antigen was detected with the absorbed sera. The present investigation did not demonstrate any stage-specific antigens in the prehatching stages.

INTRODUCTION

It is generally believed that the morphology of the embryo is determined by both structural and functional macromolecules synthesized as a result of differential gene function. Consequently, a qualitative and a quantitative determination of the embryonic chemical composition is of importance in the study of development. With modern techniques and equipment the detailed chemical composition of cells and subcellular particles has been elucidated.

Transcription into various RNA types in the early development of the amphibian and echinoderm embryos has been reviewed by Davidson (1968). Kafiani (1970) similarly has discussed the transcription process for the teleost embryo (loach).

Protein synthesis as reflecting the differential gene activity in early development at the translational level has also been studied. Twenty-three proteins have been separated and identified by enzymatic analysis during the course of development of a mollusc Ilyanassa (Morrill, 1961; Morrill and Norris, 1965).

Electrophoretic analysis has been used in the study of hydrolytic enzymes in embryos of the mollusc Limnaea palustris (Morrill et al. 1964), and in the study of the electrophoretically mobile phosphatase band pattern of the embryo of the pulmonate snail, Physa acuta (Morrill, 1965). Data on the distribution of lactate dehydrogenase isomers in the embryo of the prosobranch gastropod Argobuccinum oregonense during blastulation and subsequent differentiation were obtained by Goldberg and Cather (1963). Protein synthesis prior to organogenesis has also been reported for the molluscan embryo by Collier (1961). Protein synthesis in the development of Xenopus laevis has also been reviewed by Deuchar (1972).

Since macromolecules generally are antigenic, the study of antigens of a particular embryonic stage as compared to another, may reveal stage-specific stable or changing antigenic patterns. Immunochemical analysis, particularly immunodiffusion and immunoelectrophoresis, are two very sensitive techniques used for the detection of the antigenic patterns.

Antigenic patterns in embryonic development have been studied by a number of investigators. The early work on the appearance of antigens and proteins during development has been reviewed by Cooper (1946, 1948) Ebert (1952) and Tyler (1955).

Attempts have been made to demonstrate organ-specificity of antigens in development. Brain specific antigens have been studied in the chick by Schalekamp (1961), McCallion and Langman (1964), McCallion and Trott (1964, 1965); in the hamster by La Velle and Van Alten (1969); in the rat by Sviridov and Polyakova (1969) and in the teleost by Laale and Singh (1972).

Other organ antigens in ontogeny, such as: Lens antigens (Ten Cate and Van Doorenmaalen, 1950; Langman, 1959a, 1959b; Maisel and Langman, 1961; Barabanov, 1966a, 1966b, 1967; Kirzon et al, 1969), skin antigens (Ben-Or and Bell, 1965), mammary gland antigens (Shchekolodkin, 1967), kidney antigens (Okada and Sato, 1963, Lahti and Saxen, 1966, Linder, 1969), spleen antigens (Maiskii and Shchekolodkin, 1967), liver antigens (Croisille, 1960; Mutolo et al, 1965; Raftell and Perlmann, 1968) and serum albumin antigens (Zaccheo and Grossi, 1967; Afanas'eva, 1966; Tatarinov and Afanas'eva, 1965, Tatarinov et al, 1967; Monjour and Mariage, 1969) have been demonstrated.

In most of these studies organ-specific antigens have not been observed prior to the onset of organogenesis and histogenesis. Some exceptions however can be cited. Schechtman (1948) and Ebert (1950) demonstrated the presence of adult chick brain, heart and spleen antigens in the blastoderm. Immunologically reactive groups specific for cardiac myosin have been reported to be restricted to two bilateral regions of the chick embryo at the head process stage (Ebert, 1953; Ebert, et al 1955). Singh and Laale (1972) recently discovered a brain specific antigen in the zebrafish embryo which occurs as early as the cleavage stage.

Antisera against early stages are needed for the analysis of early stage-antigenic profiles. Any early embryonic antigen would be directly or indirectly related to the early ontogenetic mechanism. Many investigations have been directed toward the elucidation of the early antigenic patterns in embryonic development.

Telfer (1954) reported that an antigen in the adult female blood of Cecropia silkworm is present in the yolk of the unfertilized egg. This antigen was not detected in the larvae and the adult males.

Studying sea urchin hybrid embryos, Harding et al, (1954) reported that the "paternal" antigens appear before the morphological traits can be observed.

Perlmann and Gustafson (1948) found common antigens to be present from the fertilized eggs to the 48 hour pluteus stage of the sea urchin. Absorption tests also revealed that some antigens that are present in the 48 hour embryo are not detectable in the early sea urchin stages tested (unfertilized eggs, 4 hour - 12 hour embryos). None of the antigens detectable in the eggs and early embryos appear to be lost in the pluteus larva. By using the Ouchterlony method, Perlmann (1953) reported

that the extracts of different developmental stages of the sea urchin possessed mostly common antigens of similar concentration. Subsequently Westin, Perlmann and Perlmann (1967) combining radioisotope tracing techniques with immunoelectrophoresis showed that antigens are synthesized at different early developmental stages.

Clayton (1953) and Spar (1953) have demonstrated an increasingly antigenic complexity in the development of the amphibian embryo.

Schechtman (1947) has studied chick serum-like-antigens in the egg yolk and in the extract of embryos at various developmental stages using antisera against the euglobulin, pseudoglobulin, albumin fractions and whole serum. He also demonstrated that the antigens from the extracts of the early embryos are common to those of the yolk (vitelloid). Non-vitelloid antigens are first detected in the blood of 5-day chick embryos (Nace and Schechtman, 1948).

Although work has been done on the biochemical differentiation of invertebrate, amphibian and chick embryos with immunological methods, similar investigations on the early development of the teleost embryos are rare. A comparative study of the antigenic structure of oocytes and developing embryos of the Black Sea garfish and the sevruga fish has been made by Apekin (1964, 1965).

The purpose of this investigation was to study the antigenic patterns of the early developmental stages of the zebrafish, Brachydanio rerio and to determine antigenic changes in the course of development.

MATERIAL AND METHODS

I. Maintenance of fish stock

Adult male and female zebrafish, Brachydanio rerio obtained from the Hudson Bay Co. Ltd., were kept separately at 24°C in dechlorinated water. A variety of preserved fish foods were used to secure optimal health. The photoperiod was a 14 : 10 hours alternate light-dark cycle.

II. Collection of developmental stages

Two female and three male adult fish were placed in each of three spawning tanks. Eggs were subsequently removed by siphoning. The eggs were washed on a screen (mesh-hole diameter slightly less than that of the egg) with dechlorinated water and with phosphate buffer solution (0.02M, pH 6.6) (Appendix I). Eggs in lots of 30 to 50 were transferred into Petri-dishes containing buffer and allowed to incubate at 26°C . Stages according to Hisaoka and Battle (1958) namely: Cleavage, High Blastula, Mid-gastrula, Embryonic-shield (11-12 hours of incubation at 26°C), Optic Cup and Hatching stages were collected. The Cleavage and High Blastula stages were collected on ice to prevent further development. The eggs were subsequently stored at -25°C . The ovary stage was obtained by dissection of females under precooled conditions.

III. Immunological Techniques

A. Preparation of Antigens (stage-extracts)

Stage-specific embryos were homogenized in phosphate buffer solution at 4°C with a 5-ml capacity glass Porter's homogenizer.

Homogenates were centrifuged at 3000 x g for 30 minutes at -2°C and the precipitate discarded. The protein concentration of the supernatant antigens of the different stages was determined by the method of Lowry et al (1951). Antigens were stored at -25°C in aliquots of 50-75 microlitre quantities.

B. Injection of antigens

Stage-specific supernatants were diluted with phosphate buffer to 0.5 ml and mixed with an equal volume of complete Freund's adjuvant (Difco Laboratories). A 0.25 ml of the resulting antigen adjuvant emulsion was injected subcutaneously at each of four positions dorsally. Three male, nonimmunized New Zealand albino rabbits obtained from the Canadian Breeding Laboratories, Quebec, were injected for each stage specific antigen extract. Three injections per animal were administered over a period of 37 days according to the method of McCallion and Trott (1964).

C. Production of rabbit antisera to zebrafish embryonic stages

Preimmune serum from each rabbit was obtained prior to immunization. Ten days after the last injection the rabbit was bled by cutting the marginal ear vein. The blood was incubated at 36°C for 1-1½ hours, refrigerated overnight and centrifuged at 3000 x g for 30 minutes at 0°c. The supernatant antiserum was stored in aliquots containing a concentration of 0.1 per cent methiolate as a preservative agent. Antisera against the same stage were pooled prior to testing.

D. Preparation of agarose gel and application to slides

Slides were washed with soap and rinsed in running distilled water. The clean slides were dried in an oven at 66°C. A solution of

0.3 per cent agar was prepared with distilled water on a boiling water bath. The slides were immersed in the hot agar solution and removed for drying. The agar coating serves as a supporting layer for diffusion and electrophoresis. A 1 per cent agarose solution was prepared in a 1:1 mixture of distilled water and Sørenson phosphate buffer (see Appendix II) at pH 7.0. The agarose solution was heated on a boiling water bath. Two ml. of agarose solution was applied with a 2-ml blow-out serological pipette to each of the agar-coated slides on a leveling board. Antisera troughs and antigen wells were cut manually by means of a double-bladed scalpel and a well-making pipette with an internal pore size diameter of 1 mm(Figure 1).

E. Immunoelectrophoresis

Antigen samples were thawed and applied to the antigen wells with a capillary tube. An adaptation of Grabar's and Williams' immunoelectrophoretic analysis to microscope slides (Scheidegger, 1955) was used. A Shandon electrophoretic tank (Figure 2) containing in each of two buffer compartments 500 mls of Sørenson phosphate buffer solution pH 7.0 was employed.

Electrophoresis was carried out with a Gelman power supply at 4°C± with fresh precooled buffer. Agarose gel hanging strips served as linkages between the slides and the phosphate buffer compartments. Voltage was maintained at a constant level of 80 volts for a duration of two hours. Six slides were run for each experiment. New buffer was employed in each electrophoretic run. An average current flow of 8.5 milliamperes per slide was obtained. After electrophoresis, the gels in the antiserum troughs were removed and approximately 100 microlitres of antiserum was applied per trough. Slides were placed in Petri dishes

Figure 1. Instruments for making antigen wells and troughs for micro-immunoelectrophoresis and immunodiffusion.

Figure 2. Electrophoretic tank.

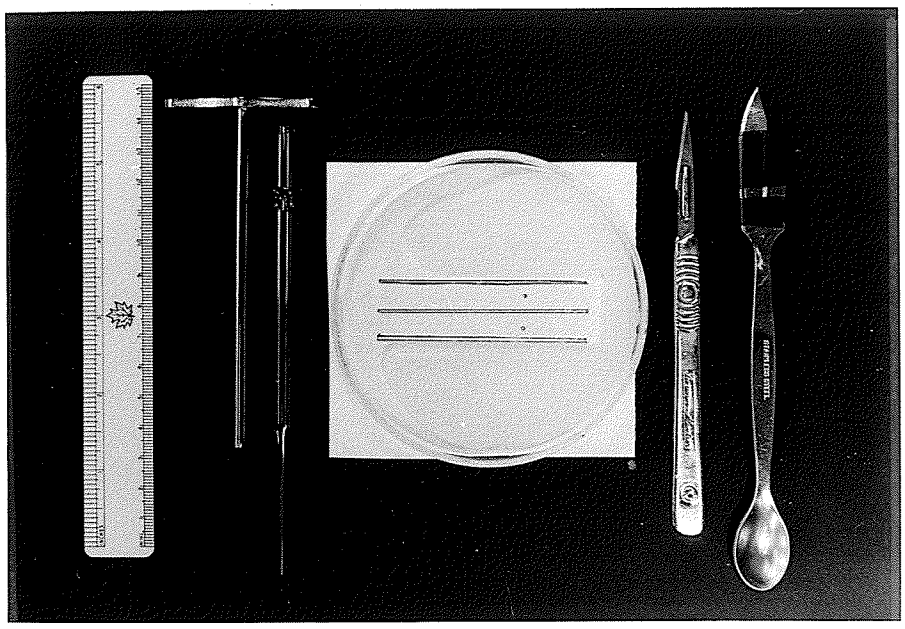


Figure 1.

Figure 2.

kept in a moist chamber at 26°C± for 48 hours for incubation.

F. Immunodiffusion

The gel used in immunodiffusion tests was prepared in the same manner as that for immunoelectrophoresis. The gel was cut as indicated (Figures 3 and 7) with a large center well for the absorbed antiserum surrounded by eight smaller antigen wells each assigned for a particular antigen.

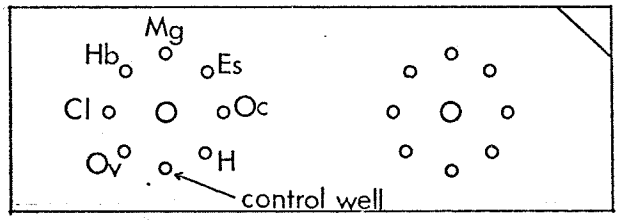


Figure 3 Immunodiffusion gel pattern
The control wells contain stage-specific antigens homologous to the antiserum used.

Plastic caps containing water were placed within the Petri dishes in order to prevent desiccation. The immunodiffusion slides were incubated at 26°C± for 48 hours.

G. Absorption of antisera

The antisera against 'Cl', 'Hb', 'Mg', 'Es', 'Oc' and the 'H' stages were absorbed with six lyophilized stage extracts, namely, 'Cl', 'Hb', 'Mg', 'Es', 'Oc' and 'H' (see Appendix III). The weights of each of the lyophilized stage extracts used in absorbing each of the antisera were recorded (Appendix IV). The absorption was done by placing weighed lyophilized stage-extract in a small serological test-tube to which 1 ml of undiluted anti-stage serum was added. After

dissolving the lyophilized stage-extracts the mixture was incubated at 37°C for two hours. The mixture subsequently was kept at 4°C overnight and then centrifuged at 4,000 x g for 30 minutes. The supernatant was removed and used for the second absorption. The same procedure was repeated for the second absorption. The fully absorbed antiserum was secured from the final supernatant (see Appendix III for abreviations employed for absorbed and unabsorbed antisera).

H. Washing of gels

Incubated slides from immunoelectrophoretic and immunodiffusion experiments were transferred to saline solution (0.85 per cent NaCl solution) and kept at 0°C for one week. Excess non-precipitated substances were removed with saline solution changed three times daily over a period of three days. The slides were washed with running distilled water to remove excess salt and dried at 37°C.

IV. Recording of the results

All the slides were photographed and drawings made independantly. Incident white light projected unto the under surface of the slides was used to facilitate the identification of bands.

A. Photography

Each slide without grid (Figure 4) was photographed three times with different shutter-speeds and with grid (Figure 5) one time. In the latter case, the slide to be photgraphed was superimposed upon a microscope slide with a standard grid. The best of three photographs taken from one slide was used in the final record as the representative photograph for that particular slide. All photographs were taken with F.P. 4 film using a blue filter.

Figure 4. Photographic record of a precipitin reaction without grid.

Figure 5. Photographic record of a precipitin reaction with grid.

Figure 6. Original 1 x scale on the microscope slide, the 2 x scale for the final combinatory line drawings and the 4 x scale of the preliminary line drawings.

Figure 7. Photographic record of an immunodiffusion absorption test.

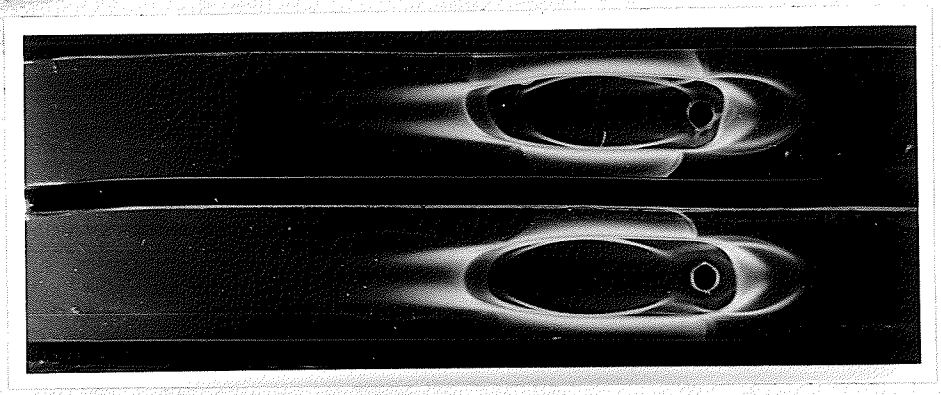


Figure 4.

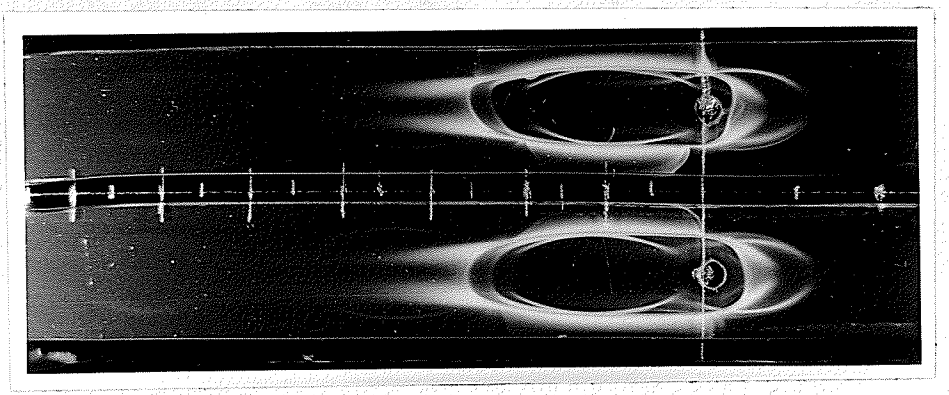


Figure 5.

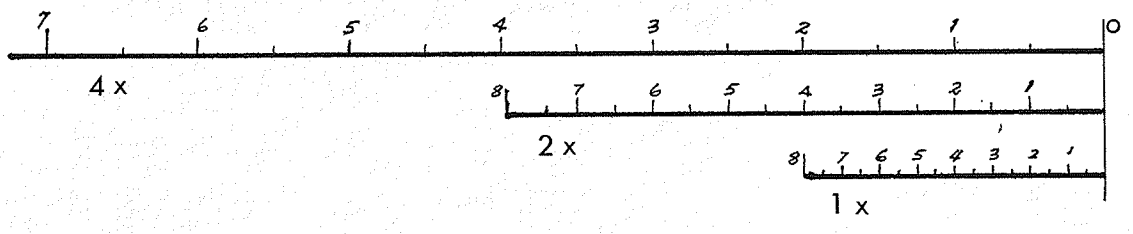


Figure 6.

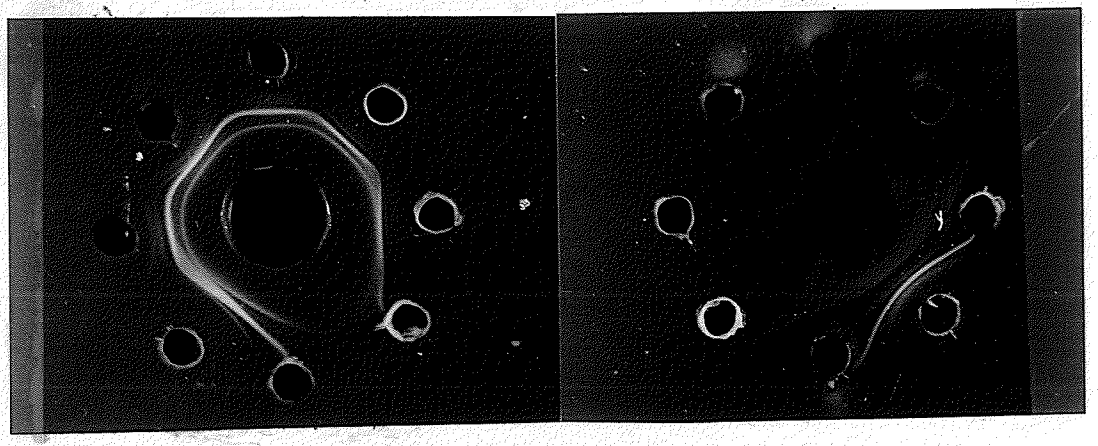


Figure 7.

B. Line drawings

Line drawings were made independently of the photographic records from a direct observation of the band pattern over a gridded slide. Preliminary drawings of the precipitin bands were recorded at 4 x the scale of the grid (Figure 6). All the line drawings were checked and approved by an independent observer. Since each precipitin reaction was repeated six times (twelve times for the control) and since all of these did not show identical precipitin patterns, a combination drawing (2 x scale) of these homologous line patterns was produced as a representative final drawing.

V. Description and classification of precipitin bands

A band is said to occupy an anterior position when it displays a high electrophoretic mobility; and a posterior position when it displays a lower electrophoretic mobility. It is said to occupy a 'lateral' position when its diffusion rate is high (i.e. when the band occupies a position near to the antiserum trough); and a 'medial' postion when its diffusion rate is low (i.e. when the band occupies a position away from the antiserum trough). The 'anterior' end of a precipitin band is the electropositive end of the precipitin arch while the 'posterior' end of the band is the electronegative or anodic end.

All the precipitin bands are named in alphabetical order from (a) to (q) commencing at the cathodic end near the antiserum trough. Faint bands like (r), (s), and (t) are also named from the cathodic end. Bands found only in the hatching stage are named in an arbitrary order. The letters of the alphabet are used to designate specific bands arising at particular electrophoretic mobilities.

The arabic number at the right lower corner of each letter denotes

the particular antiserum used (Table I).

The "prime" symbol used at the upper right corner of a letter denotes a close relationship to another band(s) signified with the same letter. The relationship that may exist between the bands is illustrated as follows:-

1. Band Continuity

Bands (c) and (c') appear in some instances as a single continuous band with two humps (Figure 11b). A similar continuity is displayed by the bands (u'), (u'') and (u''') (Figure 8b).

2. Band dichotomy

a) Non-terminal dichotomy

Band (b') is seen with its posterior arch separated from band (b) (Figure 9c).

b) Terminal dichotomy

The bands (h') and (h'') are only discernible by their posterior dichotomy, whereas the bands (1') and (1'') are discernible only by their anterior dichotomy.

The letter following the arabic number at the lower right corner implies that the band is obtained from an absorption test. For example, (d_{2a}) represents band (d) obtained with stage-absorbed Anti-Hb serum.

Table I

Symbols used for unabsorbed and absorbed anti-stage sera

Anti-stage sera	Anti-Cl	Anti-Hb	Anti-Mg	Anti-Es	Anti-Oc	Anti-H
Precipitin band detected with unabsorbed sera	a_1	a_2	a_3	a_4	a_5	a_6
Precipitin band detected with absorbed sera	m_{1a}	m_{2a}	m_{3a}	m_{4a}	m_{5a}	m_{6a}

RESULTS

Zebrafish, Brachydanio rerio antigens from seven developmental stages, namely: Ovary (Ov), Cleavage (Cl), High Blastula (Hb), Mid-gastrula (Mg), Embryonic-shield (Es), Optic-Cup (Oc) and Hatching (H) stages were analysed against the following specific anti-stage sera: Anti-Cl serum, pooled Anti-Hb serum, pooled Anti-Mg serum, pooled Anti-Es serum, pooled Anti-Oc serum and Anti-H serum.

The Ovary was chosen as a representative adult organ as well as a representative pre-fertilization stage. The ovary contains a wide spectrum of ova at different maturation stages.

Analysis of embryonic and hatching stages with anti-stage sera

I. Immunoelectrophoretic analysis (IEA) with unabsorbed rabbit anti-stage sera

A. Stages analysed with Anti-Cl serum

A resumé of the results with reference to stage antigens analysed with unabsorbed Anti-Cl serum is given in Table II and graphically presented in Figures 8a - 8g.

Eighteen bands can be identified with unabsorbed Anti-Cl serum. The bands are: (a_1), (b_1), (c_1), (c_1'), (d_1), (e_1), (g_1), (h_1'), (h_1''), (i_1), (k_1), (l_1'), (l_1''), (m_1), (n_1), (o_1), (p_1), and (s_1).

Precipitin band (a_1) present in the 'Ov' and the 'H' stages emerges from the posterior half of band (c_1) and extends its arch to the posterior extremity of the band pattern (Figure 8a). The bands (a_1) and (b_1) are the only two bands posterior to (c_1). Occasionally only a single band can be observed in the region of the precipitin bands (a_1) and (b_1). This is true for the 'Cl', 'Hb', 'Mg', 'Es',

Table II

Analysis of the precipitin band patterns of the early zebrafish developmental stages with unabsorbed Anti-Cl serum. Stages analysed: 'Ov', 'Cl', 'Hb', 'Mg', 'Es', 'Oc' and 'H'

Precipitin bands	a_1	b_1	b'_1	c_1	c'_1	d_1	e_1	f_1	g_1	h'_1	h''_1	i_1	j_1	k_1	l'_1	l''_1	m_1	n_1	n'_1	o_1	p_1	q_1	r_1	s_1	t_1
Embryonic stages																									
Ov	+	+		+	+	+	+			+	+	+		+	+	+	+	+		+	+			+	
Cl	←+→			+	+	+			+	+	+	+		+	+	+	+	+							
Hb	←+→			+	+	±			+	+	+	+		+	+	+	+	+		±	±				
Mg	←+→			+	+				+	+	+	+		←+→		+	+	+		±	±			+	
Es	←+→			+	+				+	←+→		+		←+→		+	+	+		+	±			+	
Oc	←+→			+	+				+	+	+	+		←+→		+	+	+		+	±				
H	←+→									+	+	+		←+→		+	+	+		+	±				

Symbols used:
+ Positive precipitin reaction
± Faint precipitin reaction
←+→ Precipitation could be either of two identifiable bands

Figure 8. Immunoelectrophoretic precipitin line patterns of the seven developmental stages of the zebrafish, Brachydanio rerio, analysed with unabsorbed Anti-C1 serum.

