Proc. Indian Acad. Sci. (Anim. Sci.), Vol. 93, No. 3, April 1984, pp. 179–198.

Use of electrophoresis and immunoelectrophoresis in taxonomic and pollution studies

MARIA R MENEZES* and S Z QASIM**

* National Institute of Oceanography, Dona Paula, Goa 403 004, India
** Department of Ocean Development, South Block, New Delhi 110 011, India

Abstract. Studies were conducted on the electrophoresis of blood serum and eye lens proteins of 5 fishes and immunoelectrophoresis of the soluble lens proteins of 10 fishes. The effects of a toxic pollutant (mercury) on the electrophoretic patterns of the serum, haemoglobin and eye lens proteins of a euryhaline fish *Tilapia mossambica* (Peters) has also been studied. The use of fish blood morphology as a biological index of water quality has been indicated.

Keywords. Electrophoresis; serum proteins; eyelens proteins; immunoelectrophoresis; haemoglobin; stress pattern.

1. Introduction

Rapid advances in protein biochemistry in recent years have given rise to several new concepts and techniques which could be utilised to study the phylogenetic relationships. The primary structure of a protein molecule with its amino acid sequence, is genetically determined and it is very likely that the specific folding and cross-linking of a polypeptide chain results largely, if not entirely, from the primary structure (Anfinsen 1961). The reasoning is that 'the primary structure dictates the secondary, tertiary and quaternary structures (conformation) in any given environment.' The properties of proteins show the ultimate biochemical relationships of the organisms. These properties can be regarded as the fundamentals in unravelling the evolutionary pathways and taxonomic relationships.

The existence of multiple forms of proteins has interested many biochemists and biologists (Markert and Moller 1959; Shaw 1965). In addition to the multiple forms resulting from the differences in the primary structure of the fundamental protein unit, there are also multiple forms arising due to other reasons. For example, one type of multiple molecular form results from the molecules of proteins having the same primary structure which exists in several physico-chemical forms when the structure gets influenced by the environment. These are termed 'conformational forms' (Lumry and Erying 1954).

Most important among the methodologies are the techniques of electrophoretic genetics that have become available during the last two decades. The staining of specific proteins on starch, agar, polyacrylamide and cellulose acetate strips followed by their electrophoresis reveals their characteristic migration patterns. Protein mobility is largely determined by the molecular configuration and electrical charge, which in turn are dependent on the amino acid sequence. Since the latter is a direct consequence of the genetic template, the electrophoretic phenotype lies very close to the core of the genetic information of the individuals. Electrophoretic genetics can and does reveal cryptic genetic variability with relatively little ambiguity in its interpretation. The method

detects one-locus polymorphisms of possible adaptive significance and it is of value as marker genes for the populations, stocks and races. The literature on electrophoretic genetics of fishes has been reviewed by Love (1970) and in some detail earlier by Deligny (1969): The work on specific groups such as the tuna has been summarized by Fujino (1970).

Early in the twentieth century, two events occurred which have only recently begun to affect the course of fishery research. Firstly, the observation of Landsteiner (1901) that the red blood cells of one human could be agglutinated or clumped by the serum from another and he proposed the ABO blood group system. Secondly, the same year Nuttall (1901) published the first of a series of papers demonstrating that an antiserum which is reactive with the serum proteins of one species of an animal could also react with related species, thus introducing the science of systematic serology. The early work of Nuttall (1904) promoted a great deal of comparative research on antigens of organisms which could be studied by the use of precipitin reaction. A detailed description of the application of these techniques for the study of fish serum proteins has been provided by Ridgway *et al* (1962) and Krauel and Ridgway (1963).

2. Experimental procedure

2.1 *Electrophoretic studies*

During the last eight years, studies were conducted at the National Institute of Oceanography, on the morphometry and serology of several fishes from the Goa region. In all these studies electrophoresis was conducted on cellogel strips (2·5 × 14 cm) (gelatinized cellulose acetate). Blood serum and the eye lens proteins of five fishes, *Sardinella longiceps* Val; *S. fimbriata* (Val); *Brachirus orientalis* (Bloch); *Pseudorhombus arisus* (Ham-Buch) and *Psettodes erumei* (Bloch) were studied (Menezes 1979). Fish samples used for this study were collected from the fish landing centres of Panaji, Goa. In each species, the fishes studied were of different size and sex.

Blood collected from the caudal end of the fish in capillary tubes was allowed to clot which was then cetrifuged at 1000 rpm for 5 min to obtain a clear serum.

Eye lenses removed from the fish were freed from the aqueous and vitreous humour, pieces of retina and the capsule. The lenses from each fish was weighed and placed in a dry tube and used for analysis immediately. Protein extracts were prepared by mechanically mincing the lenses of each fish in a 0·9 % saline solution using 0·5 ml of saline for about 60 mg weight of the lens. They were then centrifuged at 3000 rpm for 5 min to obtain a clear solution.

The E_f value of each band was calculated as the ratio of the distance travelled by the band to that of the distance travelled by the fastest moving band. Human serum was applied to one strip during each electrophoretic run to serve as a mobility reference standard. The intensity of protein in each fraction was determined by a densitometer.

2.1*a* *Serum proteins*: The serum of *P. erumei* shows seven bands or fractions in the electropherogram (figure 1A). Among these bands, the major ones are the first three bands of slower mobility having E_f values of 0·13, 0·25 and 0·41 respectively (table 1). The electropherogram of *P. arsius* serum shows six bands (figure 1B), of which the first, second and the fifth bands belong to the major protein with the E_f values of 0·25, 0·41 and 0·81 respectively. The serum of *B. orientalis* shows five bands (figure 1c), of which

Table 1. Electrophoretic analysis of the serum proteins.

Species studied	Fractions (in the order from the slowest to the fastest)																		
	−1a	−1b	−1c	−1d	−1e	1	1a	1b	1c	1d	2	2a	2b	2c	2d	3	3a	3b	4	5
Human (reference)	—	—	—	—	—	0·29 (6·50)	—	—	—	—	0·49 (5·85)	—	—	—	—	0·73 (11·69)	—	—	0·85 (1·95)	0·97 (74·01)
Psettodes erumei	—	0·13 (41·15)	—	—	0·25 (19·75)	—	—	—	0·41 (24·69)	—	—	0·53 (8·23)	—	—	0·69 (2·47)	0·73 (1·65)	—	—	—	0·97 (2·06)
Pseudorhombus arsius	—	—	—	—	0·25 (52·48)	—	—	—	0·41 (21·37)	—	—	—	0·57 (10·26)	—	0·69 (3·42)	—	—	0·81 (17·09)	—	0·97 (15·38)
Brachirus orientalis	—	—	—	—	—	—	—	—	0·41 (50·4)	—	—	0·53 (12·8)	—	0·61 (4·0)	—	—	0·77 (24·8)	—	—	0·97 (8)
Sardinella longiceps	0·05 (26·29)	—	0·17 (14·86)	0·21 (24·0)	—	—	0·33 (19·43)	—	—	0·45 (4·0)	—	—	0·57 (1·14)	—	—	0·73 (1·14)	0·77 (1·14)	—	—	0·97 (8)
S. fimbriata	0·05 (21·05)	—	—	0·21 (31·14)	—	—		0·37 (33·77)	—	0·45 (1·75)	—	—	0·57 (3·51)	—	—	0·73 (4·39)	—	—	0·85 (3·51)	0·97 (0·88)

The values represent E_f values along with the percentage (in parentheses) of protein in each fraction.

