BIOCHEMICAL INVESTIGATIONS ON THE STABILITY OF BIOLOGICAL MEMBRANES

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This is to certify that the thesis entitled ***Biochemical Investigations on the Stability of Biological membranes*** is an authentic record of research work carried out by Smt. Priya.M., under my supervision and guidance in the Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, in partial fulfilment of the requirements for the degree of Doctor of Philosophy of the Cochin University of Science and Technology and no part thereof has been presented before for the award of any other degree, diploma or associateship in any University.

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Chapter 1

General Introduction and Review of Literature

SCIENTIFIC BACKGROUND

Biological membranes are organized sheet like structures forming closed compartments around cellular protoplasm consisting mainly of proteins and lipids. The plasma membranes besides permitting cellular individuality by separating one cell from another carry out functions that are indispensable for life. Membranes are highly viscous yet plastic structures forming specialized intracellular compartments leading to morphologically distinguishable organelles eg., mitochondria, endoplasmic reticulum, sarcosplasmic reticulum, golgi complexes, secretory granules, lysosomes and nuclear membrane. Functional specialization in the course of evolution has been closely linked to formation of such compartments.

Biological membranes, though diverse in structure and function, share a number of common attributes. They are sheet like structures, a few molecules thick forming closed boundaries between compartments of different composition. Membranes mainly consist of lipids and proteins, the weight ratio of protein to lipid in most biological membranes ranging from 1: 4 to 4: 1. Membrane lipids are relatively small molecules that have both a hydrophilic and hydrophobic moiety. These lipids spontaneously form closed bi-molecular sheets in aqueous media and are barriers to flow of polar molecules. Membrane proteins are embedded in lipid bilayers and specific proteins mediate distinctive functions of membranes. They are non-covalent assemblies of proteins and lipid molecules.

Membranes are asymmetric, fluid structures regarded as two-dimensional solutions of oriented proteins and lipids. They are thermodynamically stable and metabolically active.*1 In 1972, Jonathan, S.Singer and Garth Nicolson proposed a fluid mosaic model for the overall organization of biological membranes. The essence of their model is that membranes are two-dimensional solutions of oriented globular proteins and lipids. The major features of this model are (1) Most of membrane phospholipid and glycolipid molecules are arranged in a bilayer (2) The lipid bilayer plays a dual role as a solvent for integral membrane proteins

and a permeability barrier. (3) Membrane proteins are free to diffuse laterally in the lipid matrix except when restricted by specific interactions (4) They are not free to rotate from one side of the membrane to the other (Flip - Flop or transverse diffusion).

Normal cellular function obviously begins with normal membrane structure. Gross alterations of membrane structure can affect water balance and ion influx and thus every cellular process. A variety of diseases can be caused by specific deficiencies or alterations in membrane components. eg., Type II glycogen storage disease (due to lysosomal absence of acid maltase); congenital goitre (caused by lack of an iodide transporter) and accelerated hypercholesterolemia and coronary artery disease (resulting from defective endocytosis of low density lipoproteins). Different membranes within the cell and between cells have different compositions as reflected in the ratio of protein to lipid and hence their different functions. ***2**

Distinctive membrane functions such as transport, communication and energy transduction are mediated by specific proteins, some of which are deeply embedded in the hydrocarbon regions of the lipid bilayer. Membranes are structurally and functionally asymmetric as exemplified by the directionality of ion transport systems and the restriction of sugar residues to the external surface of mammalian plasma membranes. They are dynamic structures in which proteins and lipids diffuse (in the plane of the membrane) laterally unless restricted by special interactions, while the transverse diffusion or flip- flop diffusion (rotation of proteins and lipids from one face of membrane to the other) is usually very slow. The degree of fluidity of a membrane partly depends on the chain length of its lipids and the extent to which their constituent fatty acids are unsaturated. ***3**

Diffusion rates in lipid bilayers are considered a function both of temperature and composition of the membrane. Bilayers consisting of a single type of phospholipid typically show an abrupt change in physical

properties over a characteristic and narrow temperature range. In contrast to pure phospholipid bilayers, membranes isolated from cells usually undergo such phase transitions over a much broader temperature range (≥10°C). ***4**

Both length of the fatty acyl groups present and proportion of unsaturated fatty acids affect the fluidity of a biological membrane at a given temperature. Thus, in general lipids bearing short or unsaturated fatty acyl chains undergo phase transitions at lower temperatures than those containing long chain saturated fatty acids. ***5**

Broad phase transitions are a general characteristic of cellular membranes due to heterogenity of lipids in the biological membranes and decreased mobility of lipids due to the presence of integral membrane proteins. Divalent cations like Ca^{2+} and Mg^{2+} are well known stabilizers of biological membranes and their removal often leads to lysis of cells and dissociation of peripheral membrane proteins. They presumably form ionic bonds with neighbouring phosphoryl head groups; tending to the phospholipid molecules together limiting their mobility.***6** Temperature, ionic environments and fatty acid compositions of phospholipids and glycolipids and presence or absence of cholesterol can affect general physical state of biological membrane. While local mobilities of membrane components can be influenced by protein - protein, lipid - protein and lipid - lipid interactions.