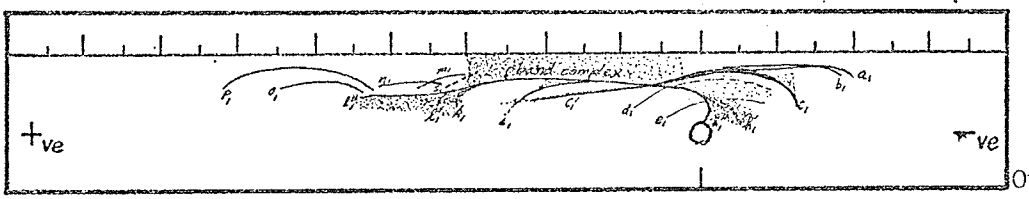


Fig. 8a Ov

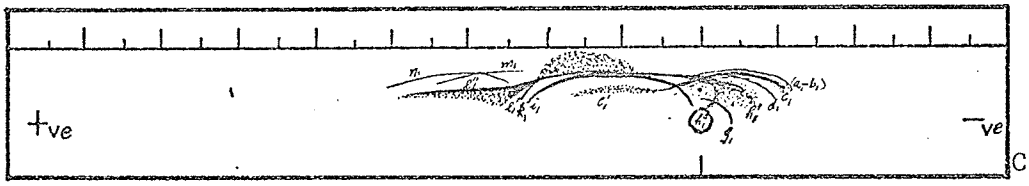


Fig. 8b C1

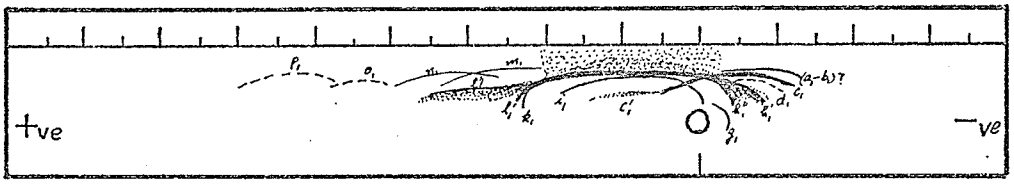


Fig. 8c Hb

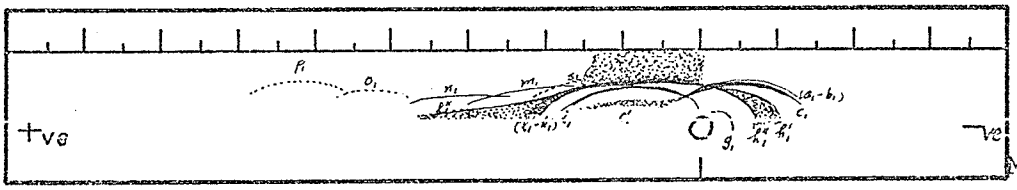


Fig. 8d Mg

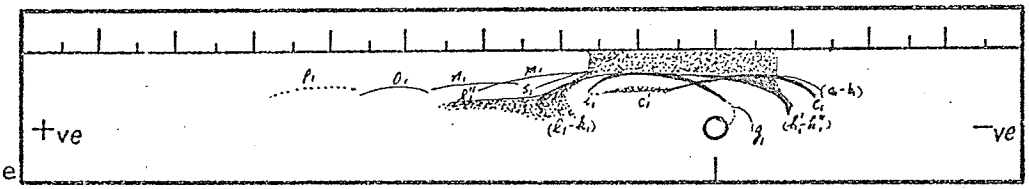


Fig. 8e Es

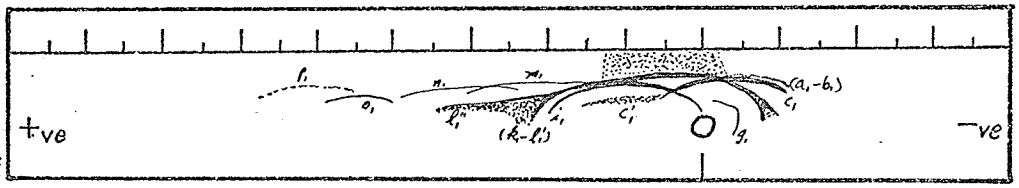


Fig. 8f Oc

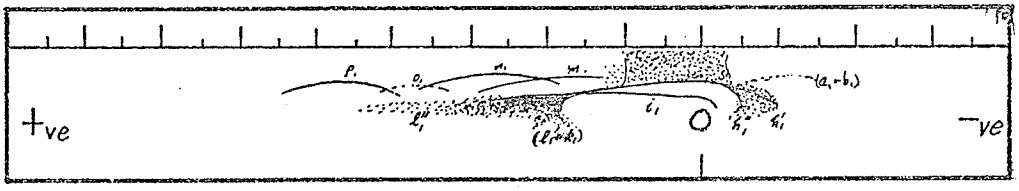
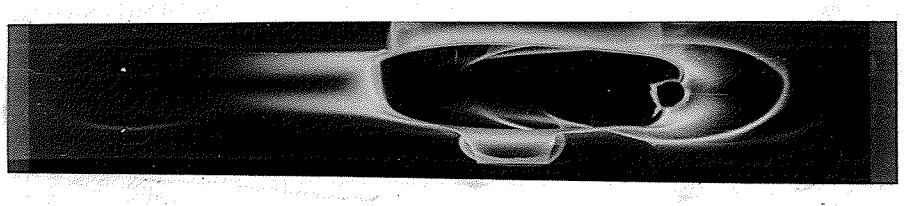


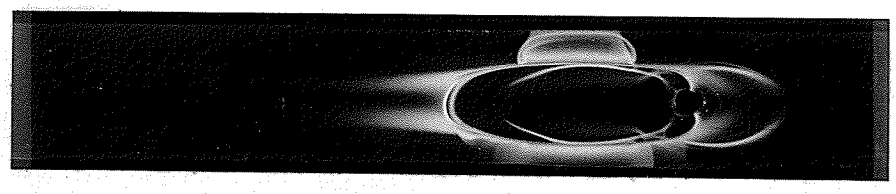
Fig. 8g H

Fig. 8a



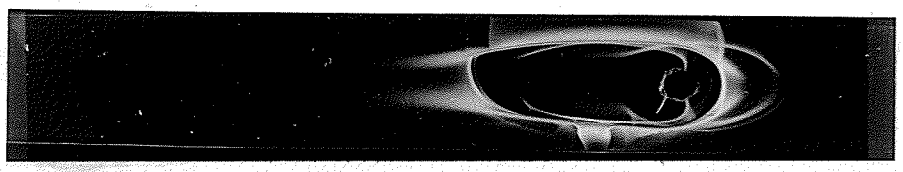
Ov

Fig. 8b



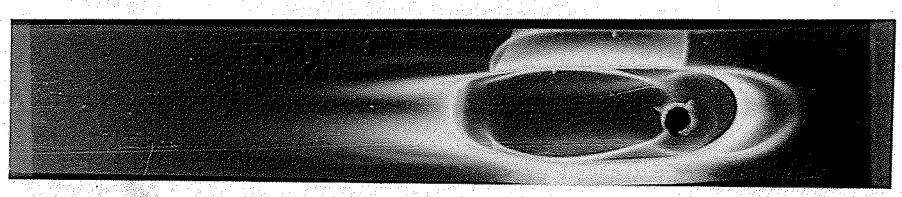
Cl

Fig. 8c



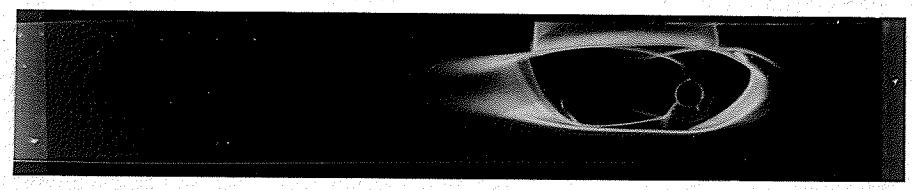
Hb

Fig. 8d



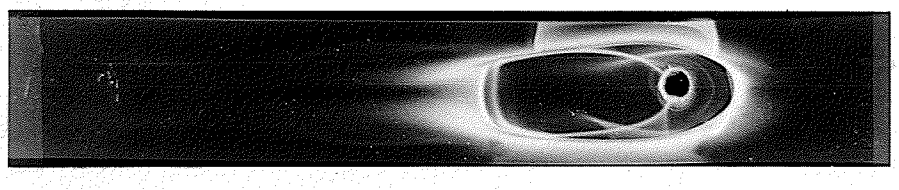
Mg

Fig. 8e



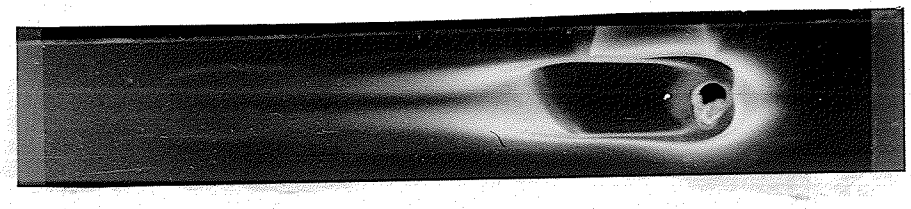
Es

Fig. 8f



Oc

Fig. 8g



H

'Oc' and 'H' stages (Figures 8b - 8f).

The (b_1) band is observed to arise near the junction of band (c_1) and band (c_1') and extends its arch to the posterior extremity. It has a higher diffusion rate than any other band in this region and consequently occupies the most lateral position from the antigen well.

Band (c_1) arches from the anterior side of the antigen well to the posterior side. Band (c_1') having a slower diffusion rate than (i_1) occurs medially to the latter. Both (i_1) and (c_1') display similar anterior electrophoretic mobilities. The posterior end of (c_1') in most cases can be seen to be continuous with the anterior end of (c_1) forming a single band with two humps. Both (c_1) and (c_1') are identified in all developmental stages except the 'H' stage.

Band (d_1) positioned medially to (c_1) is prominent in the 'Ov' stage, less so in the 'Cl', and almost invisible in the 'Hb' stage.

The (e_1) band is found in the 'Ov' stage only and is distinquished from band (d_1) by its location medial to the latter (Figure 8a).

A half band (g_1) with an electrophoretic mobility and diffusion rate close to zero is observed as a cresent shaped precipitin band near the posterior side of the antigen well. This band is not detected in the 'Ov' and 'H' stages.

Band (h_1), lateral to (i_1), shows a bifurcation at its posterior extremity in the 'Ov', 'Cl', 'Hb', 'Mg', 'Oc', and 'H' stages. Thus (h_1) consists of two closely related precipitin bands (h_1') and (h_1''). This bifurcation is not as clearly evident in the 'Es' stage (Figure 8e).

Band (i_1), found in all stages, displays a rather consistent electrophoretic mobility. Its posterior end arises at the level of the antigen well.

A 'band complex' originating from the (h_1) precipitin arch may be seen to extend anteriorly giving rise by subsequent flaring to bands (k_1), ($1_1'$) and ($1_1''$) (Figures 8a - 8g). The band (k_1) is distinct from the band ($1_1'$) in the 'Ov', 'Cl' and 'Hb' stages (Figures 8a - 8c). This distinction is not so evident in the 'Mg', 'Es', 'Oc' and 'H' stages (Figures 8d - 8g) where only one band may be discerned. Non-specific precipitation at the posterior end of band ($1_1''$) where it joins with band ($1_1'$), may explain this loss of distinction.

Band (m_1) occurs lateral to the posterior margin of the 'band complex'. It has a slightly higher anterior electrophoretic mobility than band (s_1) immediately following it (Figure 8e).

Band (n_1) shows an electrophoretic mobility intermediate between that of bands (m_1) and (o_1), and occupies a position lateral to precipitin line ($1_1''$). Bands ($1_1''$), (m_1) and (n_1) are observed in all developmental stages with Anti-Cl serum.

Band (o_1) is absent from the 'Cl' stage (Figure 8b). It appears as a faint band at the 'Hb' stage and increases in intensity during the subsequent developmental stages. The electrophoretic mobility of this band is somewhat retarded in the 'H' stage (Figure 8g).

Precipitin band (p_1) is not observed in the 'Cl' stage (Figure 8b). Subsequent to its appearance at the 'Hb' stage, it remains as a faint band anteriorly in the band pattern throughout the various developmental stages (Figures 8a - 8g).

Band (s_1) immediately following band (m_1), is identified in the 'Ov', 'Mg' and 'Es' stages. Its posterior half is usually masked by a heavy nonspecific precipitation.

When the 'H' stage is tested against Anti-Cl serum, the band pattern is quite different from that of the other stages tested (Figure 8g). By comparison, the (o_1) and (p_1) bands of the 'H' stage exhibit some retardation in anterior electrophoretic mobilities; ($1''_1$) an increased anterior electrophoretic mobility, and (i_1) an increase in both anterior as well as posterior electrophoretic mobility. The bands (c_1), (c'_1), (d_1), (e_1), (g_1) and (k_1) are not observed in the 'H' stage analysed with Anti-Cl serum.

B. Stages analysed with pooled Anti-Hb serum

A resumé of the results with reference to stage antigens analysed with unabsorbed Anti-Hb serum is given in Table III and graphically presented in Figures 9a - 9g.

Twenty four bands can be discerned with pooled Anti-Hb serum. The bands are: (a_2), (b_2), (b'_2), (c_2), (c'_2), (d_2), (e_2), (f_2), (g_2), (h'_2), (h''_2), (i_2), (j_2), (k_2), (l'_2), (l''_2), (m_2), (n_2), (o_2), (p_2), (r_2), (r'_2), (s_2) and (t_2).

Precipitin band (a_2) arising from band (c_2) in the 'Ov' and 'Hb' stages is not detectable in the 'Cl', 'Mg', 'Es' and 'Oc' stages.

Band (b_2) is present in all stages tested except the 'H' stage. A single diffuse band occupies the position of (a_2) and (b_2) at the 'H' stage (Figure 8g).

A posterior dichotomy of band (b_2) gives rise to band (b'_2). This new band is detected in the 'Ov', 'Cl', 'Hb', 'Mg', 'Es' and 'Oc' stages (Figures 9a - 9f).

No change is observed in the bands (c_2) and (c'_2) with the pooled Anti-Hb serum. These bands occur consistently throughout the early developmental stages and disappear at the 'H' stage.

Table III

Analysis of the precipitin band patterns of the early zebrafish developmental stages with pooled unabsorbed Anti-Hb serum. Stages analysed: 'Ov', 'Cl', 'Hb', 'Mg', 'Es', 'Oc' and 'H'

Precipitin bands	a_2	b_2	b'_2	c_2	c'_2	d_2	e_2	f_2	g_2	h'_2	h''_2	i_2	j_2	k_2	l'_2	l''_2	m_2	n_2	n'_2	o_2	p_2	q_2	r_2	r'_2	s_2	t_2
Embryonic stages																										
Ov	+	+	+	+	+	+	+			+	+	+	+	+	+	+	+			+	+					
Cl		+	+	+	+	+		+	+	+	+	+	±	+	+	+	+	+			+		+			+
Hb	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+		±	+		+	+		
Mg		+	+	+	+	+		+	+	+	+	+		+	+	+	+	+		±	+		+	±	+	±
Es		+	+	+	+	+		+	+	+	+	+	±	+	+	+	+	+		±	+		+		+	+
Oc		+	+	+	+				+	+	+	+	+	+	+	+	+	+		+	+				?	+
H	←±→									+	+	+		+	+	+	+	+		+	+				+	

Symbols used:

+	Positive precipitin reaction
±	Faint precipitin reaction
←+→	Precipitation could be either of two identifiable bands
?	Extremely faint band

Figure 9. Immunoelectrophoretic precipitin line patterns of seven developmental stages of the zebrafish, Brachydanio rerio, analysed with unabsorbed pooled Anti-Hb sera.

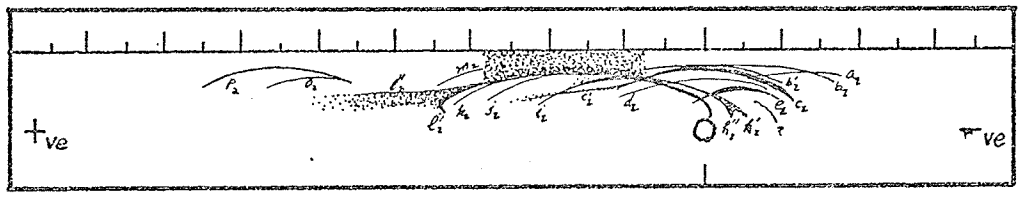


Fig. 9a Ov

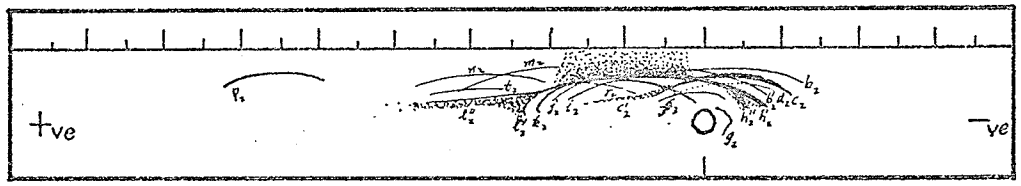


Fig. 9b Cl

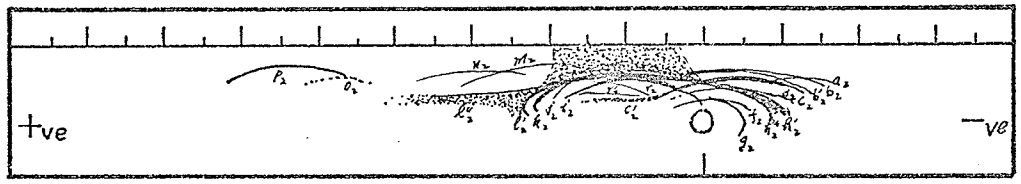


Fig. 9c Hb

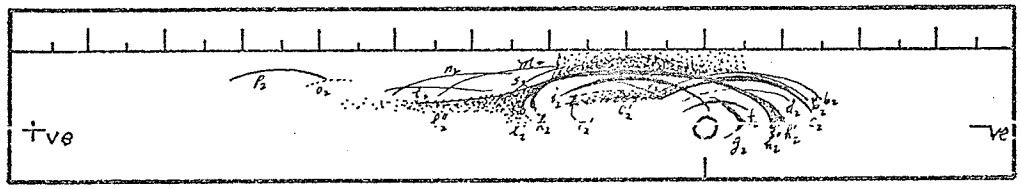


Fig. 9d Mg

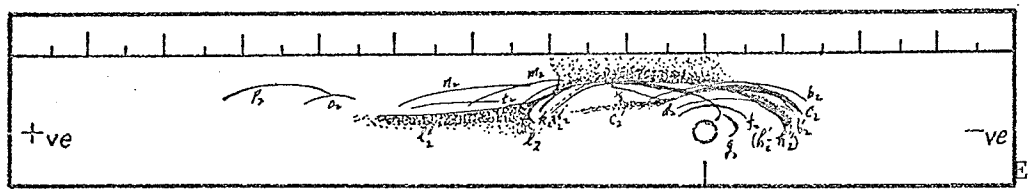


Fig. 9e Es

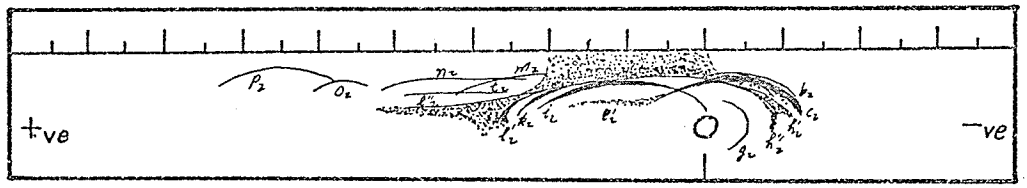


Fig. 9f Oc

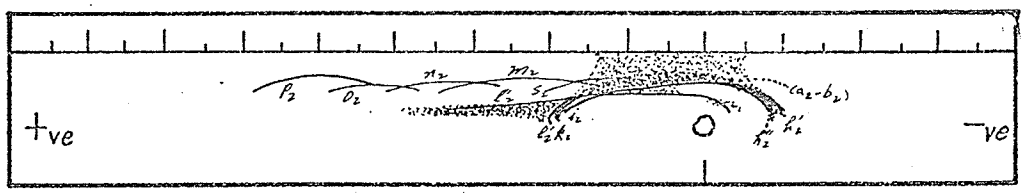
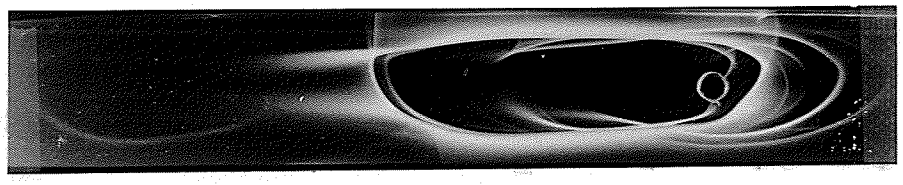


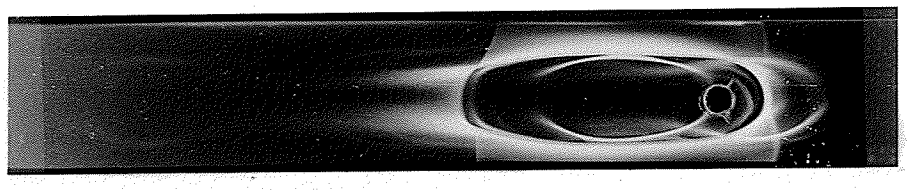
Fig. 9g H

Fig. 9a



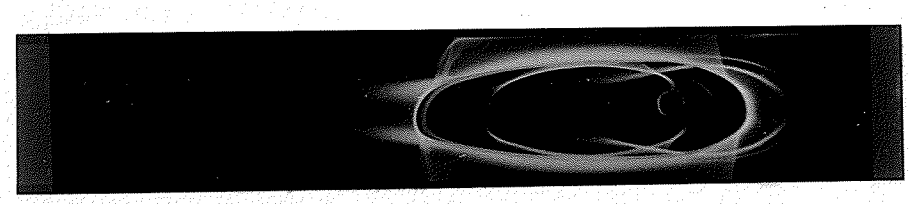
Ov

Fig. 9b



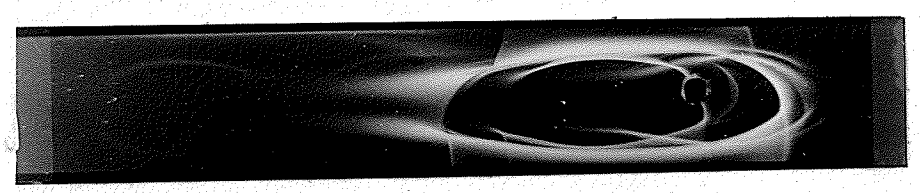
Cl

Fig. 9c



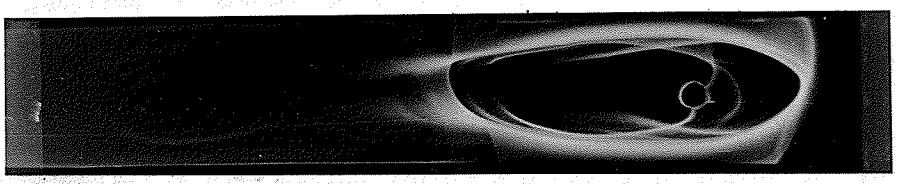
Hb

Fig. 9d



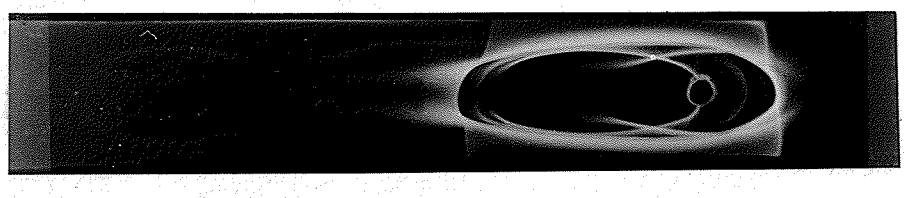
Mg

Fig. 9e



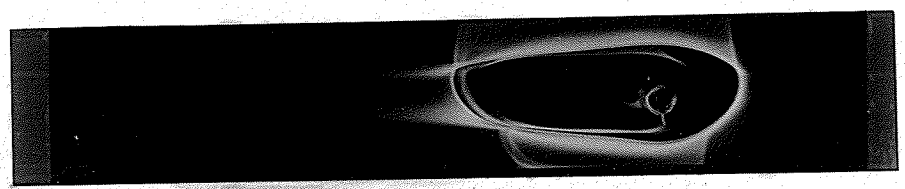
Es

Fig. 9f



Oc

Fig. 9g



H

Band (d_2) is present at all stages except the 'Oc' and 'H' stages, and band (e_2) appears at the 'Ov' stage only.

A new band (f_2) occurs medial to (e_2) in the 'Cl', 'Hb', 'Mg' and 'Es' stages (Figures 9b - 9e).

Band (g_2) is not detected in the 'Ov' and 'H' stages (Figures 9a - 9g). The posterior branches of the 'band complex', i.e. (h_2') and (h_2''), as well as band (i_2) occur at all the developmental stages tested.

The anterior arch of a new band (j_2) arises from the anterior part of the 'band complex' between (i_2) and (k_2). It is found in all stages tested except the 'Mg' and 'H' stages. It is not clearly visible in the 'Cl' and 'Es' stages (Figures 9b and 9e). In general, band (j_2) is weaker than band (k_2).

The anterior branches of the 'band complex', i.e., (k_2), (l_2') and (l_2''), as well as band (m_2) occur at all stages. These bands exhibit greater anterior electrophoretic mobilities in the 'Ov' stage when compared to the subsequent stages.

Precipitin line (n_2) is present in all stages except the 'Ov' stage (Figure 9a). Band (o_2) is not observed in the 'Cl' stage. It appears as a faint band in the 'Hb' and 'Mg' stages, becomes more pronounced in the 'Es' and 'Oc' stages and is clearly visible in the 'Ov' and the 'H' stages (Figures 9a, 9c-9g).

A slight decrease in the electrophoretic mobility of the band (p_2) at the 'H' stage is observed upon comparison with the other stages tested.

Band (r_2) appears as a new band medial to (i_2) and lateral to (c_2') in the 'Cl', 'Hb', 'Mg' and 'Es' stages. It is not present in the 'Ov', 'Oc' and 'H' stages. Another new band (r_2') detected in the 'Hb'

stage and occasionally in the 'Mg' stage occupies a position between (r_2) and (c_2'). The precipitin band (r_2) occurs posterio-laterally to band (r_2').

Precipitin line (s_2), intermediate between (m_2) and (l_2''), occurs in the 'Mg', 'Es' and 'H' stages. A heavy non-specific precipitation in this area makes it difficult to determine the presence of band (s_2) in the 'Oc' stage.

Band (t_2) appears as a strait line medially to band (n_2). It is found in the 'Cl', 'Mg', 'Es' and 'Oc' stages as a very faint band, and is not observed in any other stages tested.

C. Stages analysed with pooled Anti-Mg serum

A resumé of the results with reference to stage antigens analysed with unabsorbed Anti-Mg serum is given in Table IV and graphically presented in Figures 10a - 10g. Twenty four bands can be identified with pooled Anti-Mg serum. The bands are: (a_3), (b_3), (c_3), (c_3'), (d_3), (e_3), (f_3), (g_3), (h_3'), (h_3''), (i_3), (j_3), (k_3), (l_3'), (l_3''), (m_3), (n_3), (n_3'), (o_3), (p_3), (q_3), (r_3) and (t_3).

Band (a_3) is present in the 'Ov', 'Cl' and 'Hb' stages only. In the latter the precipitin line is very faint. Band (b_3) is not observed in the 'H' stage.

Bands (c_3) and (c_3') appear in all the stages except the 'H' stage. Precipitin line (d_3) is present in all stages except the 'Oc' and 'H' stages. Precipitin line (e_3) is found in the 'Ov' stage only.

The (e_3) band in the 'Ov' stage is distinguished from the (f_3) band in the 'Cl', 'Hb', 'Mg', 'Es' and 'Oc' stages by a slightly more negative electrophoretic mobility. Band (f_3) is not observed in the 'Ov' and 'H' stages. An electrophoretic mobility and diffusion rate

Table IV

Analysis of the precipitin band patterns of the early zebrafish developmental stages with pooled unabsorbed Anti-Mg serum. Stages analysed: 'Ov', 'Cl', 'Hb', 'Mg', 'Es', 'Oc' and 'H'

Precipitin bands	a_3	b_3	b'_3	c_3	c'_3	d_3	e_3	f_3	g_3	h'_3	h''_3	i_3	j_3	k_3	l'_3	l''_3	m_3	n_3	n'_3	o_3	p_3	q_3	r_3	r'_3	s_3	t_3
Embryonic stages																										
Ov	+	+		+	+	±	+			+	+	+	±	+	+	+	+	+		+	+	+				
Cl	±	+		+	+	±		+	±	+	+	+	←+→		+	+		+	?	+	+		+			
Hb	?	+		+	+	±		+	±	+	+	+	←+→		+	+		+	±	±	+				±	
Mg		+		+	+	+		+	±	+	+	+	←+→		+	+		+	±		+				+	
Es		+		+	+	+		+	±	+	+	+	+	←+→		+	+	+	±	±	+				+	±
Oc		?		+	+			+	±	+	+	+	←+→		+	+		+		±	+					
H										+	+	+	←+→		+	+		+	+	+	+					

Symbols used:

+	Positive precipitin reaction
±	Faint precipitin reaction
←+→	Precipitation could be either of two identifiable bands
?	Extremely faint band

Figure 10. Immunoelectrophoretic precipitin line patterns of seven developmental stages of the zebrafish Brachydanio rerio, analysed with unabsorbed pooled Anti-Mg sera.

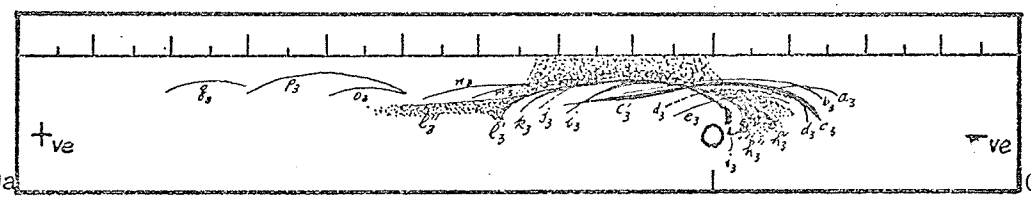


Fig. 10a Ov

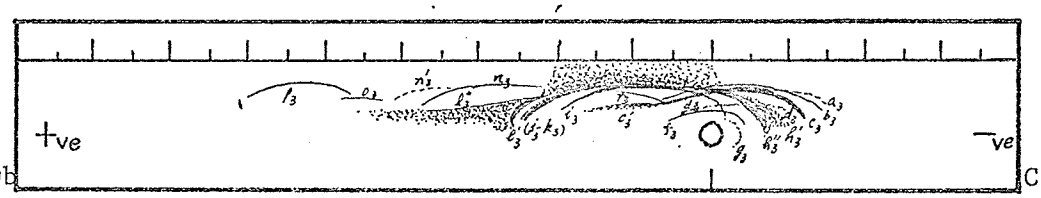


Fig. 10b C1

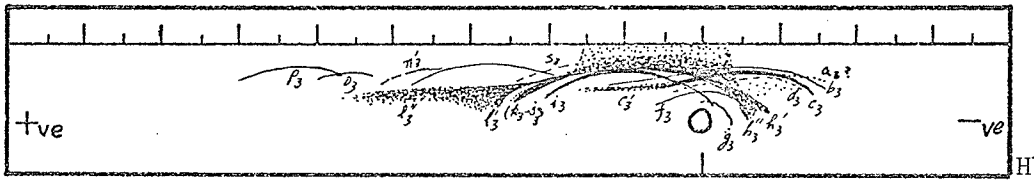


Fig. 10c Hb

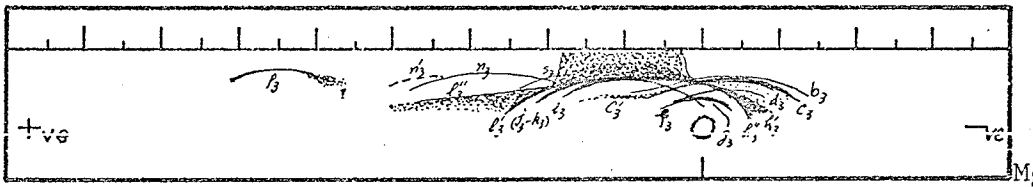


Fig. 10d Mg

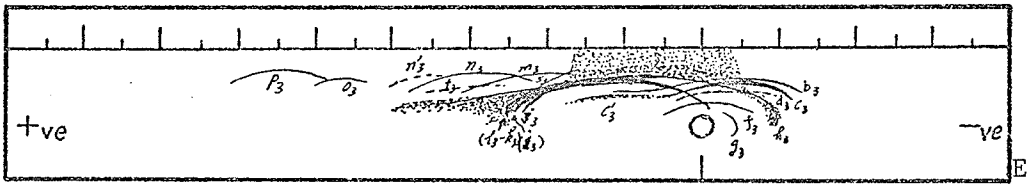


Fig. 10e Es

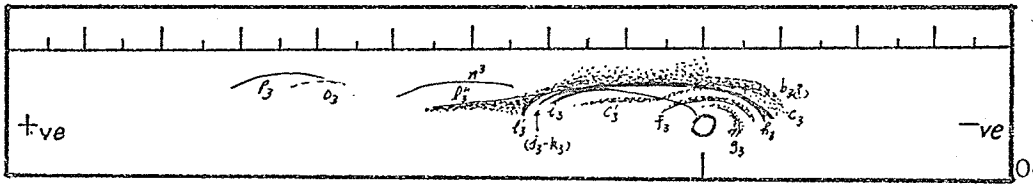


Fig. 10f Oc

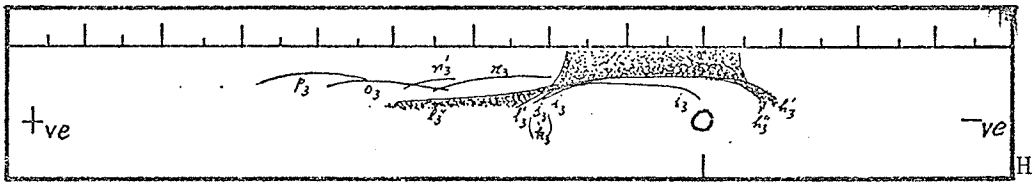


Fig. 10g H

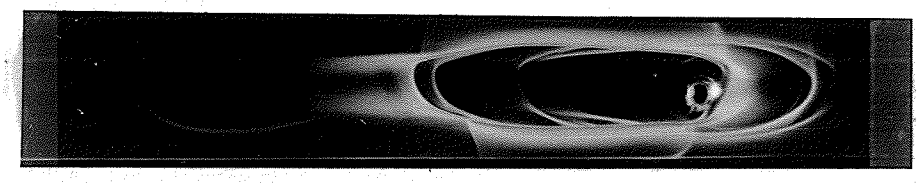
Fig. 10a  Ov

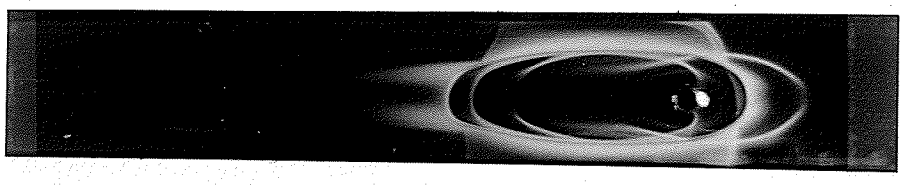
Fig. 10b  Cl

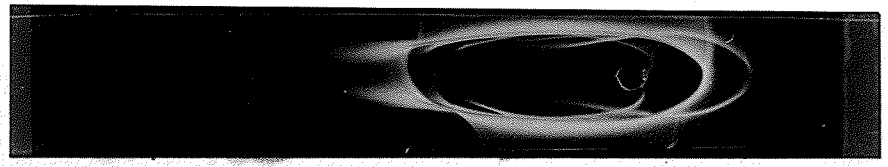
Fig. 10c  Hb

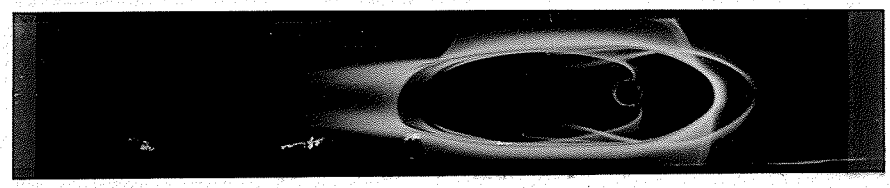
Fig. 10d  Mg

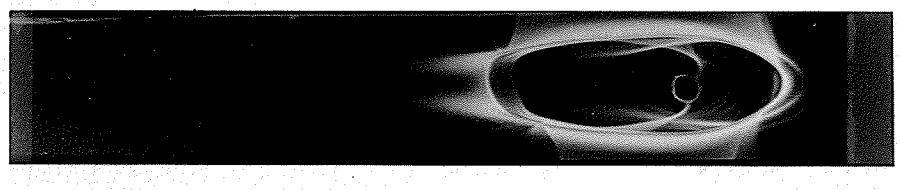
Fig. 10e  Es

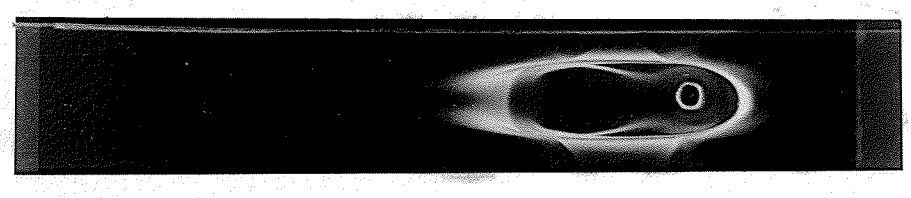
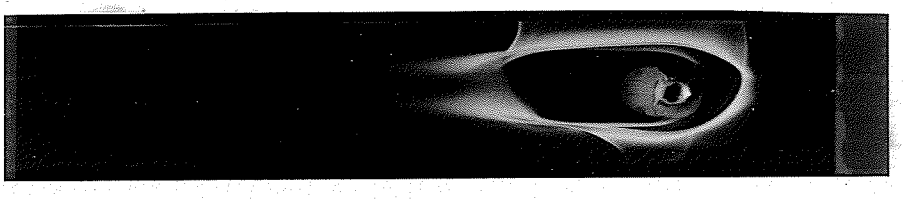
Fig. 10f  Oc

Fig. 10g  H

of almost zero is characteristic of the precipitin band (f_3). This (f_3) band is usually seen as an arch lateral to the antigen well.