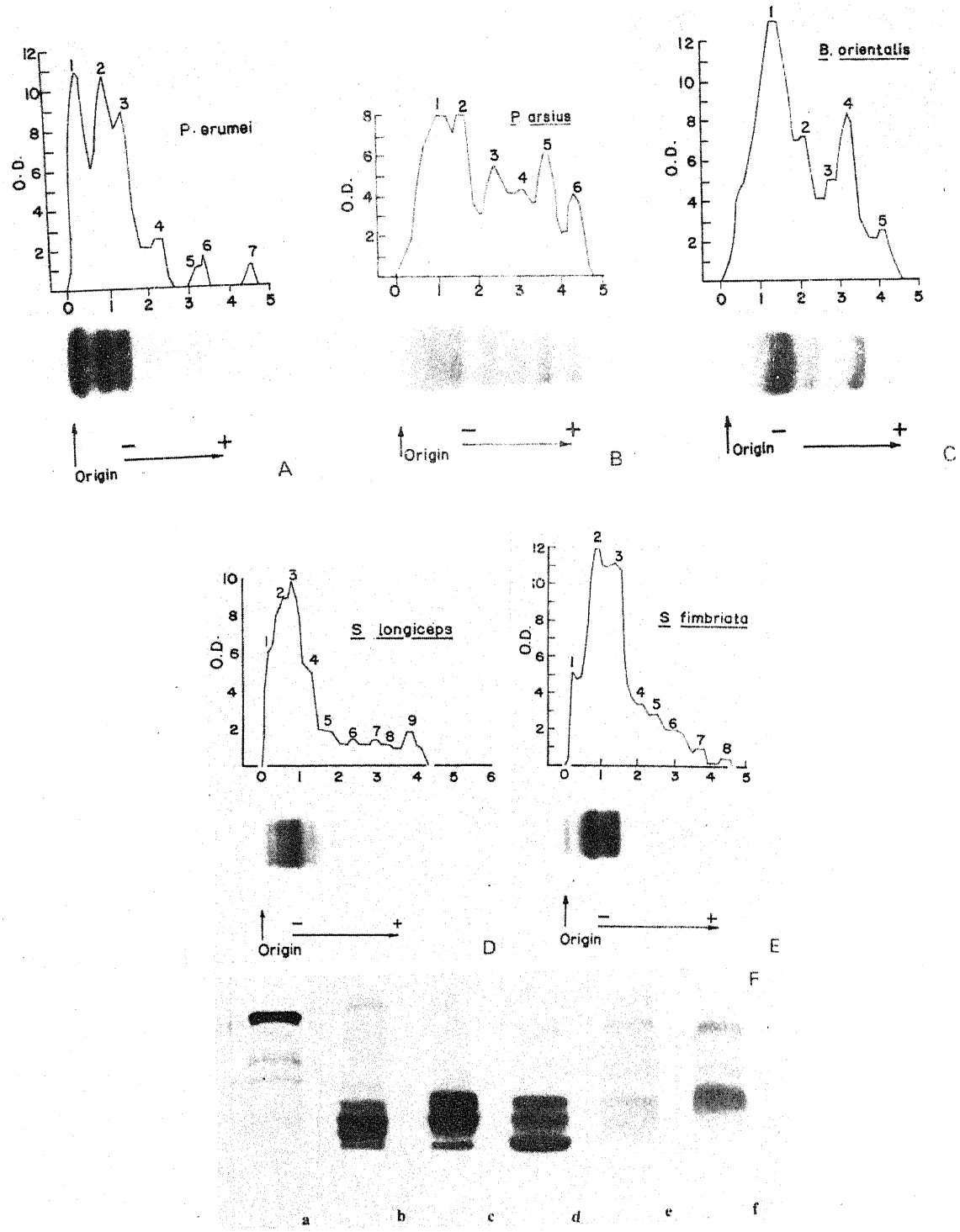


Figure 1. A–E. Electrophoretic pattern of the serum proteins in **A.** *Psettodes erumei*; **B.** *Pseudorhombus arsius*; **C.** *Brachirus orientalis*; **D.** *Sardinella longiceps*; **E.** *Sardinella fimbriata*; **F.** Comparison of the serum protein patterns of **a.** human; **b.** *S. longiceps*; **c.** *S. fimbriata*; **d.** *P. erumei*; **e.** *P. arsius*; **f.** *B. orientalis*.

the first and the fourth are the major ones with E_f values of 0·41 and 0·77 respectively. The serum of *S. longiceps* shows nine bands (figure 1D), of which the first four bands of slower mobility are the major ones with E_f values of 0·05, 0·17, 0·21 and 0·33 respectively. The serum of *S. fimbriata* has eight bands (figure 1E). Of these, the first three bands of slower mobility are the major ones with E_f values of 0·05, 0·21 and 0·37.

Seperation of the serum proteins of fish on cellulose acetate has resolved the varying numbers of bands. These range from three in the coelacanth—*Latimeria chalumnae* (Griffith *et al* 1974) to ten in the mudfish—*Labeo* sp (Hattingh 1974). In most studies, a comparison with the human serum has been used to assign the electrophoretic mobilities and/or density. In many cases, the dominant protein was a beta globulin and not albumin as was found in mammals. Similarly, in these studies, the gamma and beta globulins contained a higher percentage of proteins (table 1).

The electropherograms of the serum proteins of the three flat fishes (*Psettodes erumei*, *Pseudorhombus arisus*, and *Brachirus orientalis*) show a major band of E_f value 0·69 in common. *P. erumei* serum also shows a minor band of the E_f value 0·53 in common with that of *B. orientalis* serum. The electropherograms of the two clupeiods (*Sardinella longiceps* and *S. fimbriata*) show two major bands of E_f values 0·05 and 0·21 and three minor bands of E_f values 0·45, 0·57 and 0·73 in common. Among the three flat fishes, the serum of *P. erumei* showed a greater similarity with the serum of *P. arsius* as compared to that of *B. orientalis*.

Deutsch and Mcshan (1949) obtained electrophoretic patterns for *Salmo gairdneri* (rainbow trout), *Salvelinus namaycush* (lake trout), and found that closely related trout species showed extreme and readily recognizable variations. Sanders (1964) in a study of serum proteins of three trout species demonstrated that the patterns of serum protein are the specific characteristic of a particular species. Serum proteins of four *Tilapia* species also show characteristic patterns for each species (Badawi 1971). Other electrophoretic studies concerning the variations in the proteins of fish serum were related to: sexual differences and the degree of sexual maturity (Thomas and McCrimmon 1964), seasonal variations (Saito 1957), disease (Sindermann and Mairs 1958; Thomas and McCrimmon 1964) and changes in diet (Lysak and Wojcik 1960). In our studies, although some individual variations in the staining intensities of the various components were noted, the patterns exhibited a remarkable species specificity and no significant sexual differences were clearly evident. The curves derived from these patterns are depicted in figures 1A–E, as determined by densitometric readings. The electrophoretic fractions have been assigned numbers in the order of their increasing mobility.

A comparison of the patterns (figure 1F) illustrates that the differences between genera (*Sardinella*, *Psettodes*, *Pseudorhombus* and *Brachirus*) are greater than that between species (*S. longiceps* and *S. fimbriata*). Thus the distinctness of the patterns in different species is quite apparent, for the related species do exhibit similarities. Dobzhansky (1962) has described the basis of hereditary characteristics of organisms as follows: 'Brothers and sisters usually differ in many genes (identical twins excepted); races presumably differ in more, species and genera in many more still. All these differences arose by sorting out the genetic raw materials provided by mutations in close, remote, and very remote ancestors'. The available evidence indicates that the same hierarchy of similarities and differences in the blood proteins exists for the near and distantly related taxonomic groups. Species thought to be close relatives have similar patterns.

2.1*b* *Eye lens proteins*: The electropherogram of the eye lens proteins of *P. erumei* shows eight bands (figure 2A), of which the first two bands of slower mobility are the major bands having E_f values of 0·18 and 0·22 respectively (table 2). In *p. arsius*, the eye lens proteins also show eight bands (figure 2B), of which the first and the seventh bands are the major ones with E_f values of 0·14 and 0·70 respectively. The eye lens proteins of *B. orientalis* shows six bands (figure 2C), of which the first and the second bands are the major proteins with E_f values of 0·14 and 0·30 respectively. Some of the bands which separate faintly are not easily discernable in the photographs presented.

The eye lens proteins of *S. fimbriata* show seven bands (figure 2D) of which the first, second and the sixth are the major bands with E_f values of 0·14, 0·22 and 0·62 respectively. Two band patterns, types A and B were observed in the electropherograms of the eye lens proteins of *S. longiceps*. The eye lens proteins of the type A shows seven bands (figure 2E), of which the first and the sixth bands are the major protein bands with E_f values of 0·14 and 0·62 respectively. The electropherogram of the type B shows nine bands (figure 2F), the two extra bands seen in this type were situated at E_f values 0·01 and 0·74 respectively. The first band of the type A migrated slightly away from the point of application having an E_f value of 0·14. Repeated experiments produced the same patterns consistently and did not show any variation either due to sex or size (Menezes 1980a).