In response to environmental changes, many cells can regulate composition of their membranes to maintain the overall semi-fluid environment necessary for many membrane-associated functions. The assembly and maintenance of membrane structures in cells is a dynamic process. Components are not only synthesized and inserted into a growing membrane but are continuously degraded at a slower rate. This turnover process varies with each individual molecule type.

Generally phospholipids have shorter half-life in the membrane (high turnover) than membrane proteins, which themselves vary greatly in life expectancy depending on the specific pattern. This constant turnover allows cells to rapidly adjust membrane composition in response to changes in the environment. (Temperature, nutrition, etc.,).*7

Despite phylogenic differences, a unifying factor of all cells is that they contain many identical chemical constituents, metabolic pathways and mechanisms of cell recognition. This allows for a mode of biochemical deduction based on extrapolation of results obtained in one species (usually of lower phylogenic order) to another.

SCOPE OF THE STUDY

In this project, an attempt has been made to study the stability of erythrocyte and lysosomal membranes biochemically. The physiological and pharmacological effect of selected biochemicals on the stability of erythrocyte and lysosome membranes has been assessed.

Erythrocytes were chosen for the study because of their ready availability and relative simplicity (as they lack organelles and have only a single plasma membrane). They have been used as a model system to study the effect of toxic substances on erythrocyte membrane by measuring hemoglobin leakage.*8

Lysosomes are important in the catabolic processes occurring in the cell. Hence, a detailed study has been carried out to study the stability of isolated lysosomes. Lysosomes contain hydrolytic enzymes and are stable under normal conditions. In certain pathological conditions, the lysosomal membrane may rupture, releasing the hydrolytic enzymes into the cell and digestion of cell takes place as a whole. This is very dangerous. In normal life processes of multi cellular organisms, lysosomes rupture following the death of a cell and it may have some value as a built in mechanism for self-removal of dead cells.

Preliminary screening of selected biochemicals and natural products were carried out with the intention of identifying membrane stabilizers and destabilizers. In vitro studies were carried out by applying definite quantity of biochemical studied under controlled conditions to the red blood cells and the released hemoglobin was measured colorimetrically. In the case of lysosomes, the activity of acid phosphatase released from lysosomes was measured. When membrane of a lysosome is destabilized by chemical action, resident enzymes are released. ***9**

Destabilizers could be employed to get rid of undesirable cells like cancer cells. This technique can be employed as a preliminary screening test for potential anti- inflammatory compounds.

An attempt has also been made in this project towards developing lysosome membrane stability as an index of fish spoilage during storage. Different membranes within the cell and between cells have different compositions as reflected in the ratio of protein to lipid. The difference is not surprising given the very different functions of membranes.

The behaviour of erythrocyte membrane in different species (fish, bird and mammal) to selected biochemicals was studied and the results compared with those obtained on studying the stability of lysosome in *Oreochromis mossambicus.*

Chapter 2

Effect of selected biochemicals on the stability of erythrocyte membrane in three different species

Introduction

Erythrocytes have always been choice objects of inquiry in study of membranes because of their ready availability and relative simplicity. They lack organelles and are essentially composed of a single membrane, the plasma membrane, surrounding a solution of hemoglobin (this protein forms about 95% of the intracellular protein of RBC). An erythrocyte possesses remarkable mechanical stability and resilience due to partnership between plasma membrane and underlying meshwork called membrane skeleton, being exposed to powerful shearing forces, large changes in shape and much travel through narrow passages always during its lifetime. Since they are free from intracellular membranes and organelles, any effect of a metabolite on osmotic hemolysis can be interpreted as an effect on the plasma membrane. Thus, erythrocyte membrane is well suited for studies on action of metabolites, physiological and toxicant stress on membrane stability - since they are free from intracellular membranes and organelles. The study of erythrocyte membrane stabilization is simple, rapid, though non-specific and is useful as a preliminary screening test for the potential anti-inflammatory compounds. ***10** Brown & Mackey H.K found that non-steroidal anti-inflammatory drugs protected erythrocyte membranes from heat-induced and hypotonic hemolysis.

Changes in protein or lipoprotein structure might account for the development of erythrocyte membrane destabilization in *polyarthritis* and rheumatoid arthritis.***11** Prostaglandin E_1 (pg E) was found to act on erythrocytes in such a way that it causes phospholipid disruption.***12** At present many erythrocyte membrane stabilizers (eg. Acetyl salicylic acid, Phenylbutazone, Enfenamic acid.) and destabilizers (eg. Bile salts, Prostaglandin E_1, Penicillic acid, Acetaminophen, Vitamin A) have been identified.

Many clinically important non-steroidal anti-inflammatory drugs react with erythrocyte membrane causing membrane stabilization. The anti-inflammatory drugs tested stabilized the erythrocyte membrane against hypotonic hemolysis, whereas at higher concentration resulted in erythrocyte lysis. The stabilizing effect of