Band (g_3) occurs as a faint half-band throughout the early developmental stages except the 'H' stage. It is not found in the 'Ov' stage.

The bands (h_3') and (h_3'') appear close to the antigen well and posterio-lateral to (g_3) in the 'Ov' and the 'Oc' stages. Beginning with the 'Cl' stage, these bands exhibit an increasingly negative electrophoretic mobility throughout the succeeding stages (Figures 10a - 10f).

Band (i_3) spans from the anterio-medial region of the 'band complex' to the antigen well. The anterior extremity of the 'band-complex' gives rise to the bands (j_3), (k_3), (l_3') and (l_3''). Both (j_3) and (k_3) show clear precipitation lines between (l_3) and (i_3) in the 'Ov' stage. Only one band appears between (l_3) and (i_3) when the 'Cl', 'Hb', 'Mg', 'Oc' and 'H' stages are tested. It is possible that this band is a composite (j_3 - k_3) band. Precipitin line (j_3) appears in the 'Es' stage to be slightly more electronegative than the band (i_3) (Figure 10e). A single composite (k_3 - l_3') band is also observed at the 'Es' stage. Bands (l_3') and (l_3'') appear consistantly to occupy the same relative positions in all developmental stages.

Non-specific precipitation between the antibody trough and the 'band complex' makes the observation of the bands (b_3), (r_3), (s_3) and (m_3) difficult in most cases.

Band (m_3) is found in the 'Ov' and the 'Es' stages only, whereas band (n_3) located immediately anterior to (m_3) is found to be present at all stages.

A new band (n_3') may be observed between band (n_3) and band (o_3). It appears as a weak band in the 'Cl' stage and increases in intensity during the 'Hb', 'Mg', 'Es' and 'H' stages. It is not detectable in the 'Ov' as well as the 'Oc' stages.

Band (o_3) is found in all stages except the 'Mg' stage. It is most pronounced in the 'Ov' and 'H' stages.

The arch of band (p_3) in the 'Ov' stage extends posterio-laterally to cover band (o_3). In all other stages where (p_3) is observed, its posterior extremity bisects the anterior portion of band (o_3).

A new band (q_3) with a marked anterior electrophoretic mobility may be observed in the 'Ov' stage. This band occurs at the anterior extremity of (p_3). It is absent from all other stages tested.

Band (r_3) is identified in the 'Cl' stage only (Figure 10b). Band (s_3) is visible posterior to band (m_3) in the 'Hb', 'Mg' and 'Es' stages. When the 'Hb' and 'Mg' stages are analysed only one band with an electrophoretic mobility intermediate between (s_3) and (m_3) appears, making an exact identification of either difficult (Figures 10c - 10d). When the 'Es' stage is tested, (t_3) can be discerned very faintly medially to and intermediate between (n_3') and (m_3).

D. Stages analysed with pooled Anti-Es serum

A resumé of the results with unabsorbed Anti-Es serum is given in Table V and graphically presented in Figures 11a - 11g.

Twenty three bands can be identified with unabsorbed Anti-Es serum. The bands are: (a_4), (b_4), (c_4), (c_4'), (d_4), (e_4), (f_4), (g_4), (h_4'), (h_4''), (i_4), (j_4), (k_4), (l_4'), (l_4''), (m_4), (n_4), (n_4'), (o_4), (p_4), (r_4), (s_4) and (t_4).

Table V

Analysis of the precipitin band patterns of the early zebrafish developmental stages with pooled unabsorbed Anti-Es serum . Stages analysed: 'Ov', 'Cl', 'Hb', 'Mg', 'Es', 'Oc' and 'H'

Precipitin bands	a_4	b_4	b'_4	c_4	c'_4	d_4	e_4	f_4	g_4	h'_4	h''_4	i_4	j_4	k_4	l'_4	l''_4	m_4	n_4	n'_4	o_4	p_4	q_4	r_4	r'_4	s_4	t_4
Embryonic stages																										
Ov	+	+		+	+	+	+			+	+	+	+	+	+	+	+	±		+	±					
Cl	±	+		+	+	±		±	+	+	+	+		+	+	+	+	+	+	+	+		±			
Hb	+	+		+	+	±		+	+	+	+	+		+	+	+	+	+	+	+	+		+			
Mg	+	+		+	+			+	+	+	+	+		+	+	+	+	+	+	+	+		+			
Es	+	+		+	+	±		+	+	←+→		+	+	+	+	+	+	+	+	+	+		+		+	
Oc	+	+		+	+			±	+	←+→		+	+	+	+	+	+	+	+	+	+					±
H										+	+	+		+	+	+	+	+	+	+	+				±	

Symbols used:
+ Positive precipitin reaction
± Faint precipitin reaction
←+→ Precipitation could be either of two identifiable bands

Figure 11. Immunoelectrophoretic precipitin line patterns of seven developmental stages of the zebrafish, Brachydanio rerio, analysed with unabsorbed pooled Anti-Es sera.

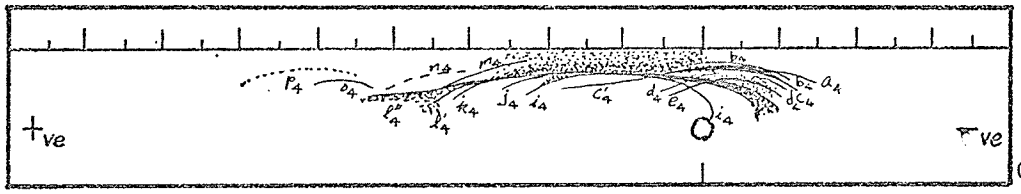


Fig. 11a Ov

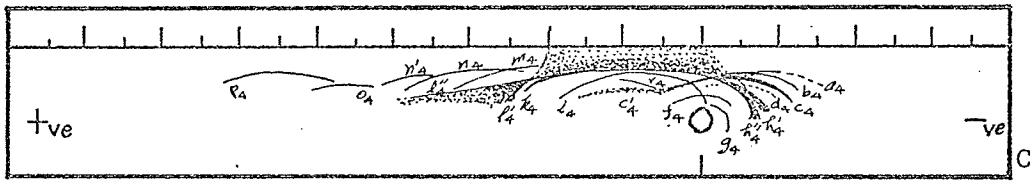


Fig. 11b Cl

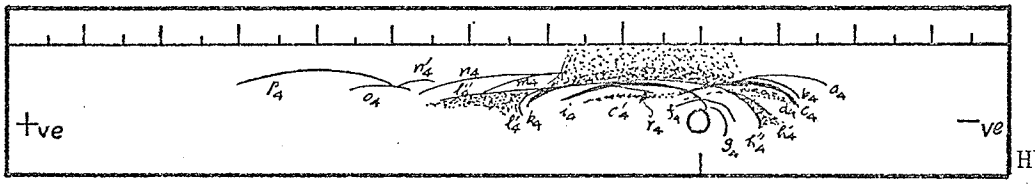


Fig. 11c Hb

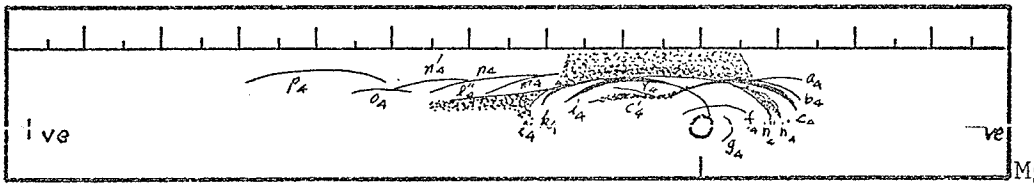


Fig. 11d Mg

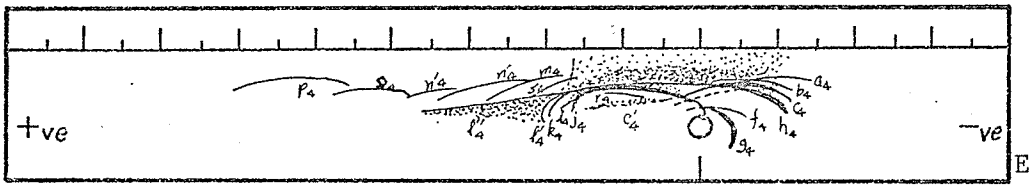


Fig. 11e Es

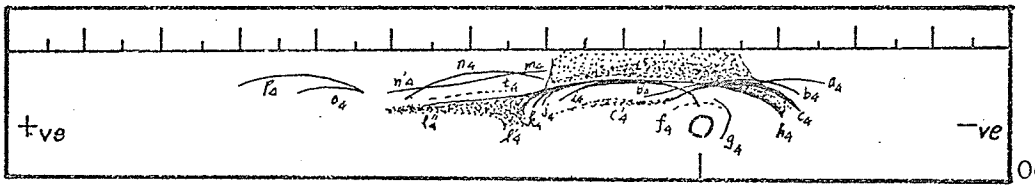


Fig. 11f Oc

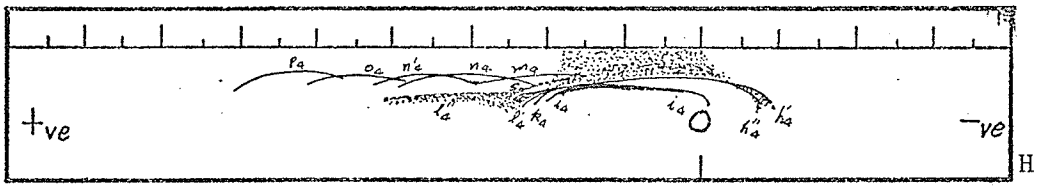


Fig. 11g H

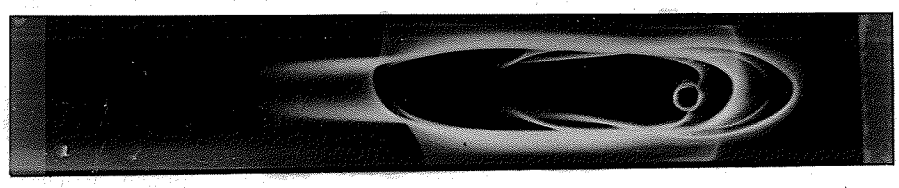


Fig. 11a Ov

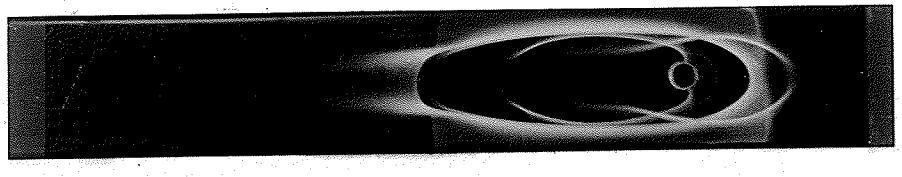


Fig. 11b Cl

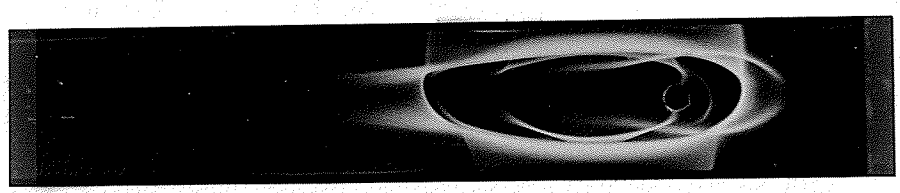


Fig. 11c Hb

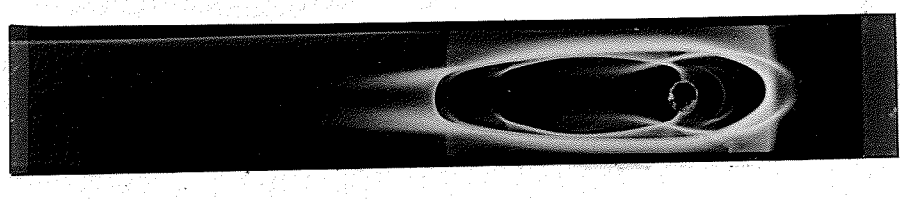


Fig. 11d Mg

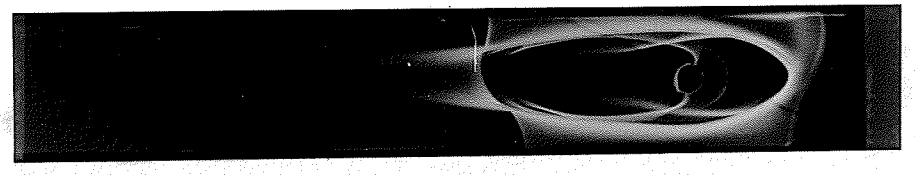


Fig. 11e Es

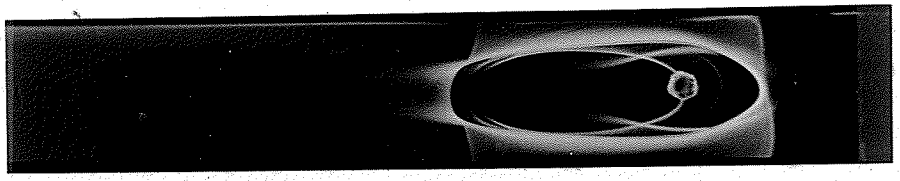


Fig. 11f Oc

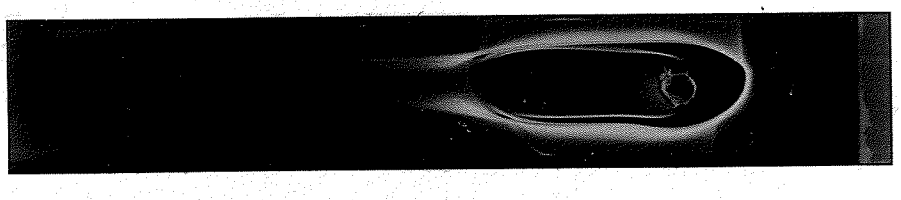


Fig. 11g H

Bands (a_4) and (b_4) are found in all stages tested except the 'H' stage. The former band decreases in intensity with development.

Bands (c_4) and (c_4') similarly occur at all stages except the 'H' stage. They are continuous, suggesting a common antigenic determinant.

Precipitin line (d_4) is observed in the 'Ov' stage and faintly in the 'Cl', 'Hb' and 'Es' stages.

Band (e_4) is found in the 'Ov' stage only, whereas (f_4) is found in the rest of the stages tested except the 'H' stage. The half band (g_4) shows consistantly in the 'Cl', 'Hb', 'Mg', 'Es' and 'Oc', but not in the 'Ov' stages (Figure 11a).

The precipitin lines (h_4') and (h_4'') are separate and distinct in the 'Ov', 'Cl', 'Hb', 'Mg' and 'H' stages, however, in the 'Es' and 'Oc' stages only one band can be discerned in the position of (h_4') and (h_4'') (Figures 11e - 11f).

Band (i_4) occurs consistantly throughout all stages. (j_4) is a weak band identified in the 'Ov', 'Es' and 'Oc' stages. It arises from the 'band complex' intermediate between (i_4) and (k_4) in the 'Ov' and 'Oc' stages and from the 'band complex' posteriorly to band (i_4) in the 'Es' stage (Figure 11e). The bands (k_4), (l_4'), (l_4''), (m_4) and (n_4) are observed in all the stages tested. In the 'Ov' stage, as compared to other stages, these precipitin bands display a greater anterior electrophoretic migration (Figure 11a).

Band (n_4') occurring between (o_4) and (n_4) is not seen in the 'Ov' stage (Figure 11a). In the 'Oc' and 'H' stages (n_4') is continuous with (m_4) (Figures 11f - 11g), suggesting the presence of common antigenic determinants.

Bands (o_4) and (p_4) are present in all the stages tested. Band (o_4) when compared to the relatively stable band (p_4), displays some variation in its electrophoretic behaviour (Figures 11a - 11g). The band (o_4) normally positioned intermediate between (n'_4) and (p_4) in the 'Ov', 'Cl', 'Hb', 'Mg', 'Es' and 'H' stages, arises from the posterior arch of (p_4) in the 'Oc' stage (Figure 11f).

Band (r_4) seen at the 'Cl', 'Hb', 'Mg' and the 'Es' stages appears as a weak band in the 'Cl' stage (Figure 11b).

The anterior extremity of band (s_4) is visible in the 'Es' and 'H' stages (Figures 11e - 11g). The remainder of the band is masked by the 'band complex'. It is not certain if band (s_4) is present in the other stages tested since a heavy non-specific precipitation frequently obscures this area.

In the 'Oc' stage an extremely faint band (t_4) is observed intermediate to (n'_4) and ($1''_4$) and medial to (n_4) (Figure 11f).

E. Stages analysed with pooled Anti-Oc serum

A resumé of the results with reference to stage antigens analysed with unabsorbed Anti-Oc serum is given in Table VI and graphically presented in Figures 12a - 12g.

Twenty five bands can be identified with unabsorbed Anti-Oc serum. The bands are: (a_5), (b_5), (c_5), (c'_5), (d_5), (e_5), (f_5), (g_5), (h'_5), (h''_5), (i_5), (j_5), (k_5), ($1'_5$), ($1''_5$), (m_5), (n_5), (n'_5), (o_5), (p_5), (q_5), (q'_5), (r_5), (s_5) and (t_5).

Band (a_5) is found in the 'Ov', 'Cl', 'Es' and 'Oc' stages. Band (b_5) occurs in all stages except the 'H' stage. A faint band occupying the position of (a_5) and (b_5) may be seen at the 'H' stage.

Table VI

Analysis of the precipitin band patterns of the early zebrafish developmental stages with pooled unabsorbed Anti-Oc serum. Stagesanalysed: 'Ov', 'Cl', 'Hb', 'Mg', 'Es', 'Oc' and 'H'

Precipitin bands	a_5	b_5	b'_5	c_5	c'_5	d_5	e_5	f_5	g_5	h'_5	h''_5	i_5	j_5	k_5	l'_5	l''_5	m_5	n_5	n'_5	o_5	p_5	q_5	r_5	r'_5	s_5	t'_5
Embryonic stages																										
Ov	+	+		+	+	+	+			+	+	+		+	+	+	+	+	+	+	+	+	±			
Cl	+	+		+	+	+		±	+	+	+	+	←+→		+	+	+	+		+	+	±	+			
Hb		+		+	+	±		+	±	+	+	+	←+→		+	+	+	+	?	±	+	+				±
Mg		+		+	+			+	+	←+→		+	←+→		+	+	+	+	+	+	+	+	+			+
Es	+	+		+	+			+	+	←+→		+	←+→		+	+	+	+	+	+	+	+				+
Oc	+	+		+	+	+		+	+	+	+	+	←+→		+	+	+	+	+	+	+	+				+
H	←±→									+	+	+			+	+	+	+	+	±	+	+			+	

Symbols used:

+	Positive precipitin reaction
±	Faint precipitin reaction
←+→	Precipitation could be either of two identifiable bands
?	Extremely faint band

Figure 12. Immunoelectrophoretic precipitin line patterns of seven developmental stages of the zebrafish, Brachydanio rerio, analysed with unabsorbed pooled Anti-Oc sera.

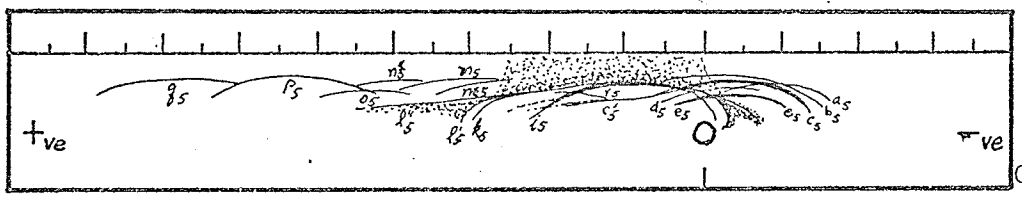


Fig. 12a Ov

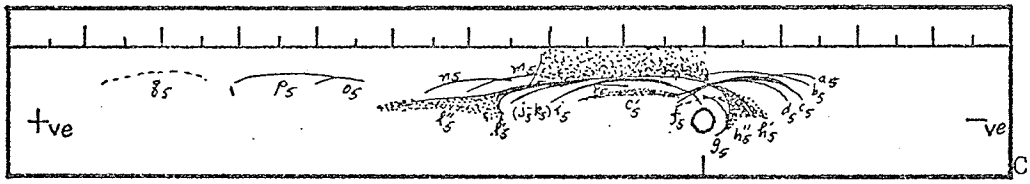


Fig. 12b Cl

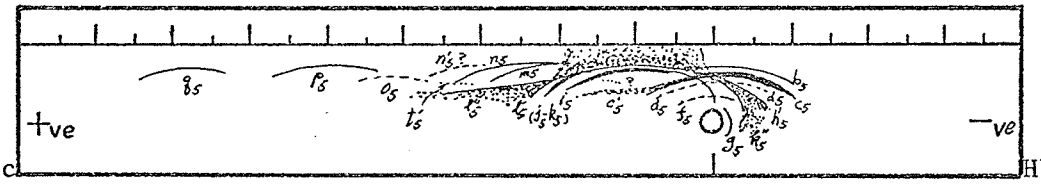


Fig. 12c Hb

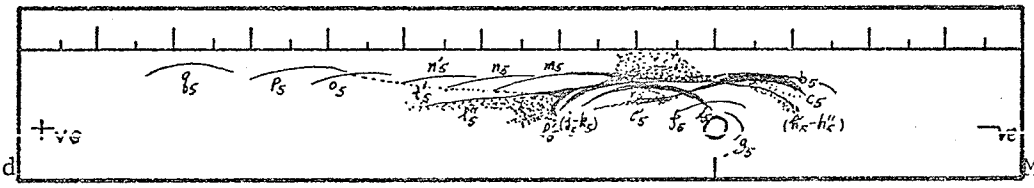


Fig. 12d Mg

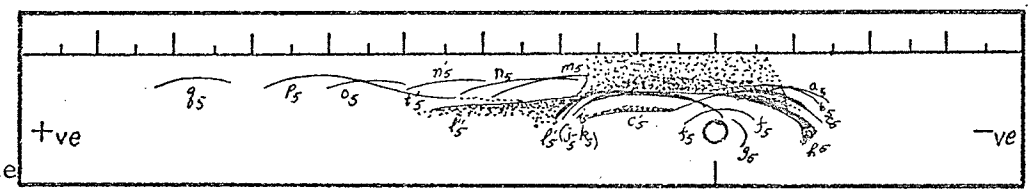


Fig. 12e Es

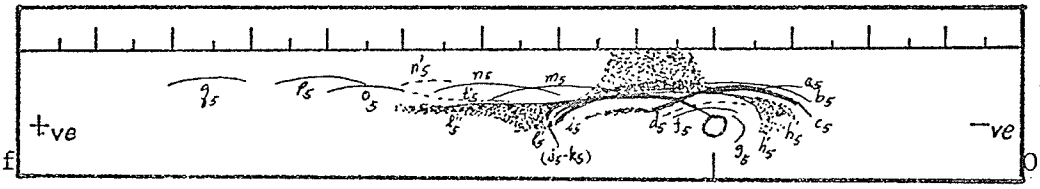


Fig. 12f Oc

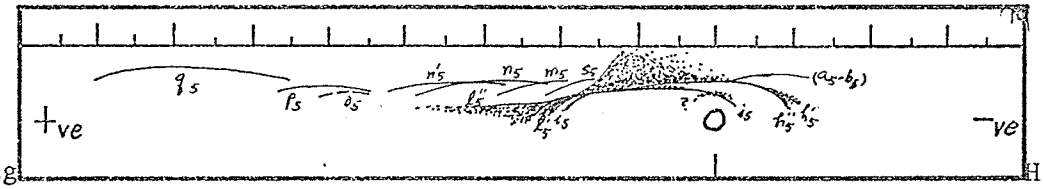


Fig. 12g H

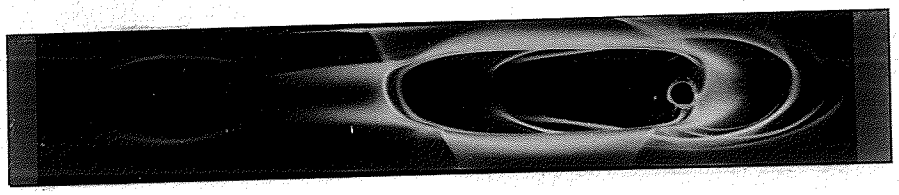


Fig. 12a Ov

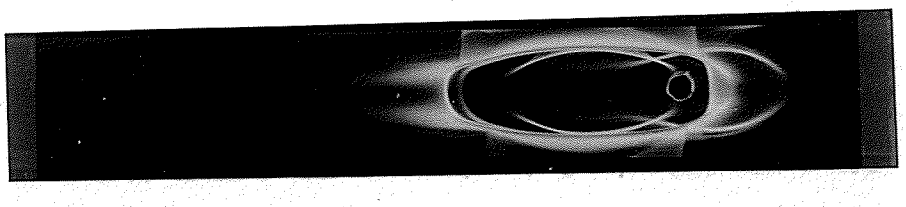


Fig. 12b C1

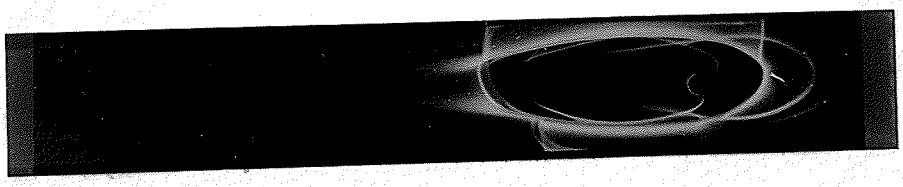


Fig. 12c Hb

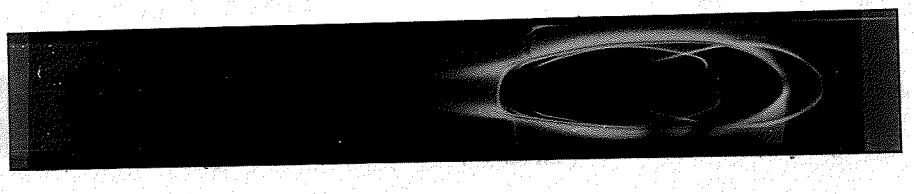


Fig. 12d Mg

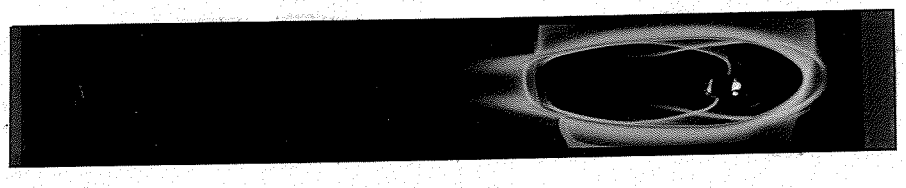


Fig. 12e Es

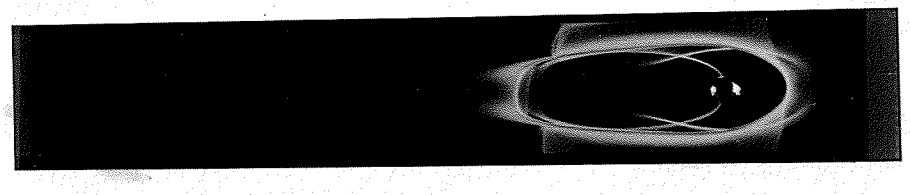


Fig. 12f Oc

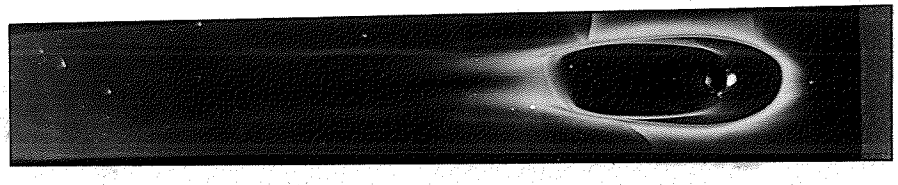


Fig. 12g H

Due to a minor fluctuation in electrophoretic mobility, the positions of the posterior extremities of bands (a_5) and (b_5) are occasionally interchanged (Figures 12a and 12e).