The electropherograms of the eye lens proteins of the three flat fishes and the two clupeiod fishes show a number of protein bands of identical mobility (figure 2G). This may be because the lens proteins are synthesized by only one type of cells present in the eye as a unicellular layer. The serum proteins, on the other hand, are synthesized by a variety of cells (Pirie and Heyningen 1956). An additional biological peculiarity of the eye lens is the manner in which it grows; the older portions get increasingly compressed into the centre—the nucleus. In this sense, the adult lens preserves the sum of its life history (Manski *et al* 1964).

The eye lenses of the fish were first studied electrophoretically at the California State Fisheries Laboratory when Smith (1962) examined the soluble lens proteins of the kelp bass (*Paralabrax clathratus*), the blue fin tuna (*Thunnus thynnus orientalis*) and the albacore (*Thunnus alalunga*). The electrophoretic patterns indicated some variation in the curves pertaining to protein concentration but failed to show clear group variations among the species. Molecular differences in the eye lens proteins of the oceanic whitefish, *Caulolatilus princeps* (Jenyns), identified by the cellulose acetate electrophoresis by Smith and Goldstein (1967), apparently reflected the intraspecific genetic variation. The lens proteins of the five scombroid were examined by microstarch gel electrophoresis (Barrett and Williams 1967). Intraspecific variation was found in the Pacific bonito, *Sarda chiliensis* (Cuvier), but was interpreted to be because of the differences in mobility of a single fraction related to ontogenetic factors. However, Eckroat and Wright (1969) indicated that this variation could instead be the result of the inheritance of the two separate fractions whose presence or absence gave rise to the differences in the fish sampled. These fish probably came from two different breeding populations to account for the distribution of the two observed phenotypes. Furthermore, these authors found no difference in the lens protein which could be attributed to the function of age in the trout. Although, genetic information about the lens proteins is very scanty, the available evidence indicates that the variations in the electrophoretic pattern have a genetic basis (Smith 1966; Eckroat and Wright 1969; Greiffendorf and Bech 1969; Smith 1971).

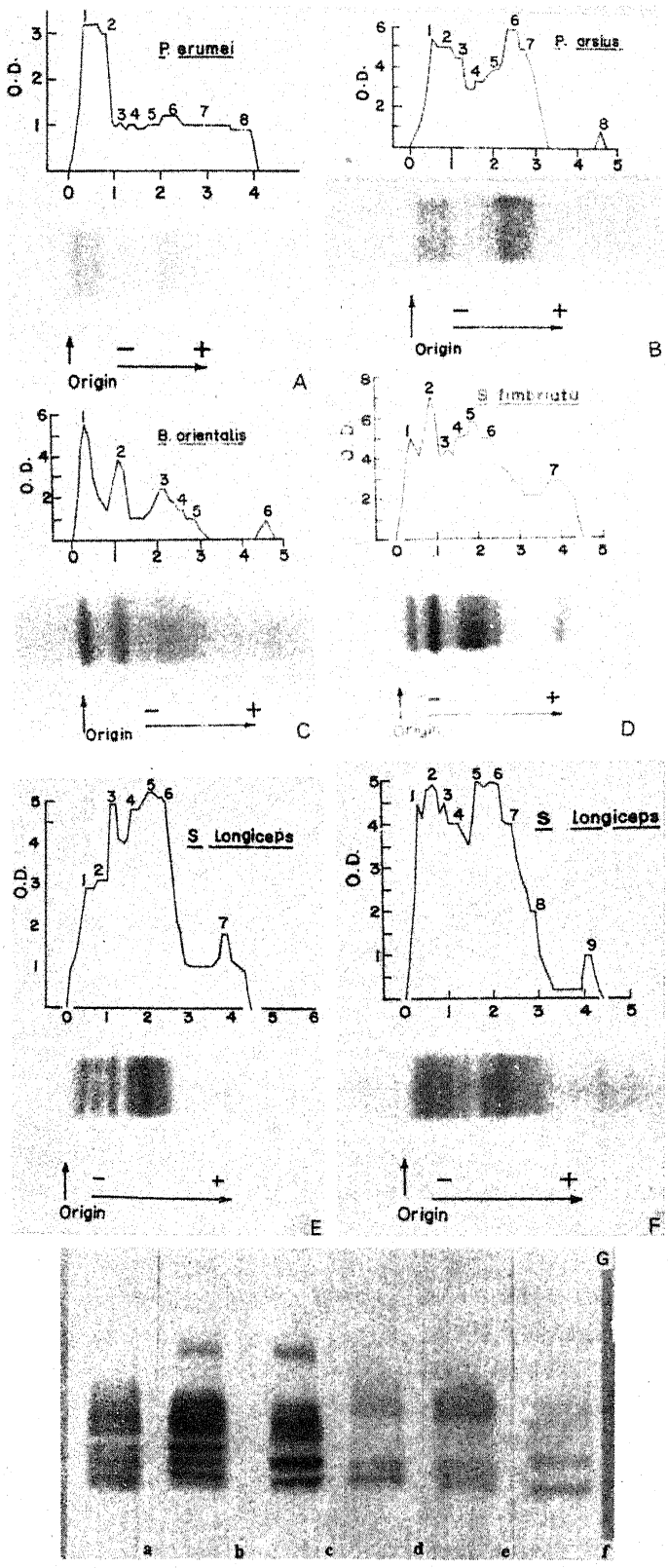


Figure 2. A–E. Electrophoretic pattern of the eyelens proteins of **A.** *Psettodes erumei*; **B.** *Pseudorhombus arisus*; **C.** *Brachirus orientalis*; **D.** *Sardinella fimbriata*; **E.** *Sardinella longiceps*; **F.** *Sardinella longiceps* (type A); **G.** Comparison of the eyelens protein patterns of **a.** *S. longiceps* (type B); **b.** *S. longiceps* (type A); **c.** *S. fimbriata*; **d.** *P. erumei*; **e.** *P. arsius*; **f.** *B. orientalis*.

Table 2. Electrophoretic analysis of eye lens proteins.

Species studied	Fractions (in the order from the slowest to the fastest)																		
	−1a	−1b	−1c	−1d	1	1a	1b	1c	1d	2	2a	2b	2c	2d	2e	3	4	4a	5
Human (reference)	—	—	—	—	0·3 (6·5)	—	—	—	—	0·5 (7·85)	—	—	—	—	—	0·74 (11·69)	0·82 (1·95)	—	0·98 (74·01)
P. erumei	—	—	0·18 (48·21)	0·22 (33·92)	0·3 (1·79)	0·34 (1·79)	—	—	0·46 (1·79)	—	—	—	0·62 (3·57)	—	—	—	—	0·86 (1·79)	0·98 (7·14)
P. arsius	—	0·14 (35·97)	—	0·22 (3·60)	0·3 (9·35)	0·34 (3·6)	—	0·42 (3·6)	—	—	0·54 (11·51)	—	—	—	0·70 (28·78)	—	—	—	0·98 (3·60)
B. orientalis	—	0·14 (38·30)	—	—	0·3 (31·91)	—	—	—	—	0·5 (13·82)	0·54 (4·26)	—	—	0·66 (6·38)	—	—	—	—	0·98 (5·32)
S. longiceps (Type A)	—	0·14 (23·08)	—	0·22 (1·10)	0·3 (8·79)	—	0·38 (6·59)	—	—	0·5 (4·40)	—	—	0·62 (41·75)	—	—	—	—	—	0·98 (14·29)
S. longiceps (Type B)	0·10 (29·31)	—	0·18 (6·03)	0·22 (6·9)	0·3 (4·31)	—	—	0·42 (12·93)	—	0.5 (7·76)	—	—	0·62 (12·93)	—	—	0·74 (12·93)	—	—	0·98 (6·9)
S. fimbriata	—	0·14 (30·53)	—	0·22 (21·37)	0·3 (1·53)	—	0·38 (6·87)	—	0·46 (5·34)	—	—	—	0·62 (19·85)	—	—	—	—	—	0·98 (14·5)

The values represent E_f values along with the percentage (in parentheses) of protein in each fraction.