The bands (c_5) and (c_5') show consistantly in all stages except the 'H' stage. Band (d_5) is detected in the 'Ov', 'Cl', 'Hb' and 'Oc' stages only. The precipitin line (e_5) only occurs in the 'Ov' stage (Figure 12a). (f_5) first appears as a weak band at 'Cl' and is present subsequently as a well defined band in the 'Hb', 'Mg', 'Es' and 'Oc' stages. Band (g_5) is observed as a faint band in all stages except the 'Ov' and 'H' stages (Figures 12a - 12g).

A composite (h_5' - h_5'') precipitin line may be seen in the 'Mg' and 'Es' stages (Figures 12d and 12e). In all other stages, the bands (h_5') and (h_5'') are observed as separate and distinct bands at the posterior extremity of the 'band complex'. Band (i_5) is found in all stages. It is seen in close contact with the anterior arch of the 'band complex' in the 'Ov' and 'H' stages (Figures 12f - 12g).

Band (j_5) is absent in the 'Ov' stage. In the 'Cl', 'Hb', 'Mg', 'Es' and 'Oc' stages, a single band, may be discerned. This band is possibly a (j_5 - k_5) composite band. Both (j_5) and (k_5) are indistinct in the 'H' stage (Figure 12g). The bands (l_5') and (l_5'') are seen at all stages tested. Bands (m_5) and (n_5) are found in all the developmental stages while band (n_5') is only identified in the 'Ov', 'Hb', 'Mg', 'Es', 'Oc' and 'H' stages (Figures 12a, 12c - 12g). The (n_5') band is barely visible at the 'Hb' stage (Figure 12c).

Precipitin band (o_5) is observable in all stages. It exhibits a fluctuating electrophoretic mobility which decreases from the 'Cl' stage through the 'Mg' stage and then increases up to the 'H' stage

(Figures 12a - 12g). Band (o_5) is found to bisect the posterior part of precipitin line (p_5) in all the prehatching stages tested (Figures 12a - 12f). Only a faint trace of band (o_5) may be observed medial to (p_5) in the 'H' stage (Figure 12g).

Precipitin line (p_5) is present in all stages. Its posterior region appears to be continuous with an anterior extension of band (t_5) in the 'Ov', 'Mg', 'Es' and 'Oc' stages (Figures 12a, 12d - 12f).

Band (q_5) is present in all stages. In the 'Ov' and the 'H' stages it arises at the anterior extremity of band (p_5), whereas in the 'Hb', 'Mg', 'Es' and 'Oc' stages it arises some distance anterior to that.

Band (r_5) is identified in the 'Ov', 'Cl' and 'Mg' stages. Precipitin band (s_5) is detected in the 'Oc' and 'H' stages only (Figures 12f - 12g).

A band occupying the position lateral to ($1''_5$) and medial to (n'_5) can be observed in the 'Hb', 'Mg', 'Es' and 'Oc' stages. It also occurs slightly anterior to the (t) position and is found to be continuous with band (p_5) in most cases. This new band is named (t_5) (Figures 12c - 12f).

F. Stages analysed with Anti-H serum

A resumé of the results with reference to stage antigens analysed with the unabsorbed Anti-H serum is given in Table VII and graphically presented in Figures 13a - 13g. A total of eighteen bands can be identified with unabsorbed Anti-H serum. The bands are: (a_6), (b_6), (g_6), (h'_6), (h''_6), (i_6), ($1'_6$), ($1''_6$), (m_6), (o_6), (p_6), (u'_6), (u''_6), (u'''_6), (v_6), (w_6), (x_6) and (y_6).

Table VII

Analysis of the precipitin band patterns of the early zebrafish developmental stages with unabsorbed Anti-H stage serum. Stages analysed: 'Ov', 'Cl', 'Hb', 'Mg', 'Es', 'Oc' and 'H'

Precipitin bands	a_6	b_6	b'_6	c_6	c'_6	d_6	e_6	f_6	g_6	h'_6	h''_6	i_6	j_6	k_6	l'_6	l''_6	m_6	n_6	n'_6	o_6	p_6	q_6	r_6	r'_6	s_6	t_6
Embryonic stages																										
Ov	←+→									+	+	+			+	+				+	±					
Cl	←+→								+	+	+	+			+	+	+									
Hb	←+→								+	+	+	+			+	+	+									
Mg	←±→								+	←+→		+			+	+	+									
Es									+	←+→		+			+	+	+									
Oc	←±→								+	←+→		+			+	+	+									
H	←±→									←+→		+			+	+	+									

Precipitin bands	u'_6	u''_6	u'''_6	v_6	w_6	x_6	y_6
Embryonic stages							
Ov							
Cl							
Hb							
Mg							
Es							
Oc							
H	+	+	+	+	+	+	+

Symbols used:
+ Positive precipitin reaction
± Faint precipitin reaction
←+→ Precipitation could be either of two identifiable bands

Figure 13. Immunoelectrophoretic precipitin line patterns of seven developmental stages of the zebrafish, Brachydanio rerio, analysed with unabsorbed Anti-H serum.

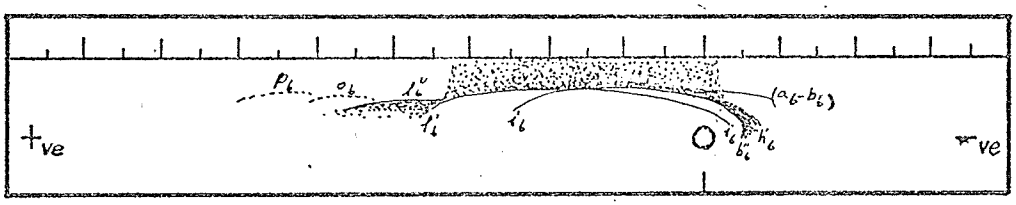


Fig. 13a Ov

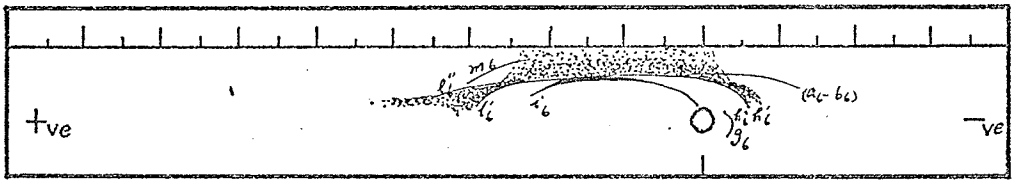


Fig. 13b C1

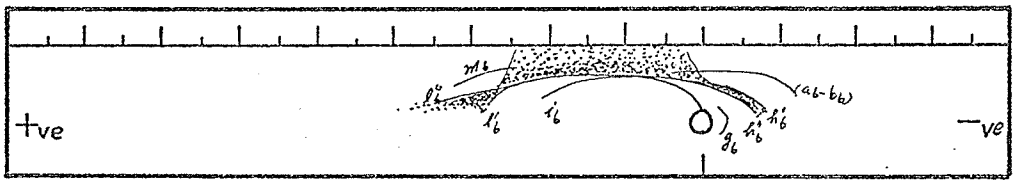


Fig. 13c Hb

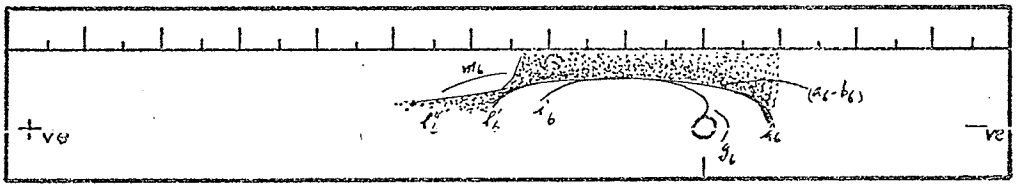


Fig. 13d Mg

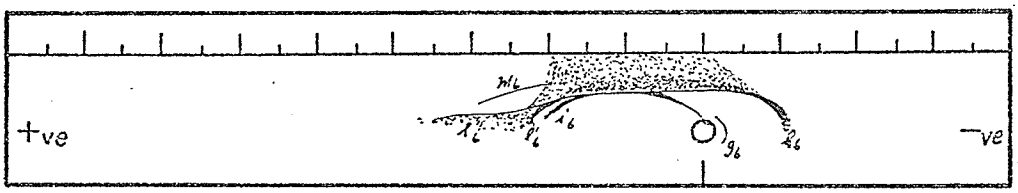


Fig. 13e Es

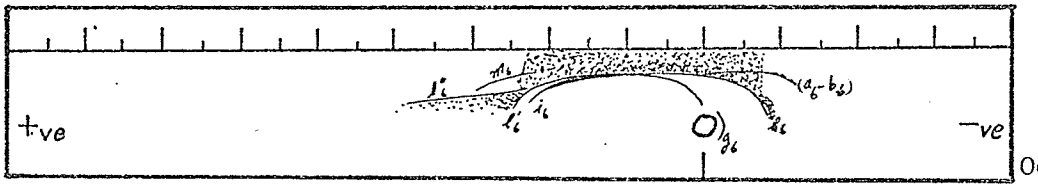


Fig. 13f Oc

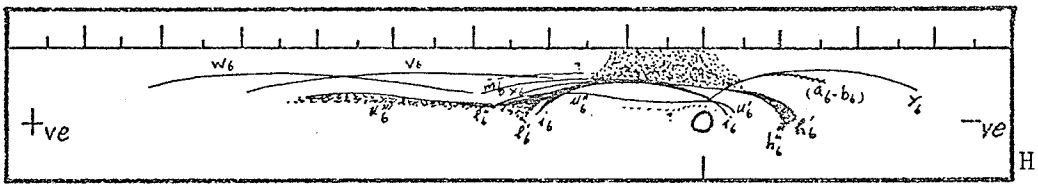


Fig. 13g H

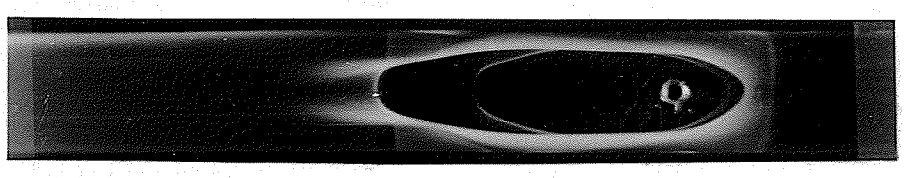
Fig. 13a  Ov

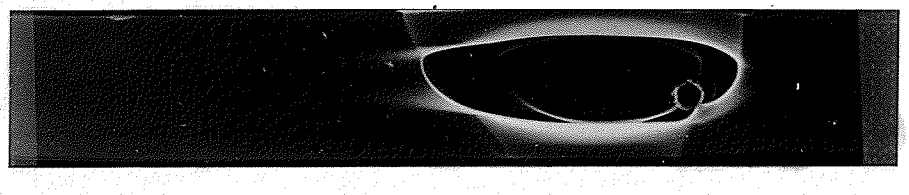
Fig. 13b  Cl

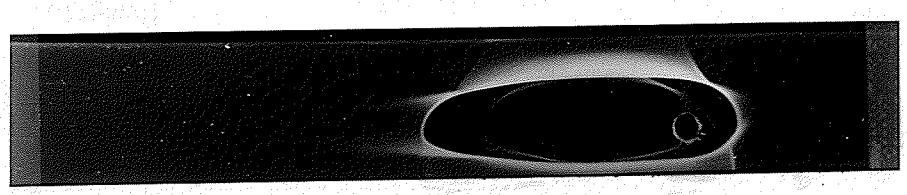
Fig. 13c  Hb

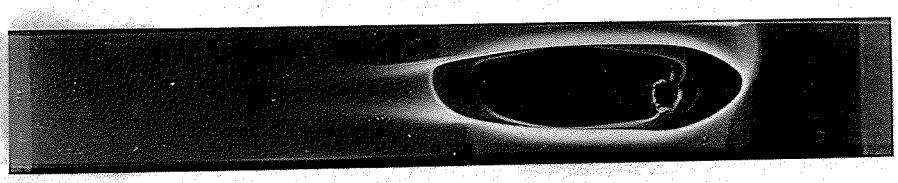
Fig. 13d  Mg

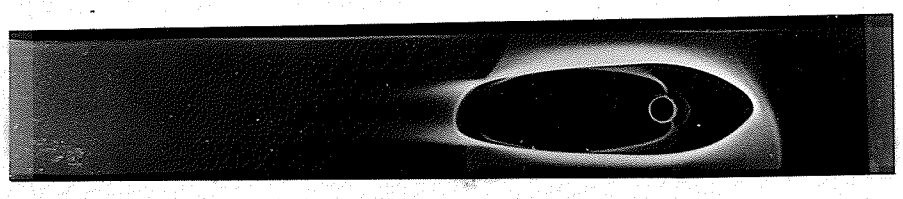
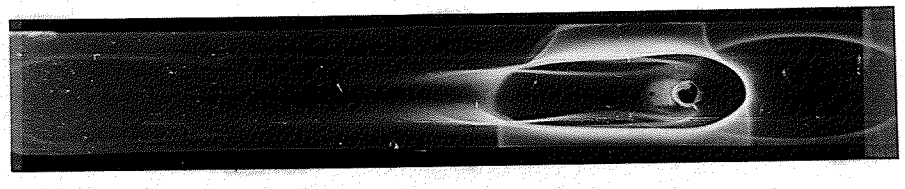
Fig. 13e  Es

Fig. 13f  Oc

Fig. 13g  H

A single composite band intermediate between (a_6) and (b_6) is found in the 'Ov', 'Cl', 'Hb' 'Mg', 'Oc' and 'H' stages. No band is found in this area at the 'Es' stage. In the latter three stages mentioned this band (a_6 - b_6) appears weaker than the one found in the earlier stages.

A crescent-shaped precipitin band (g_6) appears in the 'Cl', 'Hb', 'Mg', 'Es' and 'Oc' stages immediately posterior to the antigen well.

Bands (h'_6) and (h''_6) are separate and distinct in the 'Ov', 'Cl' and 'Hb' stages but occurs as a single precipitin line in the 'Es', 'Mg', 'Oc' and the 'H' stages.

Bands (i_6), (l'_6) and (l''_6) are seen at all stages tested. When comparing the 'Ov' stage to other developmental stages, band (l''_6) may be seen to occupy an anterior position in the 'Ov' stage and a progressively posterior position in the subsequent stages. Band (m_6), if present in the 'Ov' stage, cannot be seen due to the heavy non-specific precipitation; yet in all other stages it is clearly identifiable.

The precipitin lines (o_6) and (p_6) are identified in the 'Ov' stage only (Figure 13a).

A series of new bands are observed when the 'H' stage is tested against the unabsorbed Anti-H serum. The identification of these bands as being different from the bands already described is confirmed by the absorption tests (see page 69).

A band that appears at the relative position of the (f_6) band is identified as (u'_6). Its anterior extremity is continuous with band (u''_6). The latter merges with the anterior part of the 'Band complex' formed by (i_6), (l'_6) and (l''_6). The anterior extremity

of band ($1''_6$) of the 'Band complex' is continuous with band (u'''_6) which occupies the relative position of band (t_5) and possesses a diffusion rate and electrophoretic mobility similar to that of the latter.

The band labelled (v_6) forms an arch from the anterior extremity of band (m_6) to the band (p_6) position. It also occupies the position lateral to band (u'_6).

The most posteriorly positioned band (w_6) is seen as a precipitin line extending from the anterior region of (m_6) to the former band (q_6) position. The posterior arch of this band may be seen laterally to (u'''_6) and medially to the band (v_6). Anteriorly the arch is characterized by a very high diffusion rate and consequently occupies an extreme lateral position.

A band (x_6) arises from the 'Band complex' and extends anteriorly between the bands (m_6) and ($1''_6$).

A precipitin arch may also be seen to arise at the antigen well and to extend posterio-laterally to the posterior extremity of the band pattern. This band is identified as (y_6).

II. Immunodiffusion analysis (ID) with anti-stage sera absorbed respectively with each of the embryonic stage extracts

Preliminary immunodiffusion tests were conducted to detect stage-specific precipitin reactions.

A resumé of the results with reference to the stage antigens tested against absorbed anti-stage sera with immunodiffusion is given in the Tables VIII - XIII. Only diffusion positive tests were subjected to immunoelectrophoresis for further analysis of the bands.

Table VIII

Immunodiffusion analysis of the zebrafish embryonic stage extracts with Anti-Cl serum absorbed respectively with six stage-specific extracts

Stage Antigens	Ov	Cl	Hb	Mg	Es	Oc	H
Absorbed antisera							
Anti-Cl/Cl	0	0	0	0	0	0	0
Anti-Cl/Hb	0	0	0	0	0	0	0
Anti-Cl/Mg	0	0	0	0	0	0	0
Anti-Cl/Es	0	0	0	0	0	0	0
Anti-Cl/Oc	±	±	±	±	±	0	0
Anti-Cl/H	+	+	+	+	+	+	0

Symbols used:
+ Positive reaction
0 Negative reaction
± Weak reaction

Table IX

Immunodiffusion analysis of the zebrafish embryonic stage extracts with Anti-Hb serum absorbed respectively with six different stage-specific extracts

Stage Antigens	Ov	Cl	Hb	Mg	Es	Oc	H
Absorbed antisera							
Anti-Hb/Cl	0	0	0	0	0	0	0
Anti-Hb/Hb	0	0	0	0	0	0	0
Anti-Hb/Mg	0	0	0	0	0	0	0
Anti-Hb/Es	0	0	0	0	0	0	0
Anti-Hb/Oc	±	±	±	±	±	0	0
Anti-Hb/H	+	+	+	+	+	+	0

Symbols used:
+ Positive reaction
0 Negative reaction
± Weak reaction

Table X

Immunodiffusion analysis of the zebrafish embryonic stage extracts with Anti-Mg serum absorbed respectively with six stage-specific extracts

Stage Antigens	Ov	Cl	Hb	Mg	Es	Oc	H
Absorbed antisera							
Anti-Mg/Cl	0	0	0	0	0	0	0
Anti-Mg/Hb	0	0	0	0	0	0	0
Anti-Mg/Mg	0	0	0	0	0	0	0
Anti-Mg/Es	0	0	0	0	0	0	0
Anti-Mg/Oc	±	±	±	±	±	±	±
Anti-Mg/H	+	+	+	+	+	+	0

Symbols used:
+ Positive reaction
0 Negative reaction
± Weak reaction

Table XI

Immunodiffusion analysis of the zebrafish embryonic stage extracts with Anti-Es serum absorbed respectively with six stage-specific extracts

Stage Antigens	Ov	Cl	Hb	Mg	Es	Oc	H
Absorbed antisera							
Anti-Es/Cl	0	0	0	0	0	0	0
Anti-Es/Hb	0	0	0	0	0	0	0
Anti-Es/Mg	0	0	0	0	0	0	0
Anti-Mg/Es	0	0	0	0	0	0	0
Anti-Es/Oc	±	±	±	±	±	±	0
Anti-Es/H	+	+	+	+	+	+	0

Symbols used:
+ Positive reaction
0 Negative reaction
± Weak reaction

Table XII

Immunodiffusion analysis of the zebrafish embryonic stage extracts with Anti-Oc serum absorbed respectively with six stage-specific extracts

Stage Antigens	Ov	Cl	Hb	Mg	Es	Oc	H
Absorbed antisera							
Anti-Oc/Cl	0	0	0	0	0	0	±
Anti-Oc/Hb	0	0	0	0	0	0	±
Anti-Oc/Mg	0	0	0	0	0	0	±
Anti-Oc/Es	0	0	0	0	0	0	±
Anti-Oc/Oc	±	±	±	±	±	0	0
Anti-Oc/H	+	+	+	+	+	+	0

Symbols used: + Positive reaction
0 Negative reaction
± Weak reaction

Table XIII

Immunodiffusion analysis of the zebrafish embryonic stage extracts with Anti-H serum absorbed respectively with six stage-specific extracts

Stage Antigens	Ov	Cl	Hb	Mg	Es	Oc	H
Absorbed antisera							
Anti-H/Cl	0	0	0	0	0	0	+
Anti-H/Hb	0	0	0	0	0	0	+
Anti-H/Mg	0	0	0	0	0	0	+
Anti-H/Es	0	0	0	0	0	0	+
Anti-H/Oc	0	0	0	0	0	0	+
Anti-H/H	0	0	0	0	0	0	0

Symbols used: + Positive reaction
0 Negative reaction
± Weak reaction

Negative immunodiffusion results are obtained when 'Ov', 'Cl', 'Hb', 'Mg', 'Es', 'Oc' and 'H' stage extracts are analysed with Anti-Cl (Table VIII), Anti-Hb (Table IX), Anti-Mg (Table X), and Anti-Es (Table XI) sera absorbed respectively with 'Cl', 'Hb', 'Mg' and 'Es' stage homogenates.

When 'Ov', 'Cl', 'Hb', 'Mg', 'Es' and 'Oc' extracts are analysed with Anti-Oc serum (Table XII) absorbed respectively with the 'Cl', 'Hb', 'Mg' and 'Es' stage homogenates; as well as with Anti-H serum (Table XIII) absorbed respectively with 'Cl', 'Hb', 'Mg', 'Es', 'Oc' and 'H' stage homogenates, negative results are also obtained.

The controls, i.e., antisera absorbed with their homologous antigens, when tested against extracts from the homologous stages, in all instances except for the 'Oc' stage extract (Table XII) yield negative results indicating that the absorptions are complete.

Diffusion analysis of antigens against antisera to the 'Cl', 'Hb', 'Mg', 'Es', 'Oc' and 'H' stages respectively absorbed with the 'Oc' and 'H' stage extracts yields positive results (Tables VIII - XII). Positive reactions are obtained for all the stage extracts, except the 'H' stage extract tested against the Anti-Cl, Anti-Hb, Anti-Mg, Anti-Es and Anti-Oc sera absorbed with the 'H' stage extract (Tables VIII - XI).

When the 'Ov', 'Cl', 'Hb', 'Mg' and 'Es' stages are tested against the anti-prehatching stage sera absorbed respectively with the 'Oc' stage extract (Tables VIII - XI)a very faint positive reaction is obtained. When the 'Oc' and 'H' stage extracts are tested against these absorbed antisera, the Anti-Mg/Oc absorbed serum gives a positive reaction to the 'Oc' and 'H' stages (Table X).

The Anti-Es/Oc absorbed serum also gives a positive reaction to the 'Oc' stage extract (Table XI).

Positive reactions are also obtained when the 'H' stage extracts are tested against the Anti-Oc sera absorbed with 'Cl', 'Hb', 'Mg', and 'Es' stage homogenates (Table XII); as well as against the Anti-H serum absorbed with 'Cl', 'Hb', 'Mg', 'Es', and 'Oc' homogenates (Table XIII).

III. Immunoelectrophoretic analysis (IEA) with rabbit anti-stage sera absorbed with Hatching stage extract

Immunodiffusion positive tests were subjected to immuno-electrophoretic analysis. Extracts from six developmental stages, namely: the 'Ov', 'Cl', 'Hb', 'Mg', 'Es' and the 'Oc' stages were tested against antisera to the 'Cl', 'Hb', 'Mg', 'Es' and 'Oc' stages absorbed respectively with the 'H' stage extracts.

A resume of the results obtained is given in Tables XIVa - XIVe, and graphically presented in Figures 14 - 18.

A. Stages analysed with Anti-Cl/H serum (Figures 14a - 14f and Table XIVa)

The 'Ov', 'Cl', 'Hb', 'Mg', 'Es' and 'Oc' stages were analysed with Anti-Cl/H serum. A double-humped precipitin line similar in shape and electrophoretic mobility to that of (c_1) and (c'_1) is called (c_{1a}) and (c'_{1a}).

Band (d_{1a}) is found as a faint precipitin line in the 'Hb', 'Mg' and 'Es' stages. A half band similar to (g_1), with an electrophoretic mobility comparable to that of (h'_1) or (g_1) is named (g_{1a}). The band named (m_{1a}), with an electrophoretic mobility intermediate

Tables XIVa - XIVe

Immunoelectrophoretic analysis (IEA) of zebrafish prehatching stages with prehatching-stage antisera respectively absorbed with hatching-stage extract

	Absorbed antisera	Stage extracts	Band patterns in absorptions										
			a_{1a}	b_{1a}	b'_{1a}	c_{1a}	c'_{1a}	d_{1a}	e_{1a}	f_{1a}	g_{1a}	m_{1a}	r_{1a}	r'_{1a}
XIVa	Anti-Cl/H	Ov				+	+				+	+		
		Cl				+	+				+	+		
		Hb				+	+	+			+	+		
		Mg				+	+	+			+	+		
		Es				+	+	+			+	+		
		Oc				+	+				+	+		
			a_{2a}	b_{2a}	b'_{2a}	c_{2a}	c'_{2a}	d_{2a}	e_{2a}	f_{2a}	g_{2a}	m_{2a}	r_{2a}	r'_{2a}
XIVb	Anti-Hb/H	Ov	+	+		+	+	+	+			+		
		Cl	+	+	+	+	+	+			+	+	+	
		Hb	+	+	+	+	+	+		+	+	+	+	+
		Mg	+	+	+	+	+	+			+	+	+	+
		Es	+	+		+	+	±				+		
		Oc		+	+	+	+			+	+	+		
			a_{3a}	b_{3a}	b'_{3a}	c_{3a}	c'_{3a}	d_{3a}	e_{3a}	f_{3a}	g_{3a}	m_{3a}	r_{3a}	r'_{3a}
XIVc	Anti-Mg/H	Ov		+		+	+		+			+		
		Cl		+		+	+			+	+	+		
		Hb		+		+	+			+	+	+		
		Mg		+		+	+			+	+	+		
		Es		+		+	+			+	+	+		
		Oc		+		+	+			+		+		
			a_{4a}	b_{4a}	b'_{4a}	c_{4a}	c'_{4a}	d_{4a}	e_{4a}	f_{4a}	g_{4a}	m_{4a}	r_{4a}	r'_{4a}
XIVd	Anti-Es/H	Ov				+	+		+			+		
		Cl		±		+	+			+	+			
		Hb		+		+	+			+	+			
		Mg		+		+	+			+	+			
		Es		+		+	+			+	+			
		Oc		+		+	+			+	+			
			a_{5a}	b_{5a}	b'_{5a}	c_{5a}	c'_{5a}	d_{5a}	e_{5a}	f_{5a}	g_{5a}	m_{5a}	r_{5a}	r'_{5a}
XIVe	Anti-Oc/H	Ov		+		+	+		+		+	+		
		Cl		±		+	+				+	+		
		Hb		+		+	+	±			+	+		
		Mg		+		+	+	+		+	+	+		

Symbols used: + Positive reaction

± Weak reaction

Figure 14. Immunoelectrophoretic precipitin line patterns of six pre-hatching stages of the zebrafish, Brachydanio rerio, analysed with Anti-Cl/H serum.

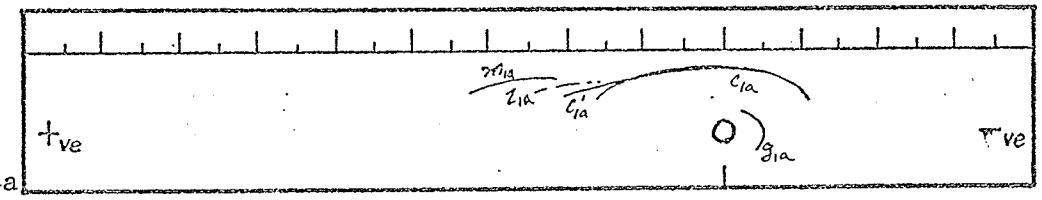


Fig. 14a Ov

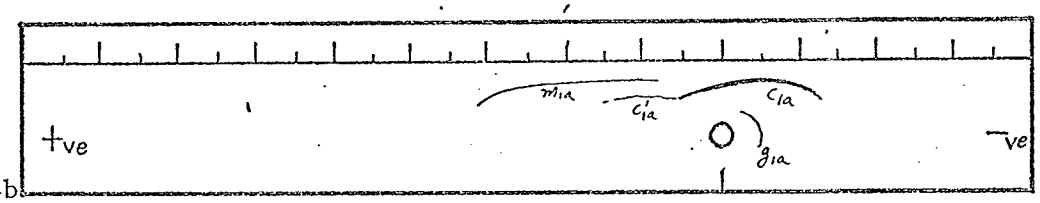


Fig. 14b C1

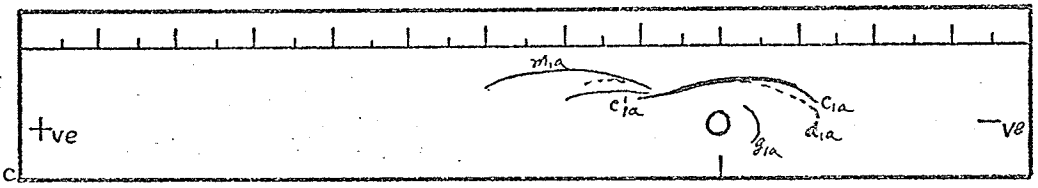


Fig. 14c Hb

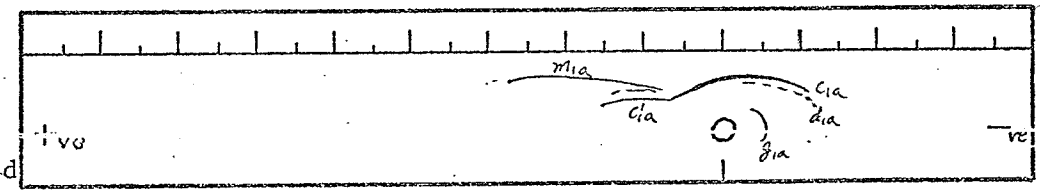


Fig. 14d Mg

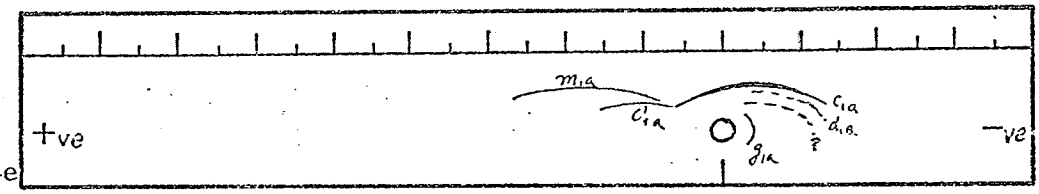


Fig. 14e Es

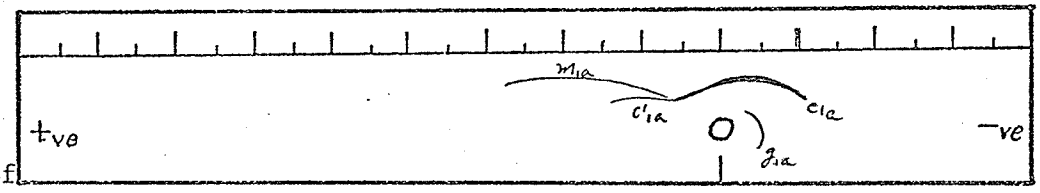


Fig. 14f Oc

Fig. 14a Ov

Fig. 14b C1

Fig. 14c Hb

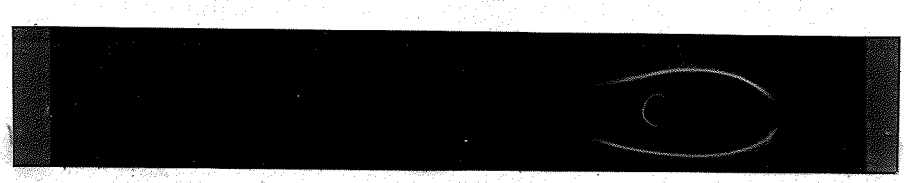


Fig. 14d Mg

Fig. 14e Es

Fig. 14f Oc

between that of (m_1) and (s_1) in the unabsorbed pattern, occurs at the anterior extremity of the absorbed pattern. Its posterior extremity arises from the junction of (c_{1a}) and (c'_{1a}) except in the 'Cl' stage (Figure 14b), where it runs parallel and lateral to band (c'_{1a}). Both (g_{1a}) and (m_{1a}) may be seen at all stages tested. A faint band, intermediate between (m_{1a}) and (c'_{1a}), located at the anterior extremity of (c_{1a}) close to the (s) position, is observed in the 'Ov', 'Hb' and 'Mg' stages. There is no band corresponding to this band in the unabsorbed pattern. This band is named (z_{1a}).

B. Stages analysed with Anti-Hb/H serum (Figures 15a - 15f and Table XIVb)

A greater complexity is obtained in pattern when all pre-hatching stages were analysed with Anti-Hb/H serum. Band (a_{2a}) arises from (c_{2a}) and extends posteriorly in the 'Ov', 'Cl', 'Hb', 'Mg' and 'Es' stages. Band (b_{2a}) is present in all stages. It is seen as a very long band extending its precipitin arch from the anterior arch of (c'_{2a}) to the posterior extremity of the pattern. The 'band complex' and the heavy non-specific precipitation usually found in the unabsorbed pattern is absent in the absorbed pattern, making the identification of this band somewhat easier. A band labelled as (b'_{2a}), found in the 'Cl', 'Hb', 'Mg' and 'Oc' stages, is easily seen to arise as a posterio-medial dichotomy of band (b_{2a}). The unabsorbed patterns (Figures 14b - 14d, 14f) suggest that (b'_{2a}) may exist in the 'Es' and 'Oc' stages as well. A continuation between (c_{2a}) and (c'_{2a}) is observed in the 'Ov', 'Cl', 'Hb', 'Mg' and 'Es' stages; however, (c_{2a}) and (c'_{2a}) occur as two separate bands in the 'Oc' stage (Figure 15f). Band (c'_{2a}) exhibits a greater anterior

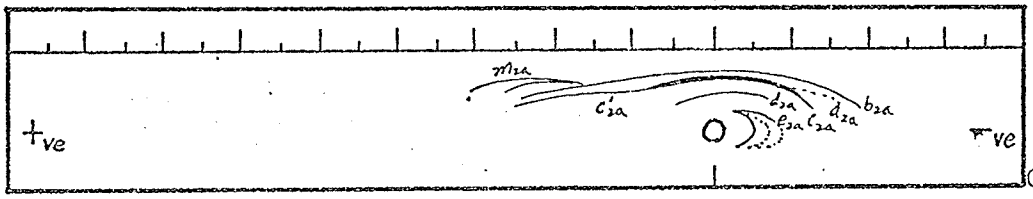


Fig. 15a Ov

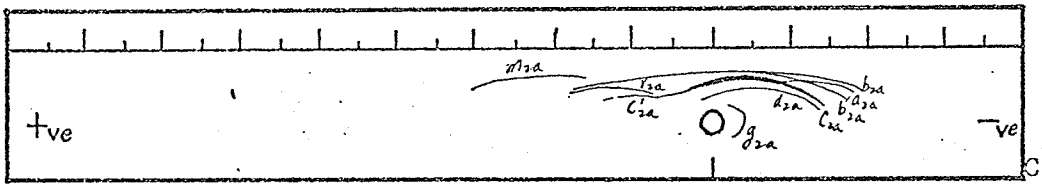


Fig. 15b Cl

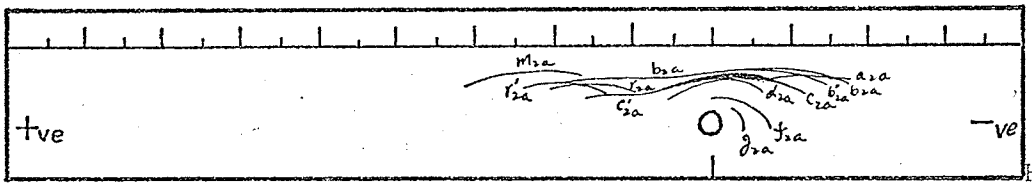


Fig. 15c Hb

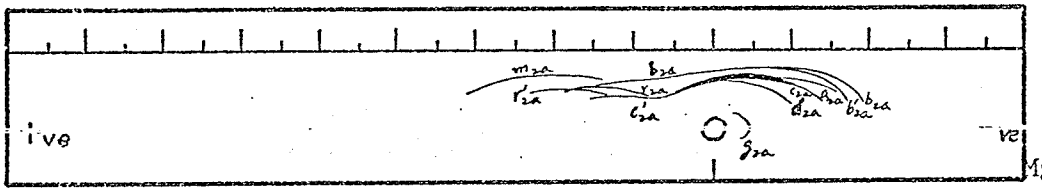


Fig. 15d Mg

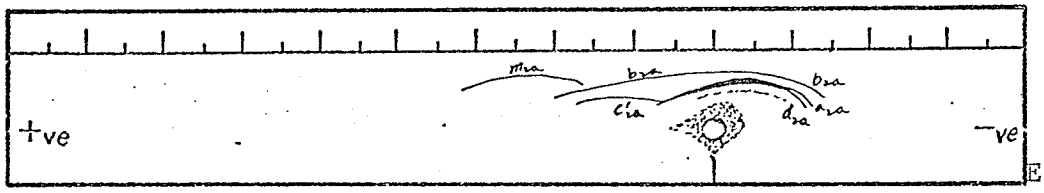


Fig. 15e Es

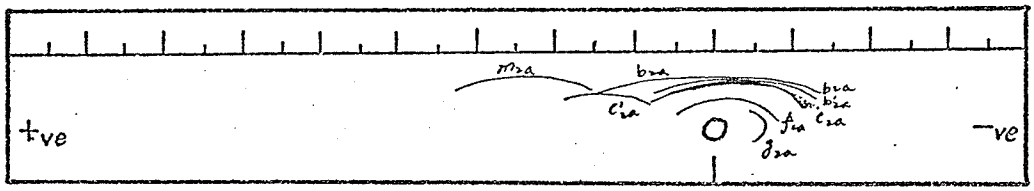


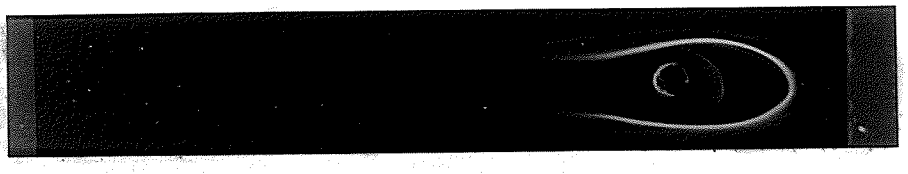
Fig. 15f Oc

Figure 15. Immunoelectrophoretic precipitin line patterns of six pre-hatching stages of the zebrafish, Brachydanio rerio, analysed with Anti-Hb/H serum.

Fig. 15a

Ov

Fig. 15b



Cl

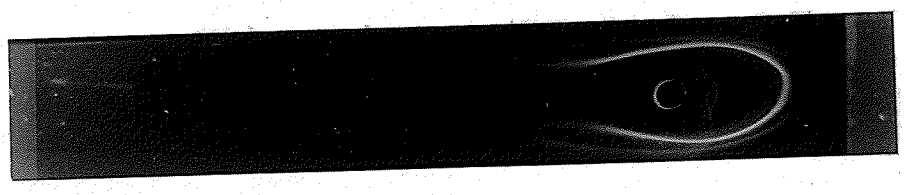
Fig. 15c

Hb

Fig. 15d

Mg

Fig. 15e



Es

Fig. 15f

Oc

migration in the 'Ov' stage as compared to the other stages. Band (d_{2a}) occurs in all stages except the 'Oc' stage. Three half bands showing their posterior arches only, are identified as (e_{2a}) in the 'Ov' stage only (Figure 15a). Precipitin line (f_{2a}) occurs only in the 'Hb' and 'Oc' stages. Band (g_{2a}), present in the 'Cl', 'Hb' 'Mg' and 'Oc' stages, cannot be discerned in the 'Es' stage due to non-specific precipitation. Precipitin line (m_{2a}) is observed at an anterior position in all the stages tested. Band (r_{2a}) is observed in the 'Cl', 'Hb' and 'Mg' stages, and (r'_{2a}) in the 'Hb' and 'Mg' stages. The former occurs intermediate between bands (c'_{2a}) and (b_{2a}) and the latter extends anteriorly to (r_{2a}). Band (r'_{2a}) posteriorly bisects the anterior part of (r_{2a}) (Figures 15c - 15d).

C. Stages analysed with Anti-Mg/H serum (Figures 16a - 16f and Table XIVc)

Band (b_{3a}) is found lateral to (c_{3a}) and (c'_{3a}) in all stages. Bands (c_{3a}) and (c'_{3a}) in most cases are separate, but occasionally these bands exhibit continuity (Figure 16c). The band found at the (d_3) and (e_3) positions of the unabsorbed pattern (Figure 16a) is identified as (e_{3a}) in the 'Ov' stage. Band (d_{3a}) is not observed with this absorbed serum. Band (f_{3a}) occurs in the 'Cl', 'Hb', 'Mg', 'Es' and 'Oc' stages and (g_{3a}) is observed in the 'Cl', 'Hb', 'Mg' and 'Es' stages. In the 'Mg' stage its position is partially obscured by band (f_{3a}) (Figure 16d). The most anterior migrating band (m_{3a}) is found in all stages tested.

D. Stages analysed with Anti-Es/H serum (Figures 17a - 17f and Table XIVd)

Band (b_{4a}) is observed in all stages except the 'Ov' stage.

Figure 16. Immunoelectrophoretic precipitin line patterns of six pre-hatching stages of the zebrafish, Brachydanio rerio, analysed with Anti-Mg/H serum.

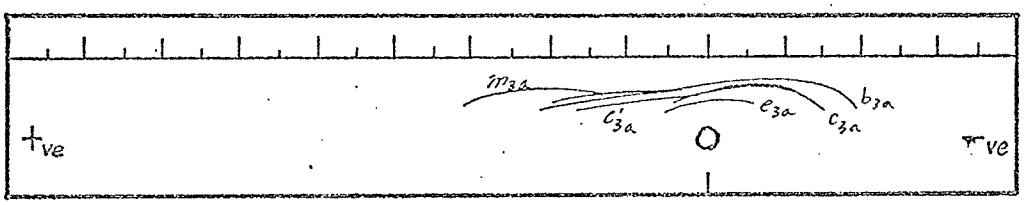


Fig. 16a Ov

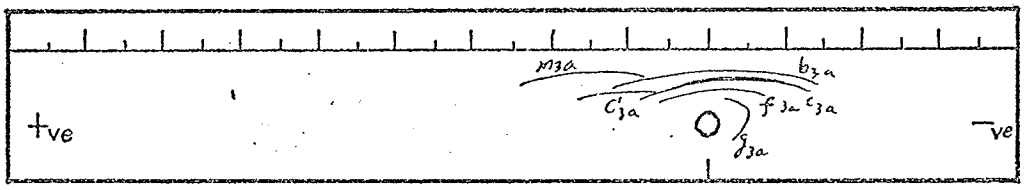


Fig. 16b C1

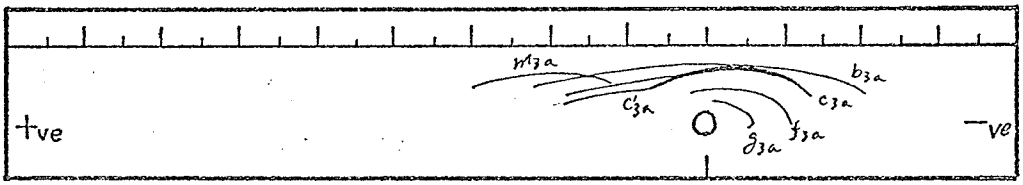


Fig. 16c Hb

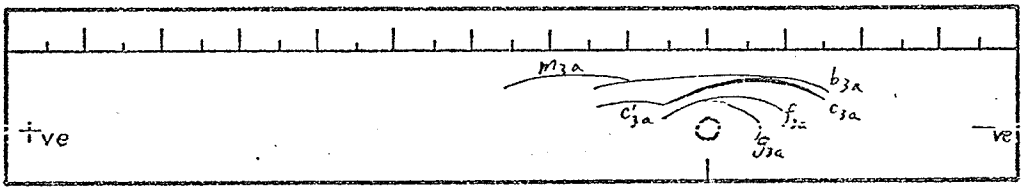


Fig. 16d Mg

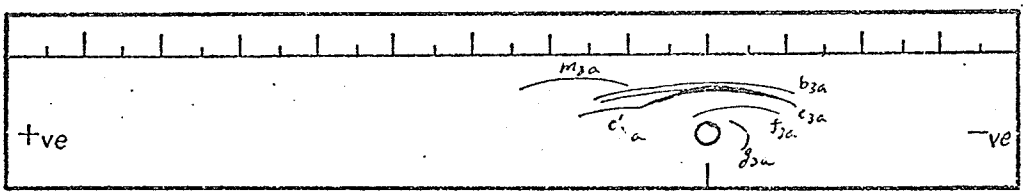


Fig. 16e Es

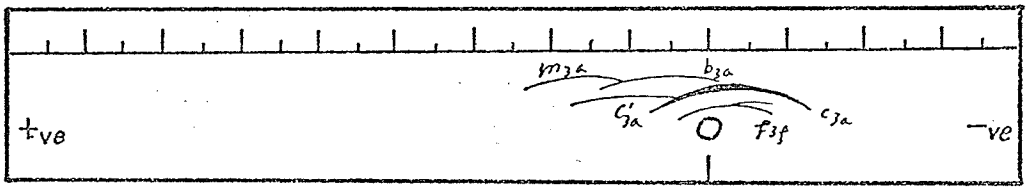


Fig. 16f Oc

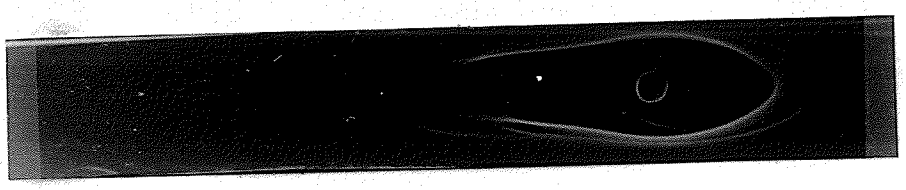


Fig. 16a Ov

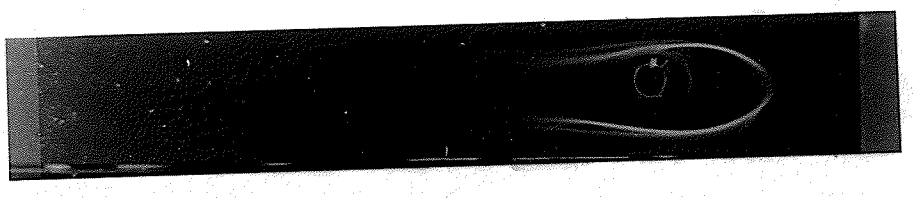


Fig. 16b Cl

Fig. 16c Hb

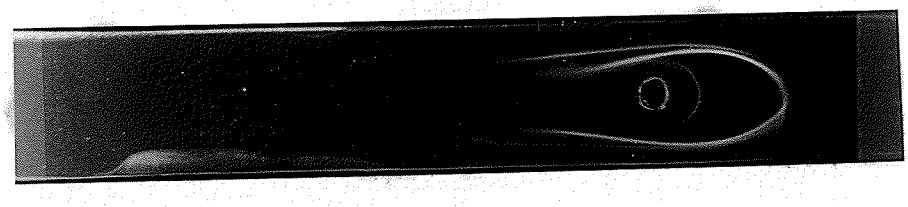


Fig. 16d Mg

Fig. 16e Es

Fig. 16f Oc

Figure 17. Immunoelectrophoretic precipitin line patterns of six pre-hatching stages of the zebrafish, Brachydanio rerio, analysed with Anti-Es/H serum.

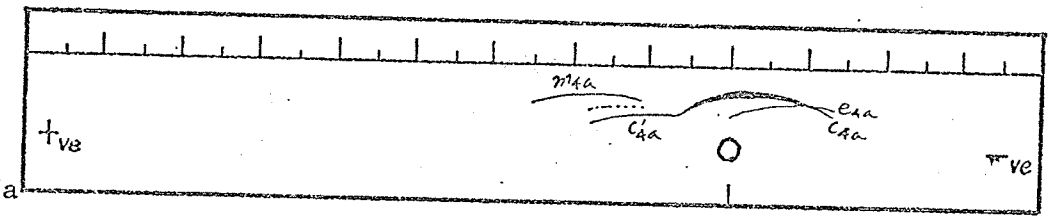


Fig. 17a Ov

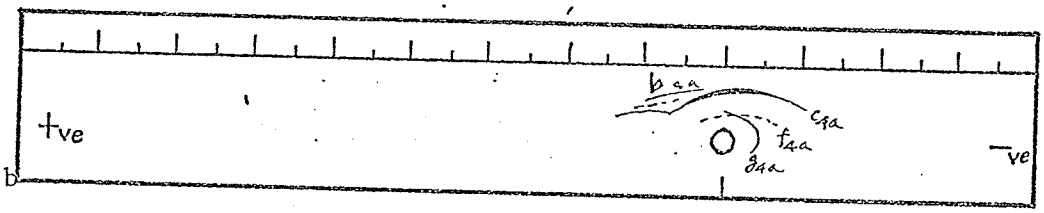


Fig. 17b Cl

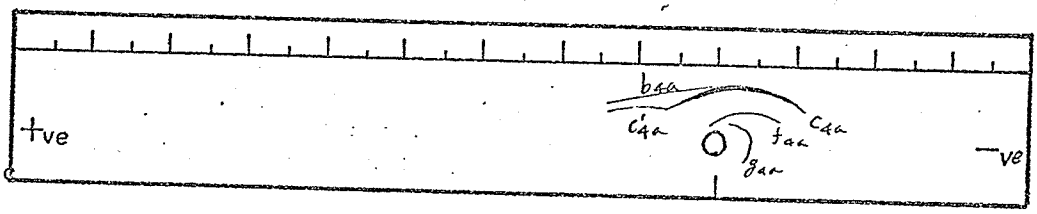


Fig. 17c Hb

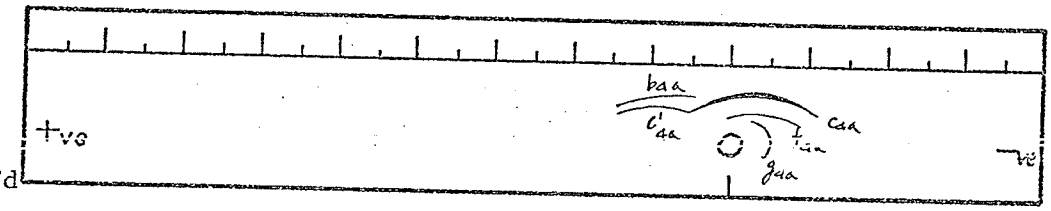


Fig. 17d Mg

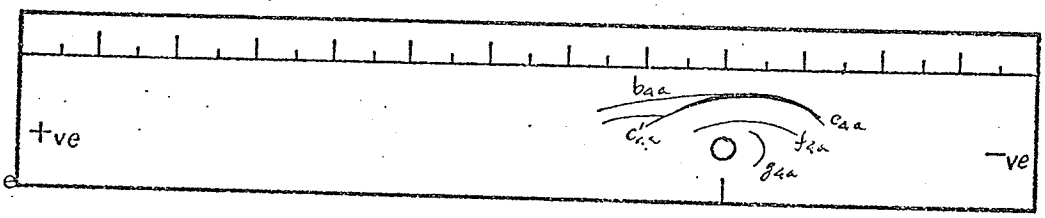


Fig. 17e Es

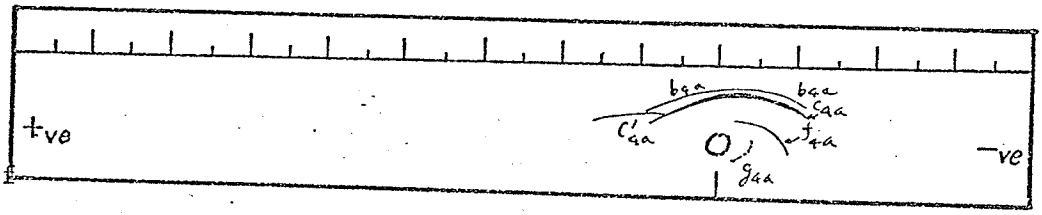


Fig. 17f Oc

Fig. 17a Ov

Fig. 17b C1

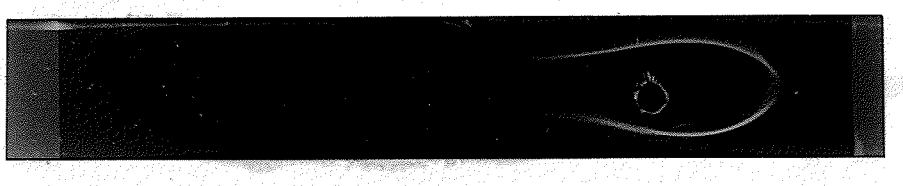


Fig. 17c Hb

Fig. 17d Mg

Fig. 17e Es

Fig. 17f Oc

In the 'Cl' stage band (b_{4a}) is faint; and in the 'Hb' and 'Mg' stages, only the anterior part of the band can be identified (Figures 17c - 17d). The bands (c_{4a}) and (c'_{4a}) are present in all stages as separate, non-continuous precipitin lines. A band identified as (e_{4a}) is observed in the 'Ov' stage only. A single band occupying the relative position of (e_4) and (f_4) of the unabsorbed pattern is known as (f_{4a}). It first appears in the 'Cl' stage and is present in all the subsequent stages tested. (g_{4a}) is also found at all stages except the 'Ov' stage. Only the 'Ov' stage shows the anterior band (m_{4a}).

E. Stages analysed with Anti-Oc/H serum (Figures 18a - 18d and Table XIVe)

The precipitin bands (b_{5a}), (c_{5a}), (c'_{5a}), (g_{5a}) and (m_{5a}) occur in the 'Ov', 'Cl', 'Hb', and 'Mg' stages. Band (d_{5a}) can be identified in the 'Hb' and 'Mg' stages. Band (e_{5a}) occurs in the 'Ov' stage only and band (f_{5a}) in the 'Mg' stage only.

Data were not obtained for the 'Es' and 'Oc' stages tested against the Anti-Oc/H serum.

IV. Immunoelectrophoretic analysis (IEA) of the hatching stage with rabbit Anti-H serum absorbed with extracts to various stages

A resumé of the results obtained when the 'H' stage antigens were tested against the Anti-H serum absorbed with 'Cl', 'Hb', 'Mg', 'Es', 'Oc' and 'H' stage homogenates, is given in Table XV and graphically presented in Figures 19a - 19f.

In this series of tests, the hatching stage was tested against aliquots of its homologous antiserum absorbed with extracts of 'Cl'

Figure 18. Immunoelectrophoretic precipitin line patterns of four pre-hatching stages of the zebrafish, Brachydanio rerio, analysed with Anti-Oc/H serum.

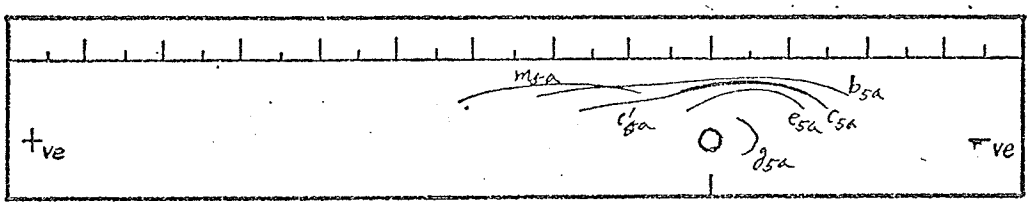


Fig. 18a Ov

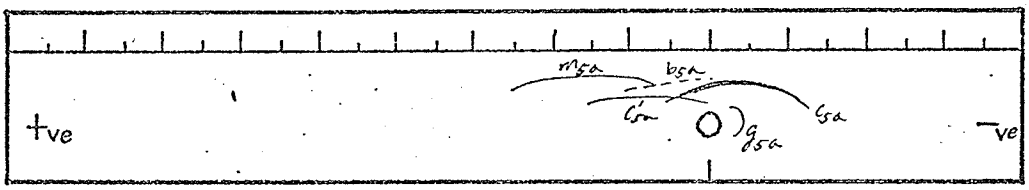


Fig. 18b Cl

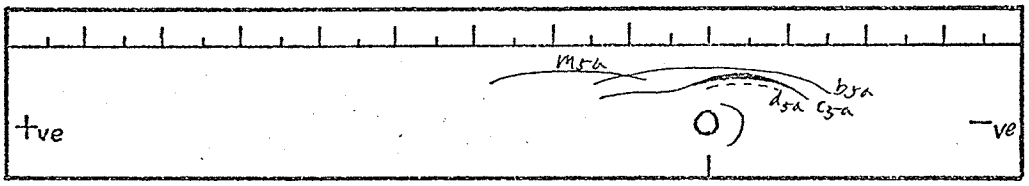


Fig. 18c Hb

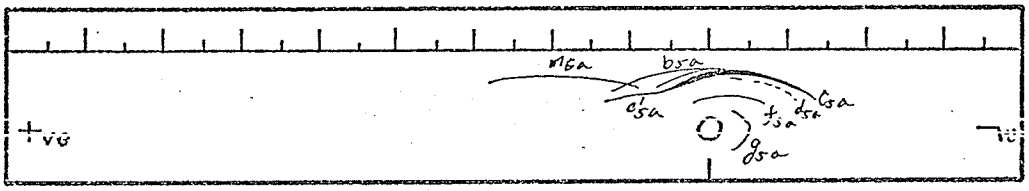


Fig. 18d Mg

Fig. 18a Ov

Fig. 18b Cl

Fig. 18c Hb

Fig. 18d Mg

Table XV

Immunoelectrophoretic analysis (IEA) of the zebrafish hatching-stage with anti-H serum absorbed respectively with six stage extracts

Stage Antigens	u'_{6a}	u''_{6a}	u'''_{6a}	v_{6a}	w_{6a}	x_{6a}	y_{6a}
Absorbed antisera							
Anti-H/Cl	+	+	+	+	0	+	+
Anti-H/Hb	+	+	+	+	±	+	+
Anti-H/Mg	+	+	+	±	+	+	+
Anti-H/Es	+	+	+	0	+	+	+
Anti-H/Oc	+	+	+	0	0	+	+
Anti-H/H	0	0	0	0	0	0	0

Symbols used:
+ Positive reaction
0 Negative reaction
± Weak reaction

Figure 19. Immunoelectrophoretic precipitin line patterns of the Hatching stage of the zebrafish, Brachydanio rerio, analysed with six stage-absorbed Anti-H sera.