In our study, patterns produced by the eye lens proteins of *S. fimbriata*, *B. orientalis*, *P. arsius* and *P. erumei* were quite uniform. There was some contrast in the lens pattern of *S. longiceps*, as the protein patterns produced by the lens of this fish revealed a conspicuous polymorphism. These polymorphisms probably result from the presence of a series of alternative genes occurring in the population (Allison 1959; Dobzhansky 1962).

The application of the principles of population genetics to the study of various polymorphic proteins has indicated that the frequencies of occurrence of the allele are often quite different within the populations of the same species (Eckroat 1971). During the past few years, much attention has been directed towards this type of studies on the commercially important fishes. The objectives of these studies have been to determine the genetic polymorphisms and to identify the units of breeding populations separately using the allele frequencies of these polymorphic systems.

The high uniformity in the patterns which the eye lens provides is an evidence in the reliability of the procedures used in the investigations. The pattern of heterogeneity as and when observed indicates that this is a result of genetic differences. In conclusion, it can be stated that the two types of patterns obtained in the lens protein of *S. longiceps* suggest the presence of two distinct populations of the fish in this region (Arabian Sea).

Our study clearly indicates that while the serum proteins may have some general usefulness in the fish taxonomy, the eye lens proteins are preferable for a precise differentiation and for distinguishing the breeding populations separately.

3. Immunoelectrophoretic studies

The soluble eye lens proteins of ten fishes, *Sardinella longiceps*, *S. fimbriata* (Clupeidae); *Hemirhamphus georgii* (Hemirhamphidae); *Lactarias lactarias* (Lactariidae); *Rastrelliger kanagurta* (Scombridae); *Parastromateus niger*, *Pampus argenteus* (Stromateidae); *Psettodes erumei* (Psettodidae); *Pseudorhombus arsius* (Bothidae) and *Brachirus orientalis* (Soleidae), collected from fish landing centres of Panaji, Goa, were studied using immunoelectrophoresis. The lens antiserum of *Sardinella longiceps* was produced in a rabbit (for methodology see Menezes 1980b).

The results of the reaction of the various test antigens with the antisera are given in figure 3. Very clear patterns of the precipitate were obtained with each of the antigen used. The pre-immune serum did not show any reaction with the test antigen.

The proteins of the lens were chosen for the investigation because they have a distinct advantage in showing an extremely wide range of serological cross-reactions (Hektoen 1922). An antiserum prepared against the lenses of any of the vertebrate species gives precipitin reactions with the lenses of all the other vertebrates (Manski *et al* 1964). This is in striking contrast to those serum proteins which may be far more intensively studied, but they have a considerably more limited range of cross-reactions (Nuttall 1904).

Antiserum prepared from the lens of *S. longiceps*, when reacted with the lens protein of the same species showed four components. With the lens of *S. fimbriata* also, it showed four components but there was a slight variation in the design of the lines of precipitates formed and in their mobilities. Reaction with the *H. georgii* lens showed five components while with *L. lactarias* it showed three, with *R. kanagurta* two, with *Parastromateus niger* and *Pampus argenteus* three each, with *Psettodes erumei* and

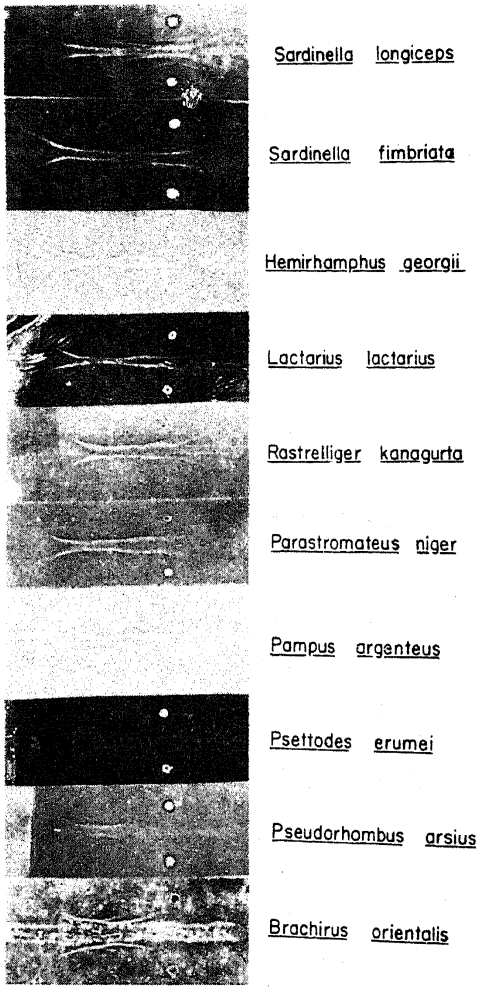


Figure 3. Immunoelectrophoretic patterns of the eyelens proteins of the fishes.

Pseudorhombus arsius one each and with *B. orientalis* two components. As the closely-related species display similar reactions and share some of the precipitate bands in their reactions of identity, it is possible to get a good idea of their relationships.

Antisera from the squid lens, prepared in the rabbit had four to five components when tested with the squid lens for the immunodiffusion assays (Manski *et al* 1961). No cross-reactions, however, were noted with a number of vertebrate lenses. Similarly the antisera against the vertebrate lens failed to show any cross-reaction with squid lens. (Manski *et al* 1964). This shows that the invertebrate lens does not share any immunochemical similarity with the vertebrate lenses and that the cephalopod eye was evolved separately as a distinct offshoot from that of the vertebrates. From the reactions obtained with the antihuman lens serum and with the lens sera of other vertebrates, it is clear that the phylogenetic relations among the vertebrate lenses show a decrease in the number of shared components when they get farther and farther removed phylogenetically (Hektoen 1922; Manski *et al* 1960; 1961).

In our studies also, the immunoelectrophoretic patterns (figure 3) clearly show a decrease in the number of shared components as the phylogenetic distance increased. It has been proved that the fishes which are more closely related on a morphological basis tend to have common antigens (Menezes 1980b).

Thus the study indicates that this technique can be used to determine the taxonomic relationships between the different species.

4. Pollution studies

4.1 *Effect of mercury*

To determine whether any alteration occurs in the normal make-up of the electrophoretic patterns of blood serum, haemoglobin and the eye lens proteins of the fishes on their exposure to adverse environmental pollutants, a study was conducted under controlled conditions. Long-term and short-term effects of low and high concentrations of mercury, on a freshwater fish *Tilapia mossambica* (see Menezes 1981) were investigated. Inorganic mercury in the form of mercuric chloride ($HgCl_2$) was introduced as a toxicant. Collateral studies included the uptake of metal by the fishes. To select the threshold concentrations of mercury for the study, some preliminary tests were conducted on the acute toxicity and growth efficiencies (Menezes and Qasim 1983a,b) of the fish in relation to some levels of the toxicant.

The potential value of electrophoresis in this study is based on the hypothesis that stress conditions may cause significant changes in the proteins of the blood serum, in the haemoglobin and in the eye lens protein. Such changes might reflect an altered antibody synthesis, protein biosynthesis, cellular leakage or perhaps other events resulting directly or indirectly from the stress.

4.1*a* *Long term-exposure*: The concentration of mercury in the fish muscle was related to the concentration of mercury in the water. At $29° \pm 1°C$, a 9 weeks exposure to two levels $-0{\cdot}01$ and $0{\cdot}04$ ppm of mercury, produced a mercury concentration of $0{\cdot}29$ and $2{\cdot}04\ \mu g\ g^{-1}$ wet weight of the muscle respectively. This was unlike the control group where the mercury concentration was $0{\cdot}024 \pm 0{\cdot}003\ \mu g\ g^{-1}$ wet weight.

During the exposure period, it was observed that the control fish fed readily whenever food was offered, but this was not the case with the experimental groups. Although feeding in 0·01 ppm mercury was not seriously affected, the response towards the food was somewhat sluggish at the end of the test period. In 0·04 ppm, the fish usually ignored the food after the 5th week of exposure.