Absorbed antisera =

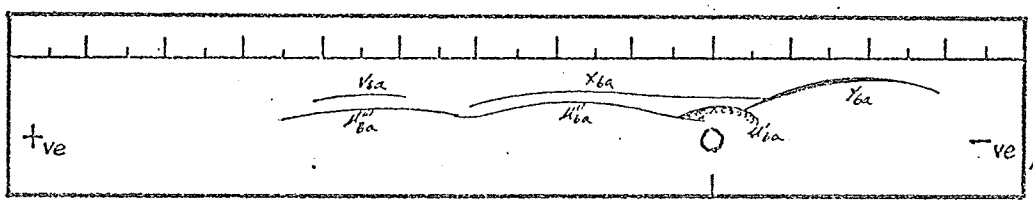


Fig. 19a Anti-H/Cl

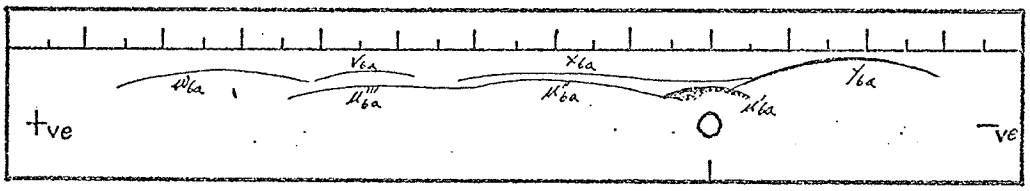


Fig. 19b Anti-H/Hb

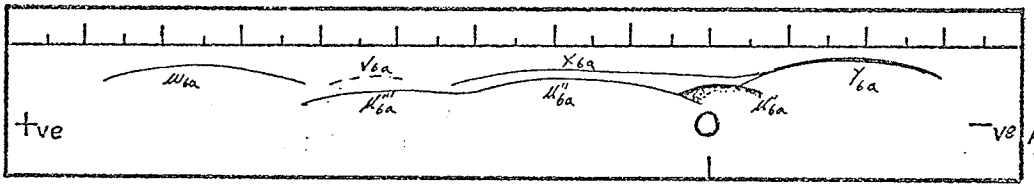


Fig. 19c Anti-H/Mg

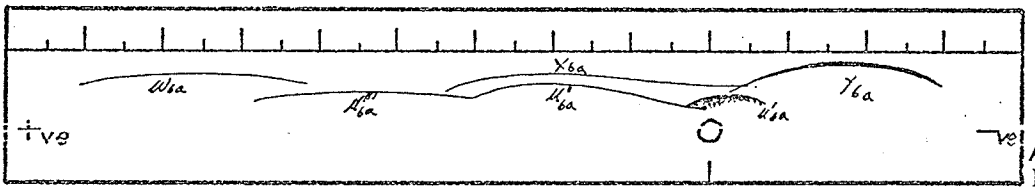


Fig. 19d Anti-H/Es

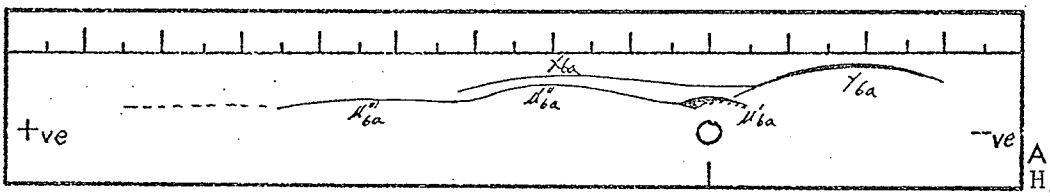


Fig. 19e Anti-H/Oc

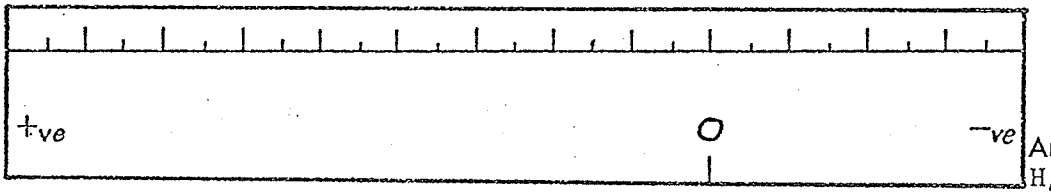


Fig. 19f Anti-H/H

Fig. 19a Anti-H/Cl

Fig. 19b Anti-H/Hb

Fig. 19c Anti-H/Mg

Fig. 19d Anti-H/Es

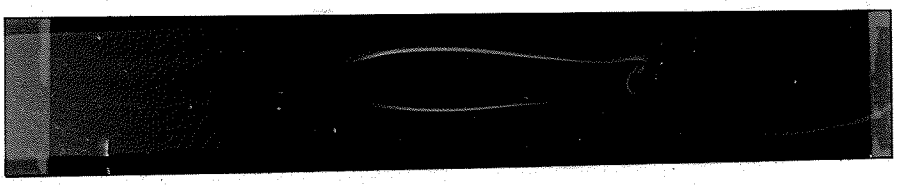


Fig. 19e Anti-H/Oc

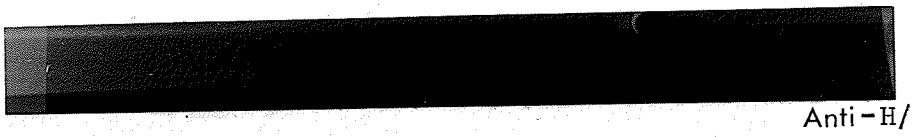


Fig. 19f Anti-H/H

(Anti-H/Cl), 'Hb' (Anti-H/Hb), 'Mg' (Anti-H/Mg), 'Es' (Anti-H/Es), 'Oc' (Anti-H/Oc) and 'H' (Anti-H/H). The Anti-H/H serum served as a control and did not show any precipitin lines (Figure 19f). A total of seven bands are observed, namely: (u'_{6a}), (u''_{6a}), (u'''_{6a}), (v_{6a}), (w_{6a}), (x_{6a}) and (y_{6a}). The precipitin lines (u'_{6a}), (u''_{6a}), (u'''_{6a}), (x_{6a}) and (y_{6a}) are present when the hatching stage is tested against Anti-H serum absorbed with different stage extracts, indicating that the bands are not common to those in earlier stages. Band (v_{6a}) shows a positive reaction in the tests with Anti-H/Cl and Anti-H/Hb sera, and a somewhat weaker precipitin reaction in the test with Anti-H/Mg serum (Figure 19c). Band (w_{6a}) is not shown with Anti-H/Cl and Anti-H/Oc sera (Figures 19a and 19f). It shows faintly in the tests with Anti-H/Hb and distinctly in tests with Anti-H/Es sera (Figures 19b and 19d).

The precipitin lines (u'_{6a}), (u''_{6a}), and (u'''_{6a}) exhibit some continuity, suggesting that these bands share certain antigenic determinant(s). Non-specific precipitation is associated with (u'_{6a}) in all cases (Figure 19a - 19e). Band (v_{6a}) is found lateral to band (u'''_{6a}). Band (w_{6a}) is the most anterior and band (y_{6a}) the most posterior band in the patterns observed. Band (x_{6a}) occurs lateral to (u'_{6a}) and (u''_{6a}).

DISCUSSION

Recent studies of the development of living organisms have been directed toward the molecular events underlying morphogenesis. Advances in molecular genetics and biochemical technology have contributed to our understanding of "differentiation" as a series of programmed gene-environmental phenomena. An analysis of translated proteins and their synthesis, metabolism, physio-chemical properties, localization, concentration levels, kinetics and specific functions all aid in our understanding of the genetic control mechanisms, and the progressive specialization of divergent cell lineages in the individual ontogeny.

Proteins possess antigenic determinants of great specificity and are particularly amenable to immunochemical analysis. In the present investigation, an analysis of seven developmental stages, namely, the 'Ov', 'Cl', 'Hb', 'Mg', 'Es', 'Oc' and 'H' stages of the zebrafish, Brachydanio rerio with their respective homologous antisera was carried out by the immunodiffusion (Ouchterlony, 1948) and immunoelectrophoretic methods (Graber and Willians, 1953). Immunoabsorption techniques were also employed. Immunoelectrophoresis separates antigens according to their net electrostatic charges and unique molecular weights while the immunodiffusion method distinguishes antigens according to molecular weight and size only. Immunoelectrophoresis was used in all analytical tests for the analysis of band patterns and immunodiffusion in the preliminary tests for positive and negative reactions in absorption analysis. Positive reactions obtained by preliminary tests were further analysed by immunoelectrophoresis with the aim of identifying specific bands in the antigenic patterns.

All the techniques employed are favourable to qualitative analysis. They are not used to directly assess the quantity of each antigenic determinant in each stage tested.

I. Problems encountered in this investigation

A. The antigen-antibody reaction is a function of the relative proportional concentration of antigens to antibodies in the test system. The relative concentrations of the heterogeneous antibodies in a given serum are fixed while the concentrations of different antigens in different stage extracts are varied. This variability was eliminated by adjusting the extracts from different stages (except the 'Ov' stage) to similar protein concentration levels by dilution with phosphate buffer adjusted to pH 7.0. The final protein concentration for each stage was measured by the method of Lowry et al, (1951) (See Appendix V).

B. According to Spar (1953), the precipitation reaction is usually positive only within a certain range of antigen and antibody concentration. Prezone (failure to precipitate when the ratio of antibody to antigen is too high) and postzone (failure to precipitate when the ratio of antibody to antigen is too low) effects may occur for some antigens in a stage extract which contains a heterogeneous mixture of antigens. The lack of detection of antigen of a low concentration may be due to the effect of "inhibition" (Boyd, 1966).

C. In the process of antibody production, one factor affecting the antibody titer is the animal's ability to respond to a certain antigenic determinant. Injection of an antigen in the same concentration into different rabbits may result in different antibody titers. This difficulty is overcome by pooling antisera from different rabbits

injected with identical stage extracts.

D. Due to the heterogeneity and fluctuating concentrations of antigenic elements in different developmental stages, a differential absorbing capacity of the stage extracts used as absorbing materials is expected (Singh, 1971).

In the present investigation, no band was found in the 'Ov', 'Cl', 'Hb', 'Mg', 'Es', 'Oc' and 'H' stages tested against the Anti-Cl, Anti-Hb, Anti-Mg and Anti-Es sera absorbed respectively with 'Cl', 'Hb', 'Mg' and 'Es' stage homogenates (Table VIII - XI). This implies that the antigenic patterns of the four developmental stages 'Cl', 'Hb', 'Mg' and 'Es' are qualitatively similar; and that stage-specific antigens, if present, are either prezone of postzone and hence not detectable. When 'Oc' homogenates were used to absorb anti-stage sera other than Anti-Oc serum, a faint precipitin reaction was observed (Tables VIII - XI). This implies that certain antigenic components in the 'Oc' stage are either absent, or present in a concentration insufficient for adequate absorption of homologous antibodies. When the 'Oc' stage extract was used to absorb its homologous antiserum, a diffuse precipitation was discovered in the immunodiffusion test (Table XII). This may be due to incomplete absorption, or to the presence of a masked form of antigenic determinant. Presumably, the masked antigen, subsequent to injection into the rabbit, is unmasked with a consequent production of its homologous antibody. The masked antigen molecules in the 'Oc' extract used to absorb the Anti-Oc serum is unable to absorb or to precipitate its homologous antibody molecules. As a result, the serum cannot be fully

absorbed, and a precipitation reaction may be obtained with other stage-extracts containing the unmasked form of this antigen.

II. Discussion of the individual antigen bands arising at different stages with the use of anti-stage sera

A. Analysis of bands (a), (b) and (b')

The appearance of antigens (a), (b) and (b') in seven developmental stages tested with six anti-stage sera is summarized in Tables XVIa - XVIc.

Positive reactions of the precipitin band (a) in the tests analysed with all six anti-stage sera, and the negative results obtained from the absorption tests (Table VIII - XIV), strongly imply that the antigen (a) is present in all the stages analysed. Fluctuations in the appearance of (a) at different stages with different anti-stage sera may be attributed to variations in the Anti-(a) antibody titer in the anti-stage sera used. Initially it may seem that the Anti-(a) antibodies in the Anti-Es serum attain an optimal titer as indicated by the presence of (a_4) in all the stages tested except the 'H' stage. Since only one band appears in the posterior extremity of the band patterns in the analysis with Anti-Cl serum, it is difficult to distinguish between the bands (a_1) and (b_1). This band therefore, may be either band (a_1) or (b_1), or a composite (a_1 - b_1) band.

Precipitin reactions indicate that the relative concentration of (a) is highest in the 'Ov' and declines after fertilization. In the 'Cl' stage, faint precipitin lines, as shown by (a_3) and (a_4) as well as the lack of detection of (a_2) substantiate that the antigen (a) concentration is low at this stage. At the 'Hb' stage, the antigen concentration rises slightly as evidenced by the occurrence of positive

Table XVI

Analysis of antigens (a), (b), and (b') in seven zebrafish embryonic stages with anti-stage sera

Table XVIa Analysis of band (a)						
Bands	a_1	a_2	a_3	a_4	a_5	a_6
Stages						
Ov	+	+	+	+	+	+
Cl	=		±	±	+	+
Hb	=	+		+		+
Mg	=			+		±
Es	=			+	+	
Oc	=			+	+	±
H	+	+			±	±

Table XVIb Analysis of band (b)						
Bands	b_1	b_2	b_3	b_4	b_5	b_6
Stages						
Ov	+	+	+	+	+	
Cl	=	+	+	+	+	
Hb	=	+	+	+	+	
Mg	=	+	+	+	+	
Es	=	+	+	+	+	
Oc	=	+	+	+	+	
H						

Table XVIc Analysis of band (b')						
Bands	b'_1	b'_2	b'_3	b'_4	b'_5	b'_6
Stages						
Ov						
Cl		+				
Hb		+				
Mg		+				
Es		+				
Oc		+				
H						

Symbols used:
+ Positive precipitin reaction
± Faint precipitin reaction
= Precipitation could be either of two identifiable bands
? Extremely faint band

precipitations for bands (a_2) and (a_4). The Anti-(a) antibody titer in the Anti-H serum may be very low since only the 'Ov' yields a positive reaction. The absence of the bands (a_{1a}), (a_{3a}), (a_{4a}), (a_{5a}) at any stage in the absorption tests when the 'H' stage extract is used as absorbant (Table XIV) indicates that the antigen (a) may be present in the 'H' stage. Thus the single bands formed at the (a_2 - b_2) positions in the 'H' stage would most likely be (a_2) and (a_5) respectively (Table XVIa, Figures 9g and 12g), and the band (a_6 - b_6) in the 'Ov' stage would be (a_6) (Figure 7a). Thus the anti-(a) antibodies in the Anti-H serum attain an antibody titer equal to that of the Anti-Es serum. The presence of band (a_{2a}) in all the pre-optic cup stages may be attributed to an incomplete absorption of the Anti-(a) antibodies in the Anti-Hb serum with the 'H' stage extract (Table XIVb, Figures 15a - 15e).

Band (b) exists in all stages with the exception of the 'H' stage (Table XVIb). The appearance of bands (b_{2a}), (b_{3a}) and (b_{5a}) in the absorption (IEA) tests (Tables XIVb - XIVe) also implies that the band (a_6 - b_6) in the 'Ov', 'Cl', 'Hb', 'Mg', 'Oc' and 'H' stages would be (a_6) (Table XVIb). It is assumed that the 'H' stage extract does not possess the antigen (b) for the absorption of the Anti-(b) antibodies in the sera used.

Antigen (b') is revealed by analysis with Anti-Hb serum. This antigen is of low antigenicity at all stages, except the 'Hb' stage. Only the Anti-Hb serum (Table XVIc) has a sufficiently high Anti-(b') antibody titer to give a positive reaction. The strong precipitin lines of (b'_2) in the 'Cl', 'Hb', 'Mg' and 'Oc' stages tested against the Anti-Hb/H absorbed serum (Table XIVb) support this interpretation.

The absence of (b'_{2a}) in the 'Es' stage with this absorbed serum may be due to a poor resolution after absorption. The possibility exists that band (b'_2) might be caused by antigenic impurities.

B. Analysis of antigens (c) and (c')

The appearance of antigens (c) and (c') in the seven zebrafish developmental stages tested with six anti-stage sera is summarized in Tables XVIIa - XVIIb.

The absence of (c_1), (c'_1), (c_2), (c'_2), (c_3), (c'_3), (c_4), (c'_4), (c_5), (c'_5), (c_6) and (c'_6) in the 'H' stage, and (c_6) and (c'_6) in all the stages (Tables XVIIa and XVIIb) suggests that (c) and (c') are absent in the 'H' stage. This is also supported by the appearance in the 'Ov', 'Cl', 'Hb', 'Mg', 'Es' and 'Oc' stages, of (c_{1a}) and (c'_{1a}) analysed with the Anti-Cl/H serum; of (c_{2a}) and (c'_{2a}) with the Anti-Hb/H serum; of (c_{3a}) and (c'_{3a}) with the Anti-Mg/H serum; of (c_{4a}) and c'_{4a}) with the Anti-Es/H serum; and of (c_{5a}) and (c'_{5a}) with the Anti-Oc/H serum (Tables XIVa - XIVe). Although no data have been obtained to show the presence of (c_{5a}) and (c'_{5a}) in the 'Es' and 'Oc' stages with the Anti-Oc/H serum, the presence of these two antigens may be deduced. In all the tests shown, (c) and (c') are closely related antigens possibly sharing a common antigenic determinant. This is seen by the continuity of these two bands in the electrophoretic patterns (Figures 8 - 12). The consistent, marked appearance of these two bands indicates that throughout the stages tested they are present in a relatively uniform and high concentration. Their stable electrophoretic mobilities also point to a rather rigid molecular structure.

Table XVII

Analysis of antigens (c) and (c') in seven zebrafish embryonic stages with anti-stage sera

Table XVIIa Analysis of band (c)

Bands / Stages	c_1	c_2	c_3	c_4	c_5	c_6
Ov	+	+	+	+	+	
Cl	+	+	+	+	+	
Hb	+	+	+	+	+	
Mg	+	+	+	+	+	
Es	+	+	+	+	+	
Oc	+	+	+	+	+	
H						

Table XVIIb Analysis of band (c')

Bands / Stages	c'_1	c'_2	c'_3	c'_4	c'_5	c'_6
Ov	+	+	+	+	+	
Cl	+	+	+	+	+	
Hb	+	+	+	+	+	
Mg	+	+	+	+	+	
Es	+	+	+	+	+	
Oc	+	+	+	+	+	
H						

Symbols used:
+ Positive precipitin reaction
± Faint precipitin reaction
= Precipitation could be either of two identifiable bands
? Extremely faint band

C. Analysis of antigens (d), (e), (f) and (g)

The appearance of antigens (d), (e), (f) and (g) in the seven zebrafish developmental stages tested with six anti-stage sera is summarized in Tables XVIIIa - XVIIId.

Antigen (d) occurs quite consistently in the 'Ov' stage when tested against all prehatching-stage sera (Table XVIIIa). In the 'Hb' stage, it appears faintly when analysed with the same antisera. The occurrence of (d_2) and (d_3) in the 'Mg' and 'Es' stages and the disappearance of (d_1), (d_4) and (d_5) in the same stages make the interpretation difficult. The absence of any positive reaction in the immunoabsorption tests with 'Ov', 'Cl', 'Hb', 'Mg' and 'Es' extracts tested against prehatching stage sera absorbed with 'Cl', 'Hb', 'Mg' or 'Es' homogenates (Tables VIII - XI) suggests that antigen (d) is present in the 'Ov', 'Cl', 'Hb', 'Mg' and 'Es' stages. The appearance of (d_5) in the 'Ov', 'Cl' and 'Hb' (Tables VI and XVIIIa), indicates that (d) antibodies, are present in the anti-Oc serum and that (d) antigens hence must be present in the 'Oc' stage. With the exception of (d_5) antigen (d) was not detected in the 'Oc' stage. This implies that antigen (d) is sufficiently antigenic to induce anti-(d) antibodies, though present in toolow a concentration in the 'Oc' stage to be detected.

Band (d) is not detected in the 'H' stage when tested against the anti-stage sera, nor is it found in any stage when tested against the Anti-H serum. This implies that antigen (d) is absent in the 'H' stage. When prehatching stages are tested against the anti-prehatching stage sera absorbed with hatching stage extracts, antigen (d) is detected

Analysis of antigens (d), (e), (f) and (g) in seven zebrafish embryonic stages with anti-stage sera

Table XVIIIa Analysis of band (d)

Bands	d_1	d_2	d_3	d_4	d_5	d_6
Stages						
Ov	+	+	±	+	+	
Cl	+	+	±	±	+	
Hb	±	+	±	±	±	
Mg		+	+			
Es		+	+	±		
Oc					+	
H						

Table XVIIIb Analysis of band (e)

Bands	e_1	e_2	e_3	e_4	e_5	e_6
Ov	+	+	+	+	+	
Cl						
Hb						
Mg						
Es						
Oc						
H						

Table XVIIIc Analysis of band (f)

Bands	f_1	f_2	f_3	f_4	f_5	f_6
Stages						
Ov						
Cl		+	+	±	±	
Hb		+	+	+	+	
Mg		+	+	+	+	
Es		+	+	+	+	
Oc			+	±	+	
H						

Table XVIIId Analysis of band (g)

Bands	g_1	g_2	g_3	g_4	g_5	g_6
Stages						
Ov						
Cl	+	+	±	+	+	+
Hb	+	+	±	+	±	+
Mg	+	+	±	+	+	+
Es	+	+	±	+	+	+
Oc	+	+	±	+	+	+
H						

Symbols used:
+ Positive precipitin reaction
± Faint precipitin reaction
= Precipitation could be either of two identifiable bands
? Extremely faint band

in the 'Ov', 'Cl', 'Hb', 'Mg' and 'Es' stages (Table XIVa - XIVe). This also confirms that antigen (d) is absent in the 'H' stage. The appearance of (d_{1a}) in the 'Mg' and 'Es' stages, and (d_{5a}) in the 'Mg' stage (Table XIVe) help to interpret the data obtained from the unabsorbed IEA tests where their homologues i.e. (d) and (d_5) are not found in the corresponding stages (Table XVIIIa). The absence of the homologues may be attributed to masking, or to a subminimal effect.

Antigen (e) is detected only in the 'Ov' stage with all sera to the prehatching stages (Table XVIIIb). Antigen (f) is detected in the 'Cl', 'Hb', 'Mg', 'Es' and 'Oc' stages (Table XVIIIc). The difference between (e) and (f) is a difference in electrophoretic mobility. Band (e) is found slightly posterior to (f) which has an electrophoretic mobility approximating zero. When 'Ov' is tested against the Anti-Cl , Anti-Hb , Anti-Mg and Anti-Es sera absorbed respectively with the 'Cl', 'Hb', 'Mg' and 'Es' extracts, no precipitin lines are observed suggesting the absorption of all antibodies including antibodies to (e). It is possible that antigens (e) and (f) might actually share a common antigenic determinant. It may also be that (e) is a precursor of (f), and that subsequent to fertilization, (e) is changed to (f). The molecule becomes more negative, that is, the molecule acquires the electrophoretic behavior of antigen (f). A low titer of antibodies to (f) in the Anti-Cl serum may explain the nondetectability of (f_1) in any of the developmental stages tested. This low titer may be due to the weak antigenicity of antigen (f) in the 'Cl' extract, or to an insufficient concentration of (f) to induce an antibody response. The absence of (f_5) in the 'Ov' stage, the presence of a weak precipitin line for (f) in the 'Cl' stage and the

presence of clear precipitin lines in the remaining prehatching stages, implies that antigen (f) is accumulating throughout the early developmental stages up to the 'Es' stage, at which time a decrease in concentration begins. This is supported by the absence of (f_2) in the 'Oc' stage (Figure 9f). Both (e) and (f) are absent in the 'H' stage (Tables XVIIIb and XVIIIc). That (f) does not occur in the hatching stage is further supported by the observation that (f_{2a}), (f_{3a}), (f_{4a}) and (f_{5a}) are identified at various stages with embryo homogenates tested against stage antisera absorbed with the hatching stage extracts (Tables XIVb - XIVe).

(e_{2a}), (e_{3a}), (e_{4a}) and (e_{5a}) are detected when the 'Ov' is tested against the Anti-Hb/H, Anti-Mg/H, Anti-Es/H and Anti-Oc/H sera (Tables XIVb - XIVe, Figures 15a, 16a, 17a, 18a, 19a) however, (e_{1a}) is not detected. This suggests that changes may take place in the antiserum in some instances as a result of absorption. What the changes are can only be conjectural.

Antigen (g) is detected in all prehatching stages analysed with all anti-stage sera (Tables XVIIId). The existence of (g_6) in several of the prehatching stages, and the absence of (g_1), (g_2), (g_3), (g_4), (g_5) and (g_6) in the 'H' stage suggest that the (g) antigen is either masked or present at a subminimal level at this stage. In the latter case, the antigenicity of (g) would have to be quite high at the hatching stage. Antigen (g) is not detected in the 'Ov' stage with any of the anti-stage sera. The bands (g_{4a}) and (g_{5a}) however are detected in the absorption IEA tests at the ovary stage (Figures 14a and 18a). It is possible that (g) exists at a low concentration level in the 'Ov' and starts accumulating after fertilization as revealed by the number of times it appears in the 'Cl', 'Hb' and 'Mg' stages.

It decreases to a subminimal level after the 'Oc' stage (Table XVIIId). Since antigen (g) is found almost uniformly in all the embryonic stages the absence of (g_3) at the 'Es' stage is difficult to interpret.

The identification of the bands (g_{1a}), (g_{2a}), (g_{3a}), (g_{4a}) and (g_{5a}) in the absorption IEA tests (Table XIVa - XIVe) also give support to the interpretation that antigen (g) at the 'H' stage is either masked, or is highly antigenic but present at a subminimal level. In either cases, (g_a) is expected to be present in the absorption tests. In the former case the result would the the non-absorption of Anti-g antibodies in the anti-prehatching stage sera whereas in the latter case, the result would be an incomplete absorption of the anti-prehatching-stage sera.

D. Analysis of the band complex antigens (h'), (h"), (j), (k), (l') and (l"), and antigen (i)

The appearance of antigens (h'), (h"), (j), (k), (l') and (l"), and (i) in the seven zebrafish developmental stages tested with six anti-stage sera is summarized in Tables XIXa - XIXg.

(h') and (h") are two closely associated antigens as indicated in the analysis by all anti-stage sera. They are identified in all developmental stages tested (Tables XIXa, XIXb). They are distinguished from each other only by a posterior flaring of the 'band complex' (Figure 8c). Their molecular structures probably have similar net electrical charges and molecular weights. The analysis of the 'Es' stage with anti-stage sera against the 'Cl', 'Es', 'Oc' and 'H' shows that there is a tendency for (h') and (h") to combine into one band (Tables II, V, VI and VII, Figure 2). In all the tests, (h') and (h") appear to accumulate electropositive charges prior to the 'Oc' stage

Table XIX

Analysis of antigens (h'), (h''), (i), (j), (k), (l') and (l'') in seven zebrafish embryonic stages with anti-stage sera

Table XIXa Analysis of band (h')

Bands	h'_1	h'_2	h'_3	h'_4	h'_5	h'_6
Stages						
Ov	+	+	+	+	+	+
Cl	+	+	+	+	+	+
Hb	+	+	+	+	+	+
Mg	+	+	+	+	=	=
Es	=	+	+	=	=	=
Oc	+	+	+	=	+	=
H	+	+	+	+	+	=

Table XIXb Analysis of band (h'')

Bands	h''_1	h''_2	h''_3	h''_4	h''_5	h''_6
Stages						
Ov	+	+	+	+	+	+
Cl	+	+	+	+	+	+
Hb	+	+	+	+	+	+
Mg	+	+	+	+	=	=
Es	=	+	+	=	=	=
Oc	+	+	+	=	+	=
H	+	+	+	+	+	=

Table XIXc Analysis of band (i)

Bands	i_1	i_2	i_3	i_4	i_5	i_6
Stages						
Ov	+	+	+	+	+	+
Cl	+	+	+	+	+	+
Hb	+	+	+	+	+	+
Mg	+	+	+	+	+	+
Es	+	+	+	+	+	+
Oc	+	+	+	+	+	+
H	+	+	+	+	+	+

Table XIX continued

Table XIXd Analysis of band (j)

Bands	j_1	j_2	j_3	j_4	j_5	j_6
Stages						
Ov		+	±	±		
Cl		±	=		=	
Hb		+	=		=	
Mg			=		=	
Es		±	+	+	=	
Oc		+	=	+	=	
H						

Table XIXe Analysis of band (k)

Bands	k_1	k_2	k_3	k_4	k_5	k_6
Stages						
Ov	+	+	+	+	+	
Cl	+	+	=	+	=	
Hb	+	+	=	+	=	
Mg	=	+	=	+	=	
Es	=	+	=	+	=	
Oc	=	+	=	+	=	
H	=	+	+	+		

Table XIXf Analysis of band (1')

Bands	$1'_1$	$1'_2$	$1'_3$	$1'_4$	$1'_5$	$1'_6$
Stages						
Ov	+	+	+	+	+	+
Cl	+	+	+	+	+	+
Hb	+	+	+	+	+	+
Mg	=	+	+	+	+	+
Es	=	+	=	+	+	+
Oc	=	+	+	+	+	+
H	=	+	+	+	+	+

Table XIXg Analysis of band (1")

Bands	$1''_1$	$1''_2$	$1''_3$	$1''_4$	$1''_5$	$1''_6$
Stages						
Ov	+	+	+	+	+	+
Cl	+	+	+	+	+	+
Hb	+	+	+	+	+	+
Mg	+	+	+	+	+	+
Es	+	+	+	+	+	+
Oc	+	+	+	+	+	+
H	+	+	+	+	+	+

Symbols used:
+ Positive precipitin reaction
± Faint precipitin reaction
= Precipitation could be either of two identifiable bands
? Extremely faint band

as evidenced by a progressive posterior migration with reference to band (c) (Figure 9e).

Antigen (i) remains at a constant concentration level throughout development and shows a constant electrophoretic mobility in the prehatching stages (Table XIXc). A slight change in the shape of the precipitin arch of antigen (i) in the 'H' stage suggests that this molecule has undergone a slight change in structure subsequent to the 'Oc' stage. The absence of precipitin band (i) in the experiments with stages tested against anti-stage sera absorbed with the 'H' stage extract (Table XIV), supports the interpretation that antigen (i) in the hatching stage, though demonstrating a slightly different electrophoretic behavior, nevertheless shares antigenic determinants with the antigen (i) of the other prehatching stages.