The natural electrophoretic pattern of haemoglobin showed two clearly-defined anodic components—a broader, denser, fast-moving band and a less mobile, slightly narrower and less dense band (figure 4A). Exposure to 0·01 and 0·04 ppm mercury for eleven weeks led to an increase in the protein in the faster moving band (figure 4B(a,b)). This was statistically significant at 1% level. The results on the mobility and the percentage of protein in the bands are summarised in table 3.

The natural eye lens pattern consisted of seven components: one component on the cathodic side, a slow component on the anodic side, three medium fast anodic components, and two fast anodic components (figure 4C). The first two components of slower mobility and the sixth one having relative mobilities of $-17{\cdot}3$, $13{\cdot}3$ and $82{\cdot}3$ from the point of application are the major protein bands (table 4). An exposure to 0·01 and 0·04 ppm mercury did not show much change in the components of the eye lens protein (figure 4D(a,b)). The results of this investigation have been summarized in table 4.

The natural electrophoretic pattern of the serum proteins consists of nine bands—

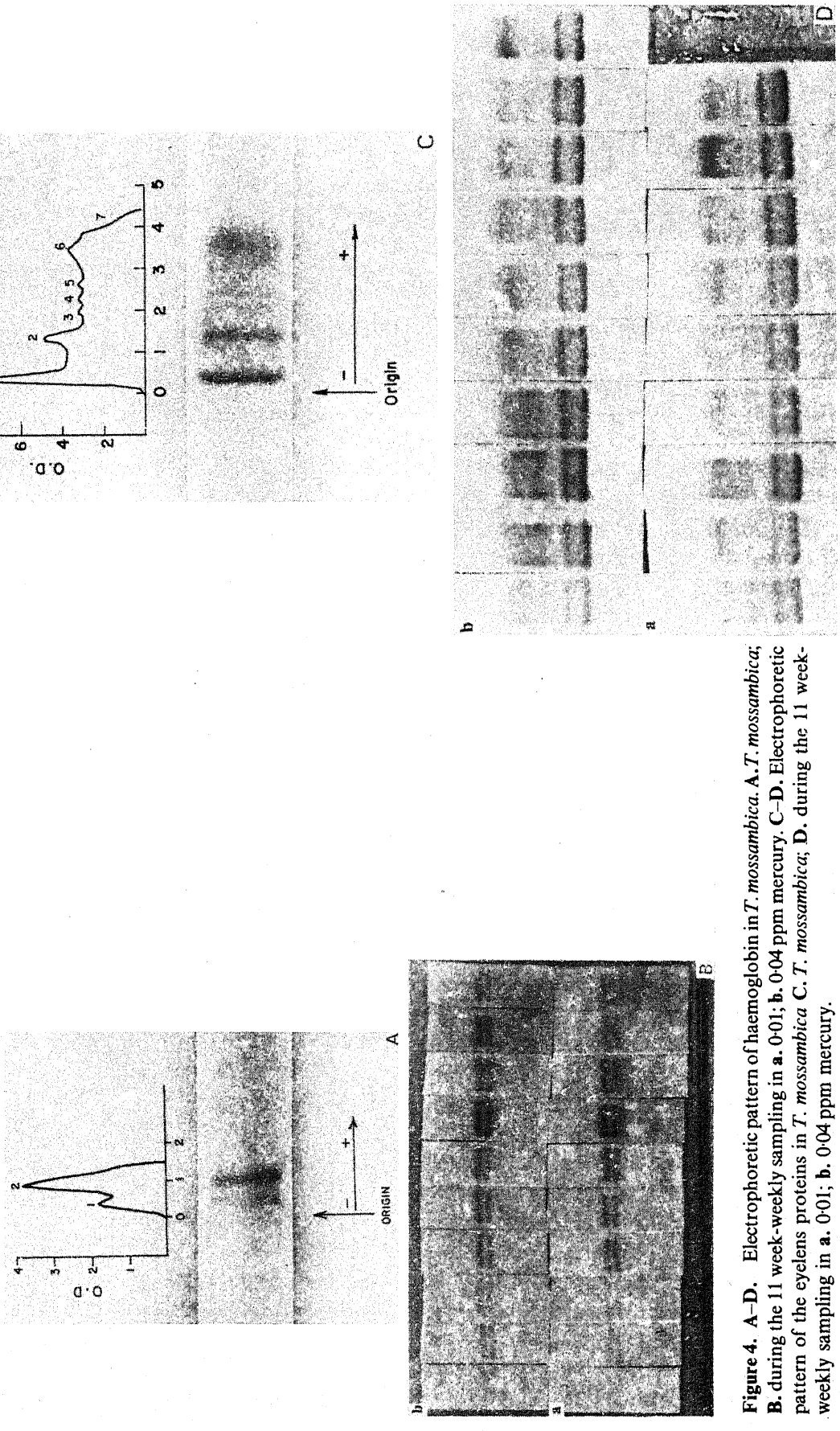


Figure 4. A–D. Electrophoretic pattern of haemoglobin in *T. mossambica*. **A.** *T. mossambica*; **B.** during the 11 week-weekly sampling in **a.** 0·01; **b.** 0·04 ppm mercury. **C–D.** Electrophoretic pattern of the eyelens proteins in *T. mossambica* **C.** *T. mossambica*; **D.** during the 11 week-weekly sampling in **a.** 0·01; **b.** 0·04 ppm mercury.

Table 3. Electrophoretic analysis of haemoglobin in *T. mossambica.*

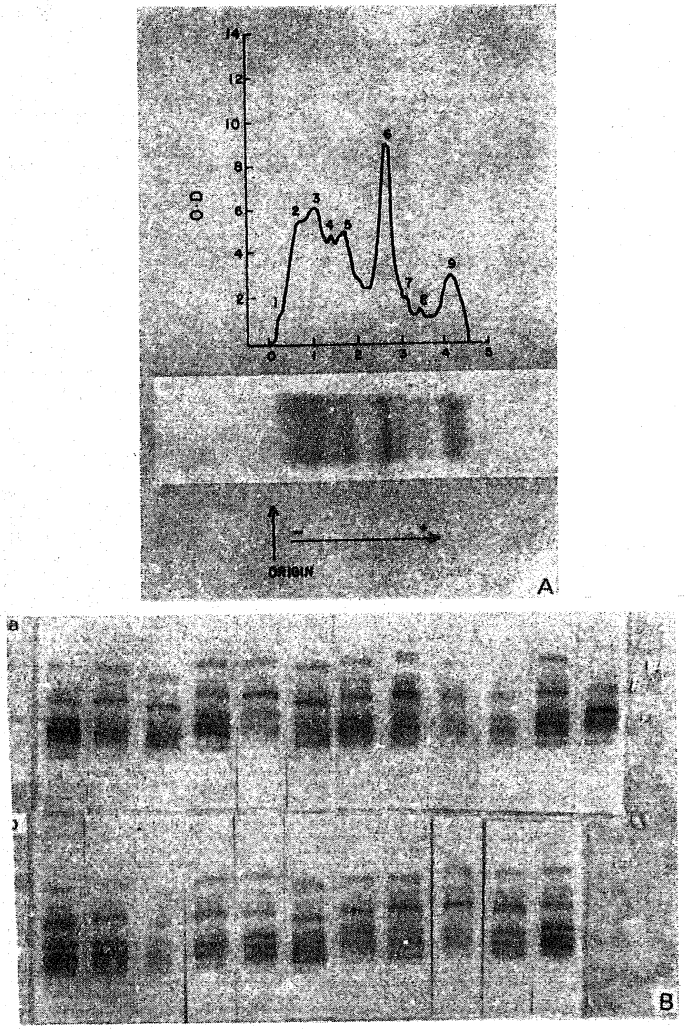
Analysis of haemoglobin	Concentration of mercury ppm	Fractions (in the order, from the slowest to fastest) 1	2
Relative mobility as percentage	Control	47·99	100
	0·01	51·74	100
	0·04	50·51	100
Percentage of protein in each fraction	Control	45·23	54·77
	0·01	40·81	59·19
	0·04	37·44	62·56

Table 4. Electrophoretic analysis of the eye lens proteins in *T. mossambica.*

Analysis of eye lens proteins	Concentration of mercury ppm	Fractions (in the order, from the slowest to the fastest) 1	2	3	4	5	6	7
Relative mobility as percentage	Control	−17·33	13·33	31·66	42·66	50·00	92·33	100
	0·01	−16·99	13·39	31·37	42·16	49·67	81·37	100
	0·04	−17·16	13·30	31·68	42·24	49·83	81·52	100
Percentage of protein in each fraction	Control	28·39	20·43	9·84	8·66	8·15	18·79	5·65
	0·01	28·50	22·42	9·2	8·89	8·03	17·6	5·27
	0·04	29·4	20·35	8·94	8·51	7·95	19·55	5·24

one located at the point of application, four are the slow anodic bands, three are the medium fast anodic bands, and one is the fast anodic band (figure 5A). The 2nd, 3rd and 6th bands have relative mobilities of 10·29, 21·34 and 58·54. They are the major protein bands (see table 5).

Exposure to 0·04 ppm mercury lasting for eleven weeks caused significant changes in the patterns of serum protein in the group of fishes exposed to this concentration. After the first week of exposure, there was a significant increase (at 1 % level) in the protein of the lower mobility bands, particularly in the 3rd band, and a decrease in the faster moving bands (figure 5B(a)). There was also a remarkable increase in the mobility from the 2nd band onwards. These changes remained somewhat similar up to the fourth week of the exposure at the 0·04 ppm. During the fifth week the concentration of mercury in the muscle of fishes was 0·65 ppm. During the eighth week of exposure, the concentration of mercury in the muscle was 1.45 ppm. In both these situations only one dense band was seen in the place of 4th and 5th bands. In the ninth week of exposure, the corresponding muscle concentration became 2.04 ppm of mercury. At this point, an extra band was seen over the 8th fast-moving band which became even more prominent after the tenth week of exposure. After the eleventh week of exposure, when all the fishes were killed and analysed, two types of patterns were seen. Some had both 4th and 5th bands along with the extra band in the same position as the others (figure 5C), while



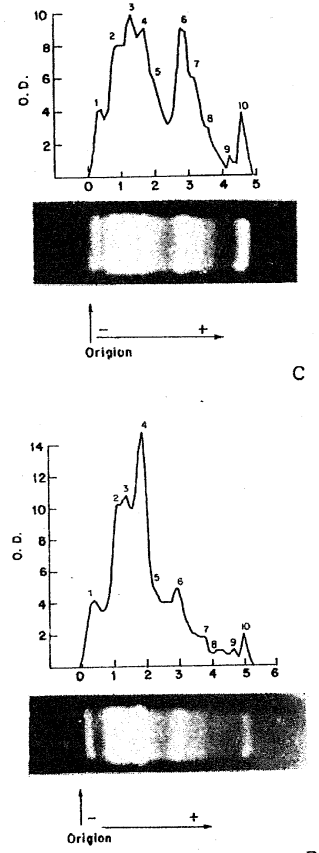


Figure 5. Electrophoretic pattern of serum proteins in *T. mossambica*. **A.** *T. mossambica*; **B.** during the 11 week-weekly sampling in **a.** 0·04; **b.** 0·01 ppm of mercury; **C–D.** in 0·04 ppm mercury after the 11 week exposure period. **C.** Type 1; **D.** Type 2.

Table 5. Electrophoretic analysis of the serum proteins in *T. mossambica.*

Analysis of serum proteins	Concentration of mercury ppm	Fractions (in the order, from the slowest to the fastest)									
		1	2	3	4	5	6	7	8a	8b	9
Relative mobility as percentage	Control	0	10·29	21·34	32·37	38·72	58·54	66·82	78·69	—	100
	0·01	0	10·02	20·48	32·59	39·33	59·41	69·47	79·32	—	100
	0·04	0	11·53	21·22	33·69	41·24	58·45	68·81	78·12	86·17	100
Percentage of protein in each fraction	Control	2·77	14·87	19·98	10·0	9·92	21·73	5·71	4·95	—	10·07
	0·01	2·90	16·35	23·62	14·77	8·76	16·68	5·66	4·48	—	6·77
	0·04	3·18	16·99	24·67	19·0	7·12	16·46	5·65	4·03	1·71	6·02

the others had only one dense band in place of the 4th and the 5th bands along with an extra band in the same position as the others (figure 5D).

The 0·01 ppm group showed an increase in the lower mobility bands and some changes were also noticed in their mobility (table 5, figure 5B(b)). Other changes seen in the 0·04 ppm group were not observed here. Moreover, the concentration of mercury in the muscle of these fishes was much lower than in the 0·04 ppm mercury group. Unlike the experimental fishes described above, the control group showed a remarkable constancy in the number, mobility and in the intensity of the protein.

Towards the end of the test period, two fishes had died in the 0.04 ppm mercury, while the others showed erratic swimming movements and had almost stopped feeding. In the batch containing 0·01 ppm mercury, the fishes responded sluggishly while the control group fed readily whenever food was offered to them.

4.1*b* *Short-term exposure*: Short-term experiments were conducted on the serum protein electrophoresis when the fishes were exposed to very high concentrations of mercury (0·4, 0·6 and 0·8 ppm). These were just below the lethal level as the 48-hr LC50 value of mercury to *T. mossambica* had previously been found to be 1 ppm. The experimental fishes showed electrophoretic patterns similar to the ones obtained in 0.04 ppm mercury after the exposure period of eleven weeks.

After 3 hr of exposure to 0·4 ppm, 0·6 ppm and 0·8 ppm mercury, the serum patterns showed some increase in the protein in the lower mobility bands and a decrease was noticed in the high mobility bands (figure 6A). The latter became more pronounced after the 24 hr exposure period (figure 6B). After the exposure of 48 and 72 hr also, a distinct extra band was seen between 8th and the 9th fast moving bands (figure 6C,D). The control groups showed a remarkable stability in the number of their protein bands as compared to the test groups. The relative mobility and percentage of protein in each band are summarized in table 6.

The electrophoretic patterns of the serum and haemoglobin in *T. mossambica* showed changes in response to low concentrations of mercury. These changes became clearly evident in the serum proteins at 0·04 ppm level, with respect to number, mobility and the intensity of fractions. Further, in 0·04 ppm mercury, the fish mostly ignored the food after the 5th week of exposure resulting in some mortality towards the end of the test period. Judging from the effects of this concentration of mercury on the blood