Antigens (j) and (k) as analysed by various anti-stage sera occasionally exhibit a slight fluctuation in electrophoretic mobility. The failure of detection of (j_1) in any of the stages (Tables II and XIXd) may be attributed to the low antigenicity of antigen (j) at the cleavage stage and consequently to an inability to induce enough anti-(j) antibodies in the Anti-Cl serum. That band (j) is not detected in the 'H' stage with any anti-stage sera (Tables II - VI) and in any stage tested against Anti-H serum (Table VI, XIXd) may imply that (j) is absent from the 'H' stage. However, when the 'H' stage extract is used to absorb anti-stage sera, band (j) is not detected (Table XIV). An alternative explanation would be that antigen (j) is present in the 'H' stage but masked by the 'band complex' when the 'H' stage is tested against anti-prehatching stage sera. Similarly, (j_2) in the 'Mg' stage (Table III) and (j_4) in the 'Cl',

'Hb' and 'Mg' stages (Table V) may overlap with (k_2) and (k_4) respectively in the unabsorbed tests. Anti-Mg serum (Table IV) and Anti-Oc serum (Table VI) poorly resolve the band (j) and (k) as indicated by the appearance of a single band at the (j - k) position in some instances. The precipitin lines (k_2), (k_3) and (k_4) in the 'H' stage equally well could be (j_2), (j_3) and (j_4) or a composite precipitin line made up of the two antigens.

Because of the proximity of these bands to the position of band (1'), they are identified as (k) rather than (j). The absence of (k_6) (Table XIXe) may be attributed to the low antigenicity of the (k) antigen in the 'H' stage. That antigen (k) may be present in the 'H' stage is confirmed by its absence in tests utilizing antisera absorbed with 'H' extracts (Table XIV).

Bands (1') and (1") occur consistantly throughout all developmental stages tested (Tables XIXf - XIXg). The former shows a minor electrophoretic fluctuation throughout the early developmental stages, with the highest positive mobility in the 'Ov' stage and the lowest mobility in the 'Es' and 'H' stages. This fluctuation is prominent in the analysis with all antisera except the Anti-Hb serum. A decrease in the positive mobility of an antigen may be indicative of an increase in positive charges.

The cathodic half of the band complex is made up of (h') and (h") and the anodic half of the complex is made up of (j), (k), (1') and (1"). The bands (1") and (h') constitute the two extremes of the band complex. It is not known if the tails of the bands (j), (k), (1') or (1") are continuous with, or identical to the anodic tails of (h') and/or (h"). Although the 'band complex' appears to be composed of

six identifiable bands, the actual band number might be reduced to four. On the other hand, the 'band complex' is thick and may be composed of a number of non-identifiable bands. The bands (h'), (h''), (k) and (l') display similar fluctuation in their electrophoretic mobilities and the possibility that they are actually composed of two antigens should not be ruled out. The presence of non-specific precipitation in addition makes an interpretation of the 'band complex' difficult.

E. Analysis of (m), (m_a), (n), (n'), (o), (p) and (q)

The appearance of antigens (m), (m_a), (n), (n'), (o), (p) and (q) in the seven zebrafish development stages tested with six anti-stage sera is summarized in Tables XXa - XXg.

Analysis of antigen (m) with various anti-stage sera (except 'Anti-Mg' serum) gives consistant results (Tables II - VII and XX). With Anti-Mg serum, (m_3) is only observed in the 'Ov' and the 'Es' stages (Tables IV and XXa). A merging of the (m_3) and (n_3) bands is possible in the 'Cl', 'Hb', 'Mg', 'Oc' and 'H' stages (Figures 10a - 10c, 10e - 10f). This merging will manifest itself in the form of a precipitin arch wider than each separately. The failure of detection of (m_6) in the 'Ov' stage (Table VII, XXa) may be due to masking by the 'band complex' (Figure 12a). All other stages tested against Anti-H serum show the presence of the antigen (m). When the 'H' extract is used to absorb sera to the prehatching stages (Tables XIVa - XIVe), band (m_a) may be observed in most cases. The implication is that two antigens with identical electrophoretic mobilities and diffusion rates occupy the same relative position in the electrophoretic tests. The antigen observed in the absorption test, which is masked by

Table XX

Analysis of antigens (m), ($m_{a'}$), (n), (n'), (o), (p) and (q) in seven zebrafish embryonic stages with anti-stage sera

Table XXa Analysis of band (m)

Bands	m_1	m_2	m_3	m_4	m_5	m_6
Stages						
Ov	+	+	+	+	+	
Cl	+	+		+	+	+
Hb	+	+		+	+	+
Mg	+	+		+	+	+
Es	+	+	+	+	+	+
Oc	+	+		+	+	+
H	+	+		+	+	+

Table XXb Analysis of band ($m_{a'}$)

Bands	m_{a_1}	m_{a_2}	m_{a_3}	m_{a_4}	m_{a_5}	m_{a_6}
Stages						
Ov	+	+	+	+	+	
Cl	+	+	+		+	
Hb	+	+	+		+	
Mg	+	+	+		+	
Es	+	+	+			
Oc	+	+	+			
H						

Table XXc Analysis of band (n)

Bands	n_1	n_2	n_3	n_4	n_5	n_6
Stages						
Ov	+		+	±	+	
Cl	+	+	+	+	+	
Hb	+	+	+	+	+	
Mg	+	+	+	+	+	
Es	+	+	+	+	+	
Oc	+	+	+	+	+	
H	+	+	+	+	+	

Table XX continued

Table XXd Analysis of band (n')

Bands	n'_1	n'_2	n'_3	n'_4	n'_5	n'_6
Stages						
Ov					+	
Cl			?	+		
Hb			±	+	?	
Mg			±	+	+	
Es			±	+	+	
Oc				+	+	
H			+	+	+	

Table XXe Analysis of band (o)

Bands	o_1	o_2	o_3	o_4	o_5	o_6
Stages						
Ov	+	+	+	+	+	+
Cl			+	+	+	
Hb	±	±	±	+	±	
Mg	±	±		+	+	
Es	+	±	±	+	+	
Oc	+	+	±	+	+	
H	+	+	+	+	±	

Table XXf Analysis of band (p)

Bands	p_1	p_2	p_3	p_4	p_5	p_6
Stages						
Ov	+	+	+	±	+	±
Cl		+	+	+	+	
Hb	±	+	+	+	+	
Mg	±	+	+	+	+	
Es	±	+	+	+	+	
Oc	±	+	+	+	+	
H	±	+	+	+	+	

Table XXg Analysis of band (q)

Bands	q_1	q_2	q_3	q_4	q_5	q_6
Stages						
Ov			+		+	
Cl					±	
Hb					+	
Mg					+	
Es					+	
Oc					+	
H					+	

Symbols used:
+ Positive precipitin reaction
± Faint precipitin reaction
= Precipitation could be either of two identifiable bands
? Extremely faint band

(m) in the non-absorbed tests, is referred to as antigen (m_a). This antigen is absent in the 'H' stage since (m_a) appears in the tests with anti-stage sera absorbed with 'H' extracts.

Although (n_6) is not detected in any of the stages with Anti-H serum, its existence at the 'H' stage however is indicated by the presence of bands (n_1), (n_2), (n_3), (n_4) and (n_5) at the 'H' stage (Table XXc). The absence of (n) antibodies in the Anti-H serum might be a consequence of a change in antigenicity of this particular molecule at the 'H' stage, or to a lack of sensitivity of the rabbit towards this injected antigen.

Analysis of the 'Ov' stage with Anti-Hb serum shows an extention of the antigen (m_2) into the (n_2) position possibly masking the latter (Figure 9a).

Antigen (n'_4) is detected in all stages except 'Ov' with Anti-Es serum (Tables V and XXd). Only (n'_5) is detected in the 'Ov' stage with Anti-Oc serum (Figure 12a). The position of (n'_5) in the 'Ov' pattern (Figure 12a), as compared with the position of its homologues in the other stage patterns, is more anterior and becomes lateral to band (o_5). Antigens (m), (n), (n') and (o) in the 'Ov' when compared to their homologous antigens obtained with other stage homogenates tested against all anti-stage sera are found to occupy a somewhat more anterior position in the gel. What causes this apparent stage-specific difference in the electrophoretic mobility of these 'Ov' antigens is not known. A strong precipitin line for (n'_3) in the 'H' stage (Figure 10g) and a less well defined precipitin line for (n'_3) in the 'Cl', 'Hb', 'Mg' and the 'Es' stages (Table XXd, Figures 10a - 10d) demonstrate that the concentration of the (n') antigen is higher at the 'H' stage. The

The absence of (n_1') and (n_2') with Anti-Cl and Anti-Hb sera points to a comparatively lower antibody level for the antigen (n') in the 'Cl' and 'Hb' stages. The accumulation of the (n') antigen in the 'Mg' stage is evident by the weak Anti-(n') titer in the Anti-Mg serum. The absence of (n_6') with Anti-H serum may be due to a low antigenicity, or to a lack of response by the animal to this particular antigen.

Precipitin bands (o_1), (o_2), (o_3) are weaker than bands (o_4) and (o_5). This suggests that Anti-Es and Anti-Oc sera may have a higher titer of anti-(o) antibodies than Anti-Cl, Anti-Hb and Anti-Mg sera. Antigen (o) has an initially higher concentration in the 'Ov' stage than in the other stages (Table XXe). The absence of (o_6) in the post-fertilization stages is due to the low Anti-(o) titer in the Anti-H serum. That an extensive change occurs in the molecular structure of antigen (o) during development is revealed by the fluctuation in the electrophoretic behavior of this antigen. Variations in anti-(o) titers in different antisera may account for the degree to which the antigens (o_2) and (o_3) are detectable.

Antigen (p) generally occurs in all developmental stages tested with all anti-prehatching stage sera. The Anti-Cl serum may have a weaker Anti-(p) titer. The absence of (p_1) in 'Cl' may be attributed to an "inhibition" effect. The identification of a weak (p_6) precipitin line only in the 'Ov' stage, implies that the Anti-H serum possesses a weak Anti-(p) titer. A slight fluctuation in the electrophoretic mobility of antigen (p) is observed in the analysis with different anti-stage sera. This fluctuation is neither uniform nor consistant, and reflects an external cause rather than an internal change in the molecular properties of the antigen (p).

The antigen (q) is the most anterior band observed with the Anti-Mg and the Anti-Oc sera. The band (q_3) of the ovary is comparatively weaker than band (q_5) of the same stage. This may be due to a lower anti-(q) titer in the Anti-Mg serum than in the Anti-Oc serum. Band (q_5) is found to be present as a stronger precipitin line in the 'Ov' stage than in the other stages tested against Anti- Oc serum. During cleavage, the concentration of antigen (q) appears to decrease slightly (Figure 12b) only to build up again commencing with the 'Hb' stage. The failure of detection of (q_1), (q_2), (q_4) and (q_6) in any of the stages suggests that only at the 'Oc' stage is the appropriate concentration level of antigen (q) reached for the production of antibodies. Band (q_5) in the 'H' stage is continuous with band (p_5) showing a different arch pattern from their non-continous homologues of the other stages (Figure 12g).

F. Analysis of (r), (r'), (s) and (t)

The appearance of antigens (r), (r'), (s) and (t) in the seven zebrafish developmental stages tested with six anti-stage sera is summarized in Tables XXIa - XXId.

Band (r) occurs from the 'Cl' stage to the 'Es' stage analysed with Anti-Hb and Anti-Es sera (Tables III and V). It appears in the 'Cl' only when Anti-Mg serum is used. A faint precipitin line at the (r) position (Figure 12a) is observed in only one instance when the 'Ov' is tested against Anti-Oc serum (Table XXIa). The failure to demonstrate this band with other antisera, and the failure to reproduce this band by testing the 'Ov' stage against the Anti-Oc serum in repeated experiments, may be attributed to a subminimal concentration of antigen (r) in the 'Ov' stage.

Table XXI

Analysis of antigens (r), (r'), (s) and (t) in seven zebrafish embryonic stages with anti-stage sera

Table XXIa Analysis of band (r)

Stages	r_1	r_2	r_3	r_4	r_5	r_6
Ov					±	
Cl		+	+	±	+	
Hb		+		+		
Mg		+		+	+	
Es		+		+		
Oc						
H						

Table XXIb Analysis of band (r')

Stages	r'_1	r'_2	r'_3	r'_4	r'_5	r'_6
Ov						
Cl						
Hb		+				
Mg		±				
Es						
Oc						
H						

Table XXIc Analysis of band (s)

Stages	s_1	s_2	s_3	s_4	s_5	s_6
Ov	+					
Cl						
Hb			±			
Mg	+	+	+			
Es	+	+	+	+		
Oc		?				
H		+		±	+	

Table XXId Analysis of band (t)

Stages	t_1	t_2	t_3	t_4	t_5	t_6
Ov						
Cl		+				
Hb					±	
Mg		±			+	
Es		+	±		+	
Oc		+		±	+	
H						

Symbols used:
+ Positive precipitin reaction
± Faint precipitin reaction
= Precipitation could be either of two identifiable bands
? Extremely faint band

Band (r) is not detected in the 'Oc' stage with the different anti-stage sera. This does not mean that antigen (r) is absent from the 'Oc' stage. Since band (r_5) is detected in the pre-Oc stages with the Anti-Oc serum, antigen (r) must be present in the 'Oc' stage either in a highly immunogenic state or in a masked form. In the former case, antigen (r) may exist in a very low concentration, yet has the ability sufficient to induce antibodies. Thus, the low (r) antigen concentration at the 'Oc' stage may not result in visible precipitation whereas the higher (r) concentration, in the pre-optic cup stages do. The appearance of (r_{2a}) in the 'Cl', 'Hb' and 'Mg' stages with the Anti-Hb serum absorbed with the hatching stage extract (Table XIVb) indicates that (r_6) is absent from the 'H' stage. This observation is at conflict with other observations made. For example, although (r_3), (r_4) and (r_5) are detected with the unabsorbed sera in the 'Cl' stage, yet (r_{3a}), (r_{4a}) and (r_{5a}) are not observed in the absorption tests (Table XXIa). This is also true for the absence of (r_{4a}) from the 'Hb', 'Mg' and 'Es' stages and for the absence of (r_{5a}) from the 'Mg' stage (Table XIVd) where their homologous appear in the corresponding stages in the non-absorption tests (Table XXa). The disappearance of antigen (r) after absorption in the instances mentioned may be due to an alteration in the Anti-(r) titers in the Anti-Mg/H, Anti-Es/H and Anti-Oc/H sera.

It is not impossible that in absorbing the anti-stage sera with the hatching stage extracts the antibody for (r) may be precipitated, not as a result of an interaction with its homologues antigen, but as a result of non-specific adsorption onto other antibody-antigen complexes which are precipitated. If so then this non-specific adsorption reaction may lower the Anti-(r) antibody titer in the Anti-Mg,

Anti-Es and Anti-Oc sera resulting in an inability to precipitate due to an inhibition effect.

Antigen (r') only appears in the unabsorbed pattern with Anti-Hb serum at the 'Hb' and 'Mg' stages (Tables III and XXIb, Figures 9c - 9d). The absence of any precipitin bands in the absorption tests with Anti-Hb serum (Table IX) and Anti-Mg serum (Table X) absorbed with 'Cl' and 'Es' extracts, suggests that the Anti-(r') antibodies in these sera may have been removed by the 'Cl' and the 'Es' extracts, and that antigen (r') therefore must be present in the 'Cl' and 'Es' stage. The appearance of (r'_{2a}) in the 'Hb' and 'Mg' stages (Table XIVb) confirms that antigen (r') is not present in the 'H' stage. From the above, it appears that antigen (r'_2) makes its first appearance at the 'Hb' stage. Since absorption of the Anti-Hb serum with 'Cl' extract removed band (r'_2) it may be concluded that this antigen is already present in the 'Cl' stage in a subminimal concentration. By the same token, the antigen (r') may be said to drop to a subminimal level after the 'Mg' stage. Antigen (r') therefore may be considered as a transient antigen occurring at the 'Hb' and 'Mg' stages.

Antigen (s) occurs in the 'Ov' stage with Anti-Cl serum (Table II, Figure 8a); in the 'Hb' stage with Anti-Mg serum; in the 'Mg' stage with Anti-Cl, Anti-Hb and Anti-Mg sera; in the 'Es' stage with Anti-Cl, Anti-Hb, Anti-Mg and Anti-Es sera; in the 'Oc' stage with Anti-Oc serum and in the 'H' stage with Anti-Hb, Anti-Es and Anti-Oc sera (Tables II - VI, XXIc). Since antigen (s) usually is closely associated with the 'band complex', the resolution of antigen (s) occasionally may be obscured. This may account for the disappearance of (s) in some tests. The presence of the (s_1) band points to the existence of the

antigen (s) in the 'Cl' stage, although this antigen has not been revealed at 'Cl' with any of the Anti-stage sera used. All the results (Table XXc) suggest that antigen (s) is present in all of the developmental stages. This suggestion is further supported by the absorption tests (Tables VIII - XII, XIV) where band (s) is not detected with any of the absorbed sera.

Antigen (t), in general, appears as an extremely weak band. Of all the anti-sera used Anti-Hb and Anti-Oc are the better antisera for the testing of the (t) antigen. Since the position of band (t_5) is found slightly more anterior to the position where (t_2), (t_3) and (t_4) are found it is not known whether (t_5) is homologous to these.

In general antigens (r), (r'), (s) and (t), as compared to other antigens, are found to give comparatively weak precipitin reactions.

G. Analysis of antigens (u'), (u''), (u'''), (v), (w), (x) and (y)

The appearance of antigens (u'), (u''), (u'''), (v), (w), (x) and (y) in the seven zebrafish developmental stages tested with six anti-stage sera is summarized in Tables XXIIa - XXIIg.

Antigens (u'), (u''), (u'''), (v), (w), (x) and (y) are found in the hatching stage only (Tables VII and XXIIa - XXIIg). The failure to identify these bands in other stages with Anti-H serum (Table VII), as well as the ability to identify these bands with hatching stage extract tested against anti-H serum absorbed respectively with the 'Cl', 'Hb', 'Mg', 'Es' and 'Oc' stage extracts (Table XV) show that these bands are not present at or prior to the 'Oc' stage. The absence of a positive reaction for band (w_{6a}) with Anti-H/Cl and Anti-H/Oc sera, as well as the absence of a positive reaction for band (v_{6a}) with Anti-H/Es and Anti-H/Oc sera (Table XV), suggest that

Table XXII

Analysis of antigens (u'), (u''), (u'''), (v) and (w) in seven zebrafish embryonic stages with anti-stage sera

Table XXIIa Analysis of band (u')

Bands	u'_1	u'_2	u'_3	u'_4	u'_5	u'_6
Stages						
Ov						
Cl						
Hb						
Mg						
Es						
Oc						
H						+

Table XXIIb Analysis of band (u'')

Bands	u''_1	u''_2	u''_3	u''_4	u''_5	u''_6
Stages						
Ov						
Cl						
Hb						
Mg						
Es						
Oc						
H						+

Table XXIIc Analysis of band (u''')

Bands	u'''_1	u'''_2	u'''_3	u'''_4	u'''_5	u'''_6
Stages						
Ov						
Cl						
Hb						
Mg						
Es						
Oc						
H						+

Table XXIId Analysis of band (v)

Bands	v_1	v_2	v_3	v_4	v_5	v_6
Stages						
Ov						
Cl						
Hb						
Mg						
Es						
Oc						
H						+

Table XXIIe Analysis of band (w)

Bands	w_1	w_2	w_3	w_4	w_5	w_6
Stages						
Ov						
Cl						
Hb						
Mg						
Es						
Oc						
H						+

Symbols used:
+ Positive precipitin reaction
± Faint precipitin reaction
= Precipitation could be either of two identifiable bands
? Extremely faint band

Table XXII continued

Table XXIIf Analysis of band (x)						
Bands	x_1	x_2	x_3	x_4	x_5	x_6
Stages						
Ov						
C1						
Hb						
Mg						
Es						
Oc						
H						+

Table XXIIg Analysis of band (y)						
Bands	y_1	y_2	y_3	y_4	y_5	y_6
Stages						
Ov						
C1						
Hb						
Mg						
Es						
Oc						
H						+

Symbols used:
+ Positive precipitin reaction
± Faint precipitin reaction
= Precipitation could be either of two identifiable bands
? Extremely faint band

in these instances antibodies for these antigens may be removed by non-specific adsorption. Since the antigen (v) and (w) overlap with the positions where bands (n), (n'), (o), (p) and (q) are usually found, it is possible that the former two may mask the latter ones (Figure 13g). Negative results with Anti-H/H serum indicate that the absorption is complete and that the appearance of (u'_{6a}), (u''_{6a}), (u'''_{6a}), (v_{6a}), (w_{6a}), (x_{6a}) and (y_{6a}) is not due to incomplete absorption.

III. Discussion of the stage antigenic patterns

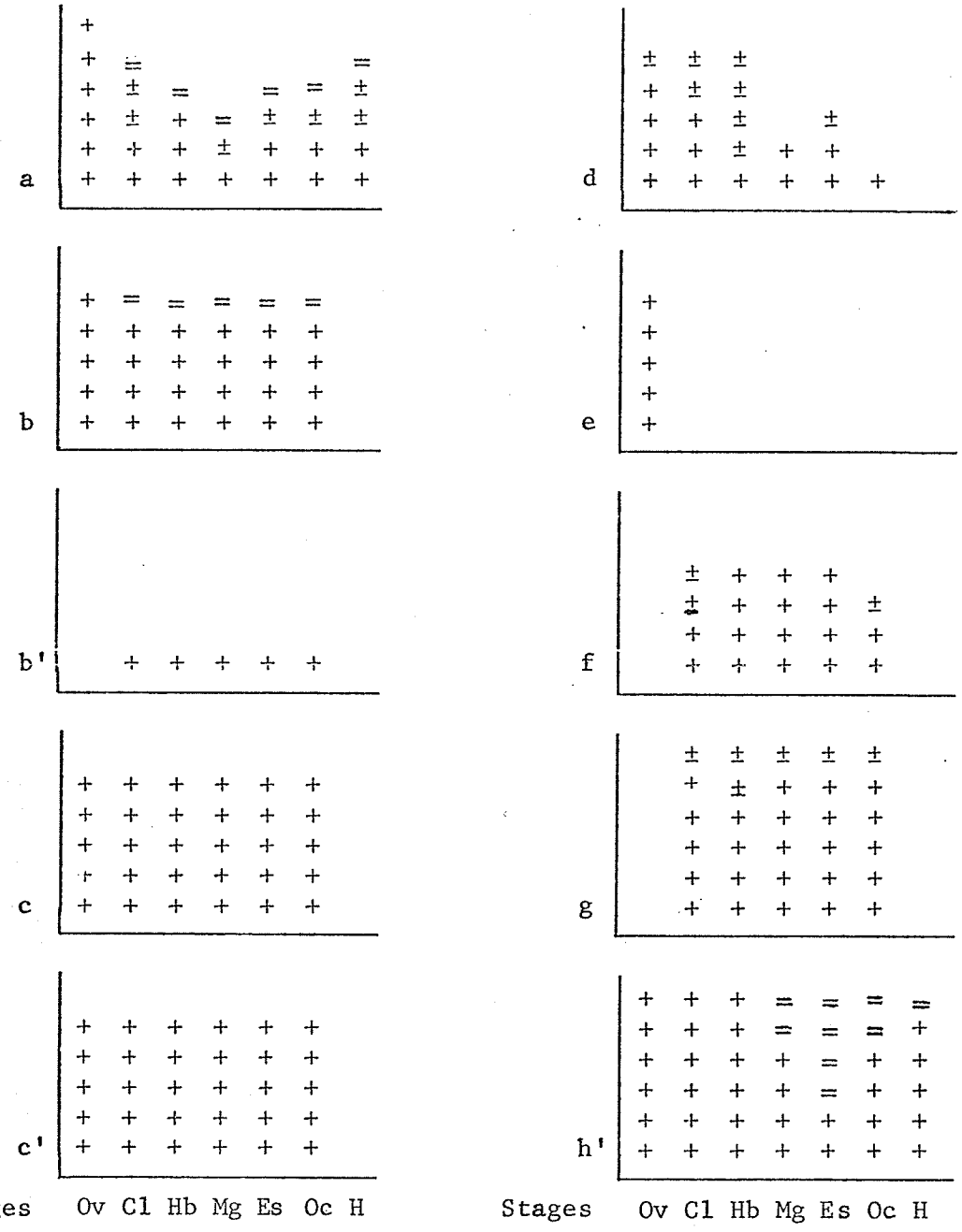
The number of times that individual antigens in the various embryonic stages are detected with anti-stage sera is summarized in Table XXIII. Quantitative methods have not been used in measuring the antigen concentration at the various developmental stages. The number of times that individual antigens are observed with all anti-stage sera, is interpreted to represent the variations in the concentration levels of the individual antigens at each developmental stage.

Immunodiffusion and immunoelectrophoretic analysis reveal thirty four bands in the 'Ov', 'Cl', 'Hb', 'Mg', 'Es', 'Oc' and 'H' stages with unabsorbed and absorbed anti-stage sera to six developmental stages. Twenty seven bands are identified in the prehatching stages, fifteen of these are common to the hatching stage. The remaining seven are found in the hatching stage only.

Twenty three antigens are present in the 'Ov' stage. Of these, twenty one are detected with unabsorbed sera and two antigens (g_{1a}) and (m_a) with absorbed sera. The antigens (b'), (f), (r') and (t) have not been observed in the 'Ov' stage.

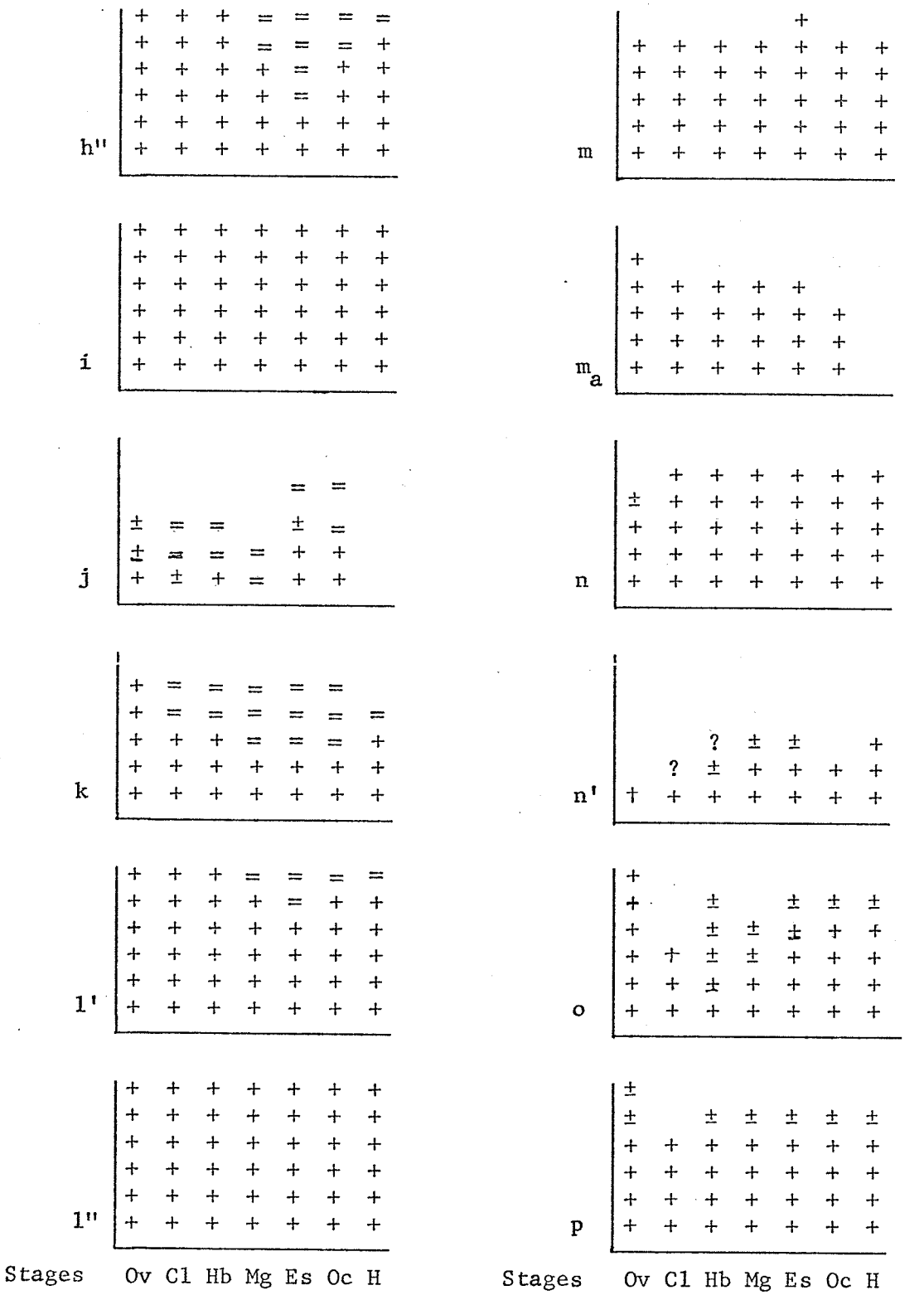
Table XXIII

The number of times that individual antigens of seven developmental stages of the zebrafish, Brachydanio rerio are detected with anti-stage sera



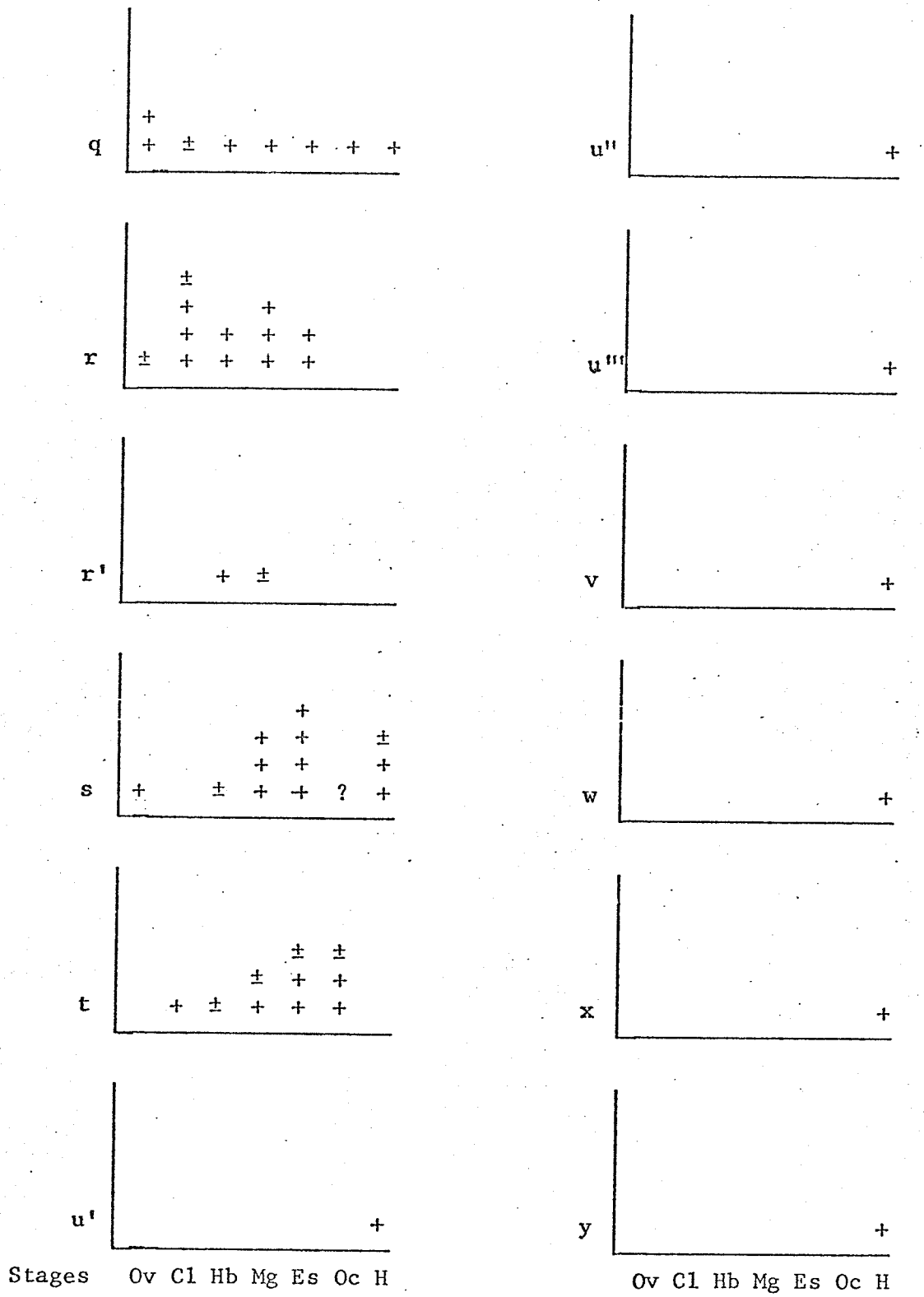
Symbols used:
+ Positive detection with anti-stage sera
± Faint detection with anti-stage sera
? Very faint detection with anti-stage sera
= Positive detection (Precipitation band either of two identifiable bands)

Table XXIII (continued)



Symbols used:
- \+ Positive detection with anti-stage sera
- ± Faint detection with anti-stage sera
- ? Very faint detection with anti-stage sera
- = Positive detection (Precipitin band either of two identifiable bands)

Table XXIII (continued)



Symbols used: + Positive detection with anti-stage sera
± Faint detection with anti-stage sera
? Very faint detection with anti-stage sera

Twenty four antigens are detected in the cleavage stage. Of these, twenty three are revealed with unabsorbed sera and one antigen (m_a) with absorbed sera. Two additional antigens (r') and (s) are deduced to be present at a subminimal level. Antigen (e) is absent at the cleavage stage and the antigens (b'), (f) and (t) make their first appearance. The number of positive reactions for each of the antigens (a), (d), (j), (o) and (q) with anti-stage sera is less at cleavage than at the 'Ov' stage. The contrary is true for the antigens (n') and (r).