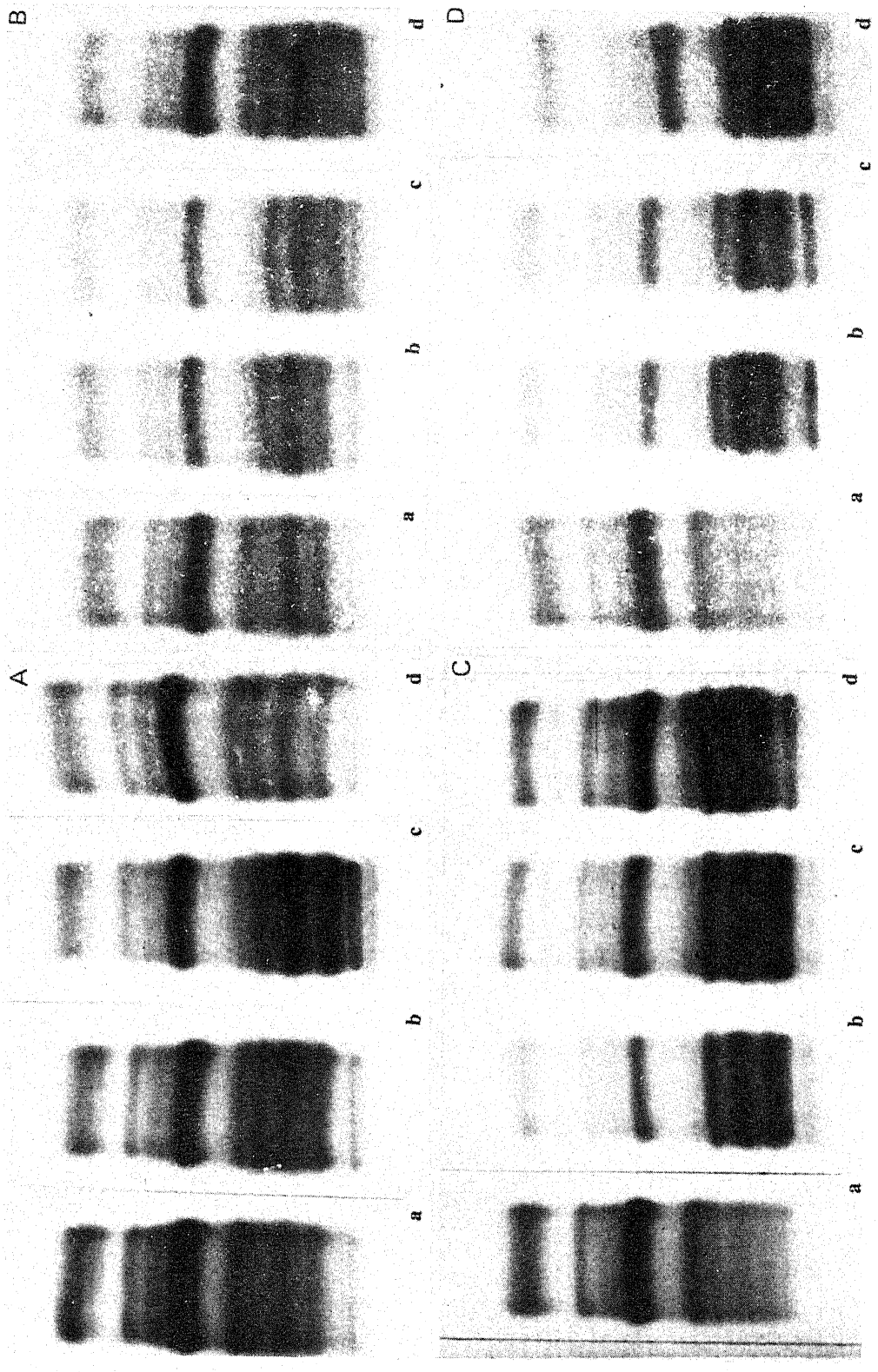


Figure 6. Electrophoretic patterns of serum proteins in *T. mossambica*. **a.** control; **b.** 0·4; **c.** 0·6; **d.** 0·8 ppm mercury after exposure (in hr) for **A.** 3; **B.** 24; **C.** 48; **D.** 72.

Table 6. Electrophoretic analysis of *T. mossambica* serum in 0·4, 0·6 and 0·8 ppm mercury.

Concentration of mercury ppm	Time in hr	Fractions (in the order, from the slowest to the fastest)									
		1	2	3	4	5	6	7	8	8a	9
Control	—	0	12·73	23·94	33·03	40·61	60·0	70·39	79·69	—	100
		(2·76)	(12·47)	(14·58)	(9·49)	(9·82)	(29·92)	(4·77)	(4·94)	—	(11·25)
0·4	24	0	12·19	21·95	34·15	41·46	60·98	70·73	80·49	—	100
		(8·33)	(10·72)	(22·59)	(11·59)	(8·47)	(18·32)	(3·98)	(6·44)	—	(9·56)
	48	0	10·45	20·91	30·60	37·32	55·24	64·95	72·40	88·81	100
		(6·62)	(19·49)	(32·35)	(13·42)	(2·39)	(12·87)	(4·04)	(3·31)	(1·47)	(4·04)
	72	0	15·55	24·44	33·33	40·0	57·78	66·67	75·55	86·67	100
		(11·67)	(25·52)	(25·83)	(12·92)	(3·29)	(10·0)	(2·71)	(2·91)	(1·67)	(4·48)
0·6	24	0	9·09	20·45	31·82	36·36	56·82	68·18	72·27	—	100
		(5·99)	(11·19)	(25·99)	(17·80)	(5·11)	(19·56)	(3·52)	(4·58)	—	(6·26)
	48	0	7·14	16·67	28·57	35·71	55·81	65·12	74·42	88·37	100
		(7·65)	(17·45)	(34·91)	(14·43)	(3·74)	(12·64)	(2·32)	(2·32)	(1·51)	(3·03)
	72	0	11·11	22·22	33·33	38·89	55·55	66·67	75·55	86·67	100
		(3·91)	(19·40)	(28·91)	(10·15)	(5·73)	(15·23)	(3·91)	(3·91)	(3·25)	(5·60)
0·8	24	0	11·36	22·73	34·04	40·91	56·82	68·18	77·27	—	100
		(7.56)	(15·49)	(17·97)	(14·67)	(4·99)	(20·81)	(3·87)	(4·56)	—	(9·89)
	48	0	9·52	19·05	30·95	38·09	54·76	66·67	76·19	88·09	100
		(3·42)	(18·43)	(26·71)	(15·01)	(5·85)	(18·98)	(2·76)	(3·86)	(1·44)	(3·54)
	72	0	8·85	18·96	31·05	36·66	55·84	65·94	76·28	88·65	100
		(11·32)	(12·26)	(34·81)	(16·89)	(1·42)	(10·85)	(2·64)	(2·64)	(2·08)	(5·09)

The values represent relative mobility as percentage along with the percentage of protein in each fraction (given in parenthesis).

proteins, feeding behaviour, swimming activity and the mercury concentration in the muscle of these fishes, it can be inferred that the observed changes in the serum protein fractions were associated with the increasing accumulation of mercury in the fish tissue.

The clinical value of the protein analysis by electrophoresis depends upon whether a given change represents an adaptation to stress conditions or a failure in the supportive physiological and biochemical mechanisms of the animals. Results from the studies conducted by Bouck (1966) showed that changes in the plasma protein were due, in parts, to the loss of protein (enzymes) from the tissues to the blood. Such a change could occur as a result of an increase in the rate of cellular degeneration or due to the leakage of proteins across the afflicted cell membranes.

Brett (1958) offered the following definition of the stress: "Stress is a state produced by an environmental or other factors which extends the adaptive responses of an animal beyond the normal range, or which disturbs the normal functioning to such an extent that, in either case, the chances of survival are significantly reduced". This definition suggests that the degree of stress could be assessed from the measurement of either a reduction in the physiological capacity or of the chances of survival of the animal, or from both.

In the present study, there was also a general increase in the intensity of the low mobility serum protein fractions—a pattern similar to that found in the mammalian species under stress. Similar changes were also seen in the serum proteins of the fish exposed to realatively higher concentrations of mercury which were just below the lethal level. There is an evidence that the low mobility proteins in the fish serum function similar to that of gamma globulins found in the human being, in their being the antibodies (Post 1963, 1966; Summerfelt 1966). Furthermore, Bouck and Ball (1967) observed that the fish species with serum protein having low levels of low mobility fractions were least likely to survive in polluted waters. If the low mobility serum proteins of *T. mossambica* function as antibodies, as in the case of other fishes, then in the experimental group, the increased levels of protein, in the low mobility serum proteins, is probably due to rapid and vigorous immune response shown by the fish when it is exposed to mercury.

Selye (1950) has categorized the stress response of mammals into three stages: the alarm reaction, the stage of resistance, and the stage of exhaustion. It appears that the batches of fish which were exposed to higher concentrations of mercury went through all these three stages, whereas those which were exposed to lower concentrations did not reach the stage of exhaustion. Rather, they were able to accommodate and adapt to the stresses. This suggests that the blood measurements made after a short exposure to a toxicant would be useful in determining the concentration of the toxicant, which under the chronic exposure will produce ill effects on a fish population.

The electrophoretic patterns of the serum obtained after the exposure of 48 and 72 hr to sublethal concentrations of mercury, were similar to the one obtained in 0·04 ppm mercury concentration when the period of exposure was 11 weeks. These features can be regarded as the 'stress pattern' of the serum of *T. mossambica* for the mercury (Menezes and Qasim 1983c). Further work is in progress using other potential pollutants to determine whether the stress pattern is similar in all cases. It would particularly be useful for testing whether or not an effluent or a body of water containing a mixture of chemicals will collectively have a stressful effect on the fish. Such a stress response would tend to reflect the summation of the effect of all the environmental contaminants present and perhaps would reveal synergistic effects

between two or more pollutants. Not only should the water be non-toxic, it should elicit no stress response in the fish.

It can thus be concluded that electrophoretic analysis provides a very useful method for certain aspects of biology, biochemistry and medicine, and that it can be used as an additional tool to evaluate environmental stress on animals with success.