Twenty six antigens are observed at the high blastula stage. Twenty five of these are revealed with unabsorbed sera and one antigen (m_a) with absorbed sera. The band pattern is similar to that of 'Cl'. The concentrations of the (d) and (r) when compared to cleavage are observed to decrease while those for the antigens (f), (n'), (o), (p) and (s) are observed to increase slightly. The antigens (r') and (s) are first detected as positive precipitin lines at the high blastula stage. The antigen (r') may be a transient antigen common to both the 'Hb' and the 'Mg' stages.

Twenty six antigens are found in the 'Mg' stage. Twenty five of these with unabsorbed sera and one antigen (m_a) with absorbed sera. The antigenic pattern of this stage is similar to that of the 'Hb' stage. When compared to the 'Hb' stage the 'Mg' antigens (a), (d), (j), (k) and (r') tend to decrease while antigens (g), (n'), (s) and (t) tend to increase in concentration. The antigens (h') and (h") first tend to combine into a single band exhibiting a greater cathodic electrophoretic mobility at this stage.

Twenty five antigens are identified in the 'Es' stage. Twenty

centration levels and electrophoretic mobilities of certain antigens are observed. The 'Hg' and the 'Mg' stages differ primarily from the other stages in the appearance of a transient antigen (r'). The pattern of the 'Oc' stage is slightly different when compared to the preceeding stages as revealed by the absorption tests (Tables VIII - XIII). This difference has not been adequately explained in the present investigation but most likely may be attributed to incomplete absorption. The disappearance of several pre-'Oc' stage antigens and a decrease in the concentration of others characterizes this particular stage.

Both the unabsorbed and absorbed patterns indicate that the ovary may possess all the antigens found in the early developmental prehatching stages except for the antigens (b'), (f), (g), (r') and (t). Antigen (e) is unique in the 'Ov' stage and is believed to have changed to (f) after fertilization. The strong precipitin lines generally obtained from tests with the 'Ov' extracts can be attributed to the higher initial concentration of these antigens at this stage. It should be borne in mind that prior to spawning, the eggs of the ovary do not take up water as do the eggs subsequent to spawning.

Furthermore, a change in molecular properties in a number of antigens occurs following fertilization as revealed by changes in electrophoretic mobilities. The antigens (k), (l'), (l"), (n) and (o) of the post-fertilization stages have acquired more posterior positions (-ve EPM) and the antigens (a) and (b) slightly more anterior positions (+ve EPM) when compared to their homologous antigens in the 'Ov'. This fluctuation in electrophoretic mobility is also found in the "group one" antigens in sea urchin development (Westin *et al*, 1967).

four of these are observed with unabsorbed sera. The antigen (m_a) is detected with the absorbed sera only. The antigen (r) of the 'Es' stage is present at a lower concentration than the same band of the 'Mg' stage. The antigens (a), (d), (j), (o), (s) and (t) of the 'Es' stage, when compared to their homologous bands in the 'Mg' stage, are present in a slightly higher concentration. Antigen (m) reaches a slightly higher concentration at the 'Es' stage than at any other developmental stages. The (r') antigen disappears at this stage.

Twenty five antigens are present in the 'Oc' stage. Twenty two of these are detected with unabsorbed sera, one antigen (m_a) with absorbed sera, and two antigens (r) and (s) are deduced to exist at subminimal levels. The antigens (d), (f) and (n') when compared to their homologues in the 'Es' stage tend to decrease while the antigens (a) and (o) increase slightly in concentration. The concentration of (r) and (s) has dropped to an undetectable level, yet these antigens retain a high enough antigenicity to induce antibody formation.

In the hatching stage, a drastic change in the antigenic pattern is observed. A total of twenty two antigens are identified. Fourteen of these are detected with unabsorbed sera and one antigen (g) is deduced. Seven new antigens, not previously identified are observed at this stage (u' - y). The antigens (b), (b'), (c), (c'), (d), (e), (f), (j), (r), (r'), (t) and (m_a) are not observed. The antigens (a), (n') and (s) are slightly stronger in their precipitin reactions than the homologous antigens of the 'Oc' stage.

The antigenic patterns of the 'Cl', 'Hb', 'Mg' and 'Es' stages are without much change. In general, slight fluctuations in con-

IV. General discussion

Spar (1953) tested blastula, gastrula and neurula extracts of Rana pipiens quantitatively against their homologous antisera and antisera absorbed with heterogeneous stage extracts in ring and precipitin tests. Six groups of antigens are reported, namely: antigens specific for glastrula (G), antigens specific for neurula (N), antigens common to both blastula and gastrula (BG), antigens common to both gastrula and neurula (GN), antigens common to both blastula and neurula (BN), and those common to all stages (BGN). No blastula stage-specific group of antigens is reported. Spar subsequently determined the relative concentrations of these antigen classes and listed them in a descending order as follows: $BNG > N > GN = BG > BN > G$. With the methods employed, he concluded that new antigen groups are first detected at the gastrula stage.

The antigenic patterns of eggs, neural plate and early neural fold stages of Rana pipiens were also analysed with antisera to neural plate and early neural fold as well as with antiserum to adult frog serum (Cooper, 1950). Five to seven antigens are described as being common to the egg, neural plate and adult frog serum. Six to eight antigens in the neural plate and neural fold stages were identified with homologous antisera. Differences in antigenic profiles were not discerned. This may be due to the insensitivity of the immunodiffusion technique used.

The Ouchterlony method was employed to show the antigenic patterns of nine developmental stages of Rana temporaria (Romanovsky, 1964a). In addition to one antigen which is common to all stages, antigens could be divided into three separate groups, each of which characterizes each of the three developmental periods as follows: the

egg to blastula period, the early to late gastrula period and the neurula to tail-bud period. In contrast to Spar's finding, a blastula stage-specific precipitin band was identified. Stage-specific antigens were also found in the late gastrula, neurula, tail-bud and larval stages. Antigens common to two neighbouring stages were observed for the early and late gastrula, the neurula and tail-bud, and the tail-bud and larval stages. Some antigens in the egg may disappear at morula or late gastrula. The earliest new antigen to be synthesized after fertilization was found at the blastula stage. Other new antigens are first detected respectively at the late gastrula and neurula stages. Romanovsky (1964a) noted that his own findings do not adequately account for the detection of qualitatively different antigens. One more antigen shared by both the neurula and tail-bud stages was subsequently observed with ring tests by Romanovsky (1964b).

The onset of rRNA synthesis in Xenopus laevis is reported to be at the gastrula stage (Davidson, 1968). This is coincident with the time of appearance of new antigens at and after gastrulation in Rana pipiens.

Combining both immunoelectrophoresis and ^{14}C-radioactive amino acid pulse labelling techniques, Westin et al, (1967) were able to detect changes in the early antigenic pattern of the sea urchin embryo, Paracentrotus lividus. Radioactive labelled extracts of unfertilized eggs as well as cleavage, hatching-blastula, gastrula and pluteus extracts were tested against antisera to unfertilized eggs, gastrula

and pluteus stage embryos. The concentration of antigen-bound radioactivity in the extracts was found to rise markedly from the fertilized egg to the pluteus stage. One of these labelled antigens found in the unfertilized egg disappears before the hatching-blastula stage while the other two become unlabelled throughout subsequent development. Some antigens become labelled after fertilization and disappear before gastrulation. Westin suggested that the same genome governing the prefertilization stage becomes active and functional again from after hatching till sometime before gastrulation. In pluteus three labelled antigens are found that are not common to any of the earlier stages. The hatching stage of this species seems to separate the embryonic development into two antigenically different periods governed respectively by separate genomes. Westin (1969) also reported that the RNA templates needed for gastrulation are synthesized at the hatching-blastula stage.

Protein patterns for developing embryos have also been studied by means of other techniques. The analysis of the hydrolytic enzyme pattern of the Ilyanassa obsoleta embryonic stages with starch-gel electrophoresis reveals twenty three bands. Seven of these are common to all stages; three are specific to the first four days of development and thirteen appear after the fourth day (Morrill, 1961). This also suggests that gastrulation in this species may divide the early development into two periods governed by different groups of mRNA. Mackentosh and Bell (1969) using acrylamide gel, similarly reported a change in pattern at the gastrula stage of the sea urchin. Exceptions exist, thus the gastrula stage may not always represent the stage that divides development into two synthetic periods. For

example, with carboxymethyl cellulose column chromatography, an acetic acid soluble protein fraction, believed to be of nuclear origin, appears at the early blastula stage in Lytechinus variegatus (Silver and Comb, 1967).

Protein synthesis immediately after fertilization is observed in the actinomycin treated and control eggs of the sea urchin (Gross et al, 1964). The assembly of proteins on templates is not blocked by actinomycin D at this stage. This implies that the templates for protein synthesis at cleavage are not synthesized 'de novo' at cleavage but are of prefertilization maternal origin. Furthermore, these experiments with actinomycin D have shown that such synthesis is necessary for the viability and mitosis of pregastrula cells as well as for development to proceed beyond gastrulation.

Activation of RNA synthesis after fertilization has been reported by Wilt (1963) who discovered that the sedimentation pattern of the RNA species before and after fertilization may be the same.

Three sedimentation patterns of rapidly synthesized RNA in purple sea urchin, Strongylocentrotus purpuratus, has been reported by Nemer (1963). Two peaks are common to both the unfertilized and the fertilized egg cells. A new 10 S peak appears just before gastrulation. It is not unlikely that this peak may account for the appearance of new antigen(s) or protein(s) arising at the gastrula stage. The post-gastrula sedimentation pattern is polydisperse indicating that a variety of proteins may be translated at this stage. It should be borne in mind that new antigens are expected in the post-gastrula stages in preparation for organogenesis and histogenesis.

Analysis of the sea urchin polyribosomes show that two classes

of polysomes, r-polysomes and s-polysomes, occur in early development. The s-polysomes are inactive (actinomycin-D insensitive) and accumulate in the pregastrula period. At gastrulation, they become active (actinomycin-D sensitive) and are referred to as r-polysomes (Infant and Nemer, 1967). This may also imply that the templates for some antigens that appear during or after gastrulation are actually pre-synthesized and maintained at a non-functional state.

DNA-RNA hydridization experiments show that in Strongylocentrotus purpuratus some of the mRNA molecules present in the unfertilized eggs are synthesized as late as the prism stage (late gastrula), (Whiteley et al, 1966). This may account for the occurrence of the common antigens that are synthesized consistently before and after fertilization up to gastrulation. Such templates, if long lived, may also account for the synthesis of common antigens occurring subsequent to gastrulation. Unique kinds of late gastrula mRNA molecules appear to be both qualitatively and quantitatively different from that of pre- and post-prism stages. This agrees with the antigenic patterns observed in the development of frog and echinoderm embryos (Spar, 1953; Romanovsky, 1964a; Westin et al, 1967). Similar mRNA species in both the unfertilized and fertilized eggs and in the blastula stage were observed by Glišin et al, (1966). They demonstrated that subsequent to the blastula stage, about 40% of the mRNA population is replaced by a different mRNA molecular species detectable at the gastrula stage and the subsequent stages.

The study of antigenic patterns and the study of transcription behaviour in the early ontogeny of echinoderms and amphibians agree with each other. The hatching-blastula stage of the echinoderms and

the gastrula of the frogs are the points of onset of embryonic gene function. New antigens are expected to be synthesized at these stages. The prehatching stage of the echinoderms and the pregastrula stages of the amphibians are under the control of the maternal genomes; hence few qualitative antigenic differences are expected.

The pregastrula period of the zebrafish seem to conform to the findings observed for the echinoderms and the amphibians. A change in the zebrafish antigenic species at fertilization as compared to pre-fertilization is noticed with the disappearance of antigen (e); a decrease in concentration of the antigens (a), (o), (p), (q) and to a small extent antigens (d) and (j); and an increase in the concentration of certain antigens such as (n), (n') and (r). A similar change in the concentration of the antigens or proteins after fertilization has also been recorded for the echinoids Hemicentrotus pulcharrinus and Pseudocentrotus depressus, and the teleost Oryzias latipes by Ishida and Yasumasu (1957).

The initial appearance of the antigens (b'), (f), (g) and (t) of the zebrafish after fertilization is supported by the finding that one group of proteins, i.e. group 6 in the sea urchin, Hemicentrotus pulchurrinus and two groups of proteins, i.e. groups 3 and 5 in Oryzias latipes make their first appearance after fertilization (Isida and Yasumasu, 1957). Protein synthesis during cleavage is implied from the study on the transcriptional level (Wilt, 1963, Gross and Cousineau, 1964; Morrill, 1965 and Collier, 1965). Such protein synthesis may not necessarily represent the synthesis of new mRNA (Gross et al , 1964).

Stage-specific antigens have not been demonstrated in the pre-

hatching zebrafish development as compared to the stage-specific antigen identified in the amphibian development (Spar, 1953; Romanovsky, 1964a). The antigen (r') in the zebrafish is a weak transient antigen at least common to the 'Hb' and the 'Mg' stages. Most other antigens are common to all prehatching stages, though variations in their concentrations at different stages are observed. Thus the antigens (a), (d) and (j) of the 'Mg' stage are present at a low level whereas the antigens (n'), (r), (s) and (t) have reached a peak at the 'Mg' and 'Es' stages. In view of the observation that variations in the concentration of proteins do occur from stage to stage, it is not unreasonable to suggest that the stage-specific antigens reported for the amphibian embryo by Spar (1953) and Romanovsky (1964a, 1964b) are in fact antigens that reach detectable levels at particular stages. Thus these antigens may well be common to stages in which their concentrations are subminimal. Transient antigens likewise are antigens that acquire detectable levels at a specific stage or stages. In the case of the antigens (a), (d) and (j) an apparent decrease in the concentration may indicate that these are residual non-functional macromolecules gradually catabolized in the course of development. In the case of the antigen (n'), (r), (s) and (t) an increasing concentration may indicate that these are formed at the expense of the others. An increase in the concentration of the antigens (n'), (r), (s) and (t) of the zebrafish is coincident with the time of onset of rapid rRNA synthesis in the loach gastrula (Kafiani et al, 1969; Kafiani, 1970). If these antigens are synthesized on newly formed rRNA at the time of gastrulation, then the translation of these antigens is controlled by the embryonic genomes responsible for

the synthesis of rRNA. Variations in the concentration of certain antigens within an embryo may be considered important in the control of differentiation. For example, Stanisstreet and Deuchar (1972) have compared the immunoelectrophoretic patterns of the neural ectodermal areas of the late Xenopus gastrula. Although no qualitative differences were noticed, quantitative differences between the dorsal and ventral areas nevertheless were observed. An increase in the concentration of the antigenic components in the neural ectoderm immediately following neural induction was observed. Whether or not the increase can be related directly to subsequent neural differentiation is not known.

The hatching stage in the zebrafish development shows fewer antigens common to the pre-hatching stages and seven new antigens namely: (u'), (u''), (u'''), (v), (w), (x) and (y). These new antigens are possibly controlled by a set of genomes which manifest themselves after the commencement of organogenesis. Since organogenesis and histogenesis in the zebrafish occurs subsequent to gastrulation, a slight variation in the antigenic pattern from that of the pregastrula and gastrula stages is expected by the 'Oc' stage. The results of the absorption immunodiffusion tests suggest that the 'Oc' stage represent a transitional period during which a shift from gastrula to post-gastrula genomic control may occur. New antigens appear at the Xenopus tail-bud stage after the onset of organogenesis (Romanovsky, 1964a). Similarly, new antigens occur after the onset of organogenesis in the echinoderm Paracentrotus (Westin et al 1967), in the mollusc Ilyanassa (Morrill, 1961) and in the teleost, Oryzias latipes (Ishida and Yasumasu, 1957).

In the study of fish lactate dehydrogenase (LDH) isozymes, only one of the five isomers is present in the prehatching stage, all other isomers are abruptly synthesized at hatching (Nakano and Whiteley, 1965).

Since in the present study non-dechorionated embryonic stages (except the 'H' stage) were injected for antibody production, some of the common antigens observed may have been localized in the perivitelline fluid. Preliminary analysis of the perivitelline fluid of some developmental stages with anti-stage antisera show that a number of antigens are present in the perivitelline fluid. A variation in the antigenic profile of the pervitelline fluid has also been observed. The disappearance of some prehatching stage common antigens at the hatching stage may be due to the loss of perivitelline fluid as a result of natural dechorionation (Law, unpublished). Similarly, some antigens in the zebrafish embryo may originate in the yolk. Thomas (1968) has discussed the incorporation of yolk material into the embryo proper during zebrafish development. Further analysis of yolk and perivitelline fluid may help to elucidate the actual antigenic patterns of the embryo-proper.

Since the production of antibodies to stage extracts depends on the antigenicity and concentration of the antigens injected as well as upon the selective response by the animal to the antigens injected, a failure to detect stage-specific antigens may not imply an actual absence of such antigens. A low concentration, or an inertness of certain stage-specific macromolecules (if present) may render such molecules non-detectable with the techniques employed in the present investigation.

SUMMARY

1. Rabbit antisera were produced to adult zebrafish developmental stages, namely: Cleavage, High Blastula, Mid-gastrula, Embryonic shield, Optic Cup and Hatching by subcutaneous injections with antigen and Freund's adjuvant. Aliquots of each of these antisera respectively were absorbed with lyophilized extracts of the six developmental stages.

2. Antigen preparations from seven zebrafish developmental stages, namely: Ovary, Cleavage, High Blastula, Mid-gastrula, Embryonic shield, Optic Cup and Hatching were analysed by immunoelectrophoresis with unabsorbed anti-stage sera, and by immunodiffusion with absorbed anti-stage sera. Positive immunodiffusion reactions were further analysed by immunoelectrophoresis.

3. Fourteen antigens (a), (h'), (h''), (i), (k), (l'), (l''), (m), (n), (o), (p) and (s) are common to all seven stages tested.

4. The Antigens (b), (c), (c'), (d), (g), (j) and (r) are found in all the prehatching stages tested including the ovary stage with unabsorbed sera. The antigens (b'), (f) and (t) are found in the post-fertilization and prehatching stages only.

5. One antigen (e) is found in the Ovary only. It changes to antigen (f) in the post-fertilization stages.

6. A transient antigen (r') is demonstrated in the High Blastula and Mid-gastrula stages.

7. One antigen (m_a) is identified in all the prehatching stages tested with the absorbed sera.

8. Seven antigens (u'), (u''), (u'''), (v), (w), (x) and (y) are observed in the Hatching stage only.

9. Slight alterations in electrophoretic mobilities were observed for the antigens (h'), (h''), (k), (l') and (o).

10. Variations in antigen concentrations during development is reported for the antigens (a), (d), (j), (n'), (r), (s) and (t).

11. The techniques employed in this investigation have not demonstrated any stage-specific antigens in the prehatching development of the zebrafish.

APPENDIX I

Phosphate buffer, pH 6.6 - 6.8 ionicity 0.02

$Na_2HPO_4.12H_2O$	12.78 gm
KH_2PO_4	12.25 gm
H_2O	1000 ml

Use 1:8 parts distilled water to make 0.02M, pH 6.6 - 6.8 solution.

APPENDIX II

Sørensen-phosphate buffer, pH 7.0, ionicity 0.05*

Na_2HPO_4: K_2HPO_4 (Sørensen) pH 5.9-8.04, 18°C

11.876 gm $Na_2HPO_4.2H_2O$ per liter

9.078 gm KH_2PO_4 per liter

To 6.0 ml of $Na_2HPO_4.2H_2O$ solution, add 4.0 ml of the KH_2PO_4 solution.

* Hale, L.J. 1965. Biological Laboratory Data. Science Paperbacks and Methuen and Co. Ltd., p. 84.

APPENDIX III

Abreviations employed in this investigation

Abreviations	Descriptions
Ov	Ovary
Cl	Cleavage
Hb	High Blastula
Mg	Mid-gastrula
Es	Embryonic shield
Oc	Optic Cup
H	Hatching

Anti-Cl	Antiserum produced to Cleavage
Anti-Hb	Antiserum produced to High Blastula
Anti-Mg	Antiserum produced to Mid-gastrula
Anti-Es	Antiserum produced to Embryonic-shield
Anti-Oc	Antiserum produced to Optic Cup
Anti-H	Antiserum produced to Hatching
Anti-Cl/Cl	Anti-Cl serum absorbed with Cleavage extract
Anti-Cl/Hb	Anti-Cl serum absorbed with High Blastula extract
Anti-Cl/Mg	Anti-Cl serum absorbed with Mid-gastrula
Anti-Cl/Es	Anti-Cl serum absorbed with Embryonic-shield extract
Anti-Cl/Oc	Anti-Cl serum absorbed with Optic Cup extract
Anti-Cl/H	Anti-Cl serum absorbed with Hatching extract
Anti-Hb/Cl	Anti-Hb serum absorbed with Cleavage extract
Anti-Hb/Hb	Anti-Hb serum absorbed with High Blastula extract
Anti-Hb/Mg	Anti-Hb serum absorbed with Mid-gastrula extract
Anti-Hb/Es	Anti-Hb serum absorbed with Embryonic-shield extract
Anti-Hb/Oc	Anti-Hb serum absorbed with Optic Cup extract
Anti-Hb/H	Anti-Hb serum absorbed with Hatching extract
Anti-Mg/Cl	Anti-Mg serum absorbed with Cleavage extract
Anti-Mg/Hb	Anti-Mg serum absorbed with High Blastula extract
Anti-Mg/Mg	Anti-Mg serum absorbed with Mid-gastrula extract
Anti-Mg/Es	Anti-Mg serum absorbed with Embryonic-shield extract
Anti-Mg/Oc	Anti-Mg serum absorbed with Optic Cup Extract
Anti-Mg/H	Anti-Mg serum absorbed with Hatching extract

Appendix III (continued)

Abreviations	Descriptions
Anti-Es/Cl	Anti-Es serum absorbed with Cleavage extract
Anti-Es/Hb	Anti-Es serum absorbed with High Blastula extract
Anti-Es/Mg	Anti-Es serum absorbed with Mid-gastrula extract
Anti-Es/Es	Anti-Es serum absorbed with Embryonic-shield extract
Anti-Es/Oc	Anti-Es serum absorbed with Optic Cup extract
Anti-Es/H	Anti-Es serum absorbed with Hatching extract
Anti-Oc/Cl	Anti-Oc serum absorbed with Cleavage extract
Anti-Oc/Hb	Anti-Oc serum absorbed with High Blastula extract
Anti-Oc/Mg	Anti-Oc serum absorbed with Mid-gastrula extract
Anti-Oc/Es	Anti-Oc serum absorbed with Embryonic-shield extract
Anti-Oc/Oc	Anti-Oc serum absorbed with Optic Cup extract
Anti-Oc/H	Anti-Oc serum absorbed with Hatching extract
Anti-H/Cl	Anti-H serum absorbed with Cleavage extract
Anti-H/Hb	Anti-H serum absorbed with High Blastula extract
Anti-H/Mg	Anti-H serum absorbed with Mid-gastrula extract
Anti-H/Es	Anti-H serum absorbed with Embryonic-shield extract
Anti-H/Oc	Anti-H serum absorbed with Optic Cup extract
Anti-H/H	Anti-H serum absorbed with Hatching extract

APPENDIX IVa

Weights of lyophilized stage-antigens used in the absorption of 6 aliquots of Anti-Cl serum (1 ml per aliquot)

		Freeze-dried weights of absorbing materials (mg)		
Aliquots of Anti-Cl serum	Absorbing Material	1st absorption	2nd absorpiton	Total Weight
1	Cl	25.4mg	10.2mg	35.6 mg
2	Hb	40.1	-	40.1
3	Mg	20.8	-	20.8
4	Es	19.7	11.8	31.5
5	Oc	25.4	8.6	34.0
6	H	15.7	7.0	22.7

APPENDIX IVb

Weights of lyophilized stage-antigens used in the absorption of 6 aliquots of Anti-Hb serum (1 ml. per aliquot)

		Freeze-dried weights of absorbing materials (mg)		
Aliquots of Anti-Hb serum	Absorbing Material	1st absorption	2nd absorption	Total Weight
1	Cl	25.3 mg	11.4mg	36.7 mg
2	Hb	41.5	-	41.5
3	Mg	21.5	-	21.5
4	Es	22.8	12.3	35.1
5	Oc	25.9	8.2	34.1
6	H	15.3	7.7	23.0

APPENDIX IVc

Weights of lyophilized stage-antigens used in the absorption of 6 aliquots of Anti-Mg serum (1 ml. per aliquot)

		Freeze-dried weights of absoring material (mg)		
Aliquots of Anti-Mg serum	Absorbing Material	1st absorption	2nd absorption	Total Weight
1	Cl	25.0 mg	11.6 mg	36.6 mg
2	Hb	43.5	-	43.5
3	Mg	21.0	-	21.0
4	Es	23.0	10.9	33.9
5	Oc	26.4	8.2	34.6
6	H	16.0	7.5	23.5

APPENDIX IVd

Weights of lyophilized stage-antigens used in the absorption of 6 aliquots of Anti-Es serum (1 ml per aliquot)

		Freeze-dried weights of absorbing material (mg)		
Aliquots of Anti-Es serum	Absorbing Material	1st absorption	2nd absorption	Total Weight
1	Cl	25.5 mg	10.6 mgm	36.1 mg
2	Hb	41.0	-	41.0
3	Mg	19.7	-	19.7
4	Es	22.1	9.7	31.8
5	Oc	26.7	9.1	35.8
6	H	15.3	7.5	22.8

APPENDIX IVe

Weights of lyophilized stage-antigens used in the absorption of 6 aliquots of Anti-Oc serum (1 ml per aliquot)

		Freeze-dried weights of absorbing material (mg)		
Aliquots of Anti-Oc serum	Absorbing Material	1st absorption	2nd absorption	Total Weight
1	Cl	25.9 mg	12.2 mg	38.1 mg
2	Hb	42.6	-	42.6
3	Mg	20.9	-	20.9
4	Es	28.8	10.4	39.2
5	Oc	25.0	10.0	35.0
6	H	16.6	7.3	23.9

APPENDIX IVf

Weights of lyophilized stage-antigens used in the absorption of 6 aliquots of Anti-H serum (1 ml per aliquot)

		Freeze-dried weights of absorbing material (mg)		
Aliquots of Anti-H serum	Absorbing Material	1st absorption	2nd absorption	Total Weight
1	Cl	26.9 mg	11.9 mg	38.8 mg
2	Hb	42.0	-	42.0
3	Mg	20.4	-	20.4
4	Es	20.5	-	20.5
5	Oc	28.5	8.2	36.7
6	H	14.9	8.7	23.6

APPENDIX V

Protein concentrations of the zebrafish embryonic stages

Stages	Initial concentration of proteins	Final concentration of proteins
Cl	13 mg/ml	13 mg/ml
Hb	14 mg/ml	14 mg/ml
Mg	11 mg/ml	11 mg/ml
Es	30 mg/ml	15 mg/ml
Oc	13 mg/ml	13 mg/ml
H	17 mg/ml	17 mg/ml

Note : The initial concentrations of the proteins were detected without diluting the original stage extracts. The final concentrations of the proteins were obtained by diluting the extracts with phosphate buffer solution (Appendix I).

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