References

Anifinsen C B 1961 The influences of three dimensional configuration on the chemical reactivity and stability of proteins; *J. Polym. Sci.* **49** 31–49

Allison A C 1959 Metabolic polymorphisms in mammals and their bearing on problems of biochemical genetics; *Am. Nat.* **93** 5–16

Badawi H K 1971 Electrophoretic studies of serum proteins of four tilapia species (Pisces); *Mar. Biol.* **8** 96–98

Barrett I and Williams A A 1967 Soluble lens proteins of some scombroid fishes; *Copeia* **1967** 468–471

Bouck G R 1966 *Changes in blood and muscle composition of rock bass (Ambloplites rupestris) as physiological criteria of stressful conditions*, Ph.D. Thesis, Michigan State University, East Lansing, Michigan. 155 p

Bouck G R and Ball R C 1967 Distribution of low mobility proteins in the blood of fishes; *J. Fish. Res. Bd. Canada* **24** 695–697

Brett J R 1958 Implications and assessments of environmental stress, in *The investigations of fish power problems, H R MacMillan lectures in fisheries*, (ed) P A Larkin, (Vancouver: British Columbia Press) pp. 69–83

Deligny W 1969 Serological and biochemical studies on fish populations; *Oceanogr. Mar. Biol. Annu. Rev.* **7** 411–513

Deutsch H F and Mcshan W H 1949 Biophysical studies of blood plasma proteins XII. Electrophoretic studies of the blood serum proteins of some lower animals; *J. Biol. Chem.* **180** 219–234

Dobzhansky T 1962 *Mankind evolving* (New Haven, Connecticut: Yale University Press) pp. 381

Eckroat L R 1971 Lens protein polymorphisms in Hatchery and Natural populations of brook trout *Salvelinus frontinalis* (Mitchell); *Trans. Am. Fish. Soc.* **100** 527–536

Eckroat L R and Wright J E 1969 Genetic analysis of soluble lens protein polymorphism in brook trout *Salvelinus fontinalis; Copeia* **3** 466–473

Fujino K 1970 Immunological and biochemical genetics of tunas; *Trans. Am. Fish. Soc.* **99** 152–178

Greiffendorf G and Bech S L 1969 A genetic investigation of eye lens protein variants among inbred mouse strains; *Am. Zool.* **9** 614

Griffith R W, Umminger B L, Grant B F, Pang K T and Pickford G E 1974 Serum composition of the coelacanth *Latimeria chalumnae* Smith; *J. Exp. Zool.* **187** 87–102

Hatting J 1974 The plasma proteins of *Labeo umbratus* (Smith) and *Labeo capensis* (Smith); *J. Fish. Biol.* **6** 439–446

Hektoen L 1922 The specific precipitin reaction of the normal and cataractous lens; *J. Infect. Dis.* **31** 72–78

Krauel K K and Ridgway G J 1963 Immunoelectrophoretic studies of red salmon (*Oncorhynchus nerka*) serum; *Int. Arch. Allergy. Appl. Immun.* **23** 246

Landsteiner K 1901 Uber agglinationsercheinungen normalen menschlichen Blutes; *Wien. Klin. Wochenschr.* **14** 1132–1134

Love R M 1970 *The chemical biology of fishes* (London: Academic Press) 547 p.

Lumry R and Eyring H 1954 Conformation changes of proteins; *J. Phys. Chem.* **58** 110

Lysak A and Wojcik K 1960 Electrophoretic investigations on the blood of carp fed with food containing various protein amounts; *Acta Hydrobiol.* vol II

Markert C L and Moller F 1959 Multiple forms of enzymes: Tissue, ontogenetic and species specific patterns; *Proc. Natl. Acad. Sci. U.S.A.* **45** 753

Manski W, Auerbach T P and Halbert S P 1960 On the phylogenetic pathways of lens proteins; *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **19** 334

Manski W, Halbert S P and Auerbach T P 1961 Immunochemical analysis of lens protein separations; *Arch. Biochem. Biophys.* **92** 512

Manski W, Halbert S P and Auerbach T P 1964 Immunochemical analysis of the phylogeny of lens proteins, in *Taxonomic biochemistry and serology* (ed.) C A Leone (New York: The Ronald Press)

Menezes M R 1979 *Morphometry and serology of some fishes from the Goa region.* M.Sc. thesis, University of Bombay, 144 p

Menezes M R 1980a Soluble lens protein polymorphism in oil sardine *Sardinella longiceps* Val; *Mahasagar–Bull. Natn. Inst. Oceanogr.* **13** 183–185

Menezes M R 1980b Immunochemical analysis of soluble lens proteins in some Marine fishes; *Indian J. Mar. Sci.* **9** 63–65

Menezes M R 1981 *Studies on some physiological changes in fishes from polluted and unpolluted environments,* Ph.D. Thesis, University of Bombay 204 p

Menezes M R and Qasim S Z 1983a Determination of acute toxicity levels of mercury to the fish *Tilapia mossambica* (Peters); *Proc. Indian Acad. Sci. (Anim. Sci.)* **92** 375–380

Menezes M R and Qasim S Z 1983b Growth efficiencies of *Tilapia mossambica* (Peters) in relation to the toxicant (mercury); *Mahasagar* (in press)

Menezes M R and Qasim S Z 1984 Effects of mercury accumulation on the electrophoretic patterns of the serum, haemoglobin and eye lens proteins of *Tilapia mossambica* (Peters); *Water Res.* **18** 153–161

Nuttall G H F 1901 The new biological test for blood in relation to zoological classification; *Proc. R. Soc. (London)* **B69** 150–153

Nuttall G H F 1904 *Blood immunity and blood relationships* (Cambridge: University Press)

Pirie A and R Van Heyningen 1956 *Biochemistry of the eye*; (Springfield: Charles C Thomas) 111 p

Post G 1963 The immune response of rainbow trout (*Salmo gairdneri*) to *Aeromonas hydrophilus*; *Utah State Dep. Fish. Game Inform. Bull.* **63** 1–82

Post G 1966 Serum proteins and antibody production in rainbow trout (*Salmo gairdneri*); *J. Fish. Res. Bd. Canada* **23** 1957–1963

Ridgway G J, Klontz G W and Matsumoto G 1962 Intraspecific differences in serum antigens of red salmon demonstrated by immunochemical methods; *Bull. No.* 8, *Int. North Pacific Fisheries Commission*

Saito K 1957 Biochemical studies on the fish blood. On the seasonal variations of serum protein components of cultured fish; *Bull. Jpn. Soc. Scient. Fish.* **22** 768–777

Sanders B G 1964 Electrophoretic studies of serum proteins of three trout species and the resulting hybrids within the family salmonidae, in *Taxonomic biochemistry and serology* (ed.) C A Leone (New York: The Ronald Press)

Selye H 1950 *Stress* (Montreal Quebec: Acta Inc.) 822 p

Shaw C R 1965 Electrophoretic variation in enzymes; *Science* **149** 936

Sindermann C J and Mairs D F 1958 Serum protein changes in diseased sea herring; *Anat. Rec.* **131** 599–600

Smith A C 1962 The electrophoretic characteristics of albacore, blue fin tuna and kelpbass eye lens proteins; *Calif. Fish. Game Bull.* **48** 199–201

Smith A C 1966 Electrophoretic studies of eye lens proteins from marine fishes Ph.D. Thesis, University of California, Irvine, *Diss. Abstr.* **28** 1968 98 p

Smith A C and Goldstein R A 1967 Variation in protein composition of the eye lens nucleus in ocean whitefish, *Caulolatilus princeps; Comp. Biochem. Physiol.* **23** 533–539

Smith A C 1971 The soluble proteins in eye lens nuclei of albacore, blue fin tuna and bonito; *Comp. Biochem. Physiol.* **39** 719–724

Summerfelt R C 1966 Homology of serum proteins of golden shiner (*Notemigonus crysoleucas*) and man; *Trans. Am. Fish. Soc.* **95** 272–279

Thomas M L H and McCrimman H R 1964 Variability in paper electrophoretic patterns of the serum of landlocked sea lamprey, *Petromyzon marinus; J. Fish. Res. Bd. Can.* **21** 239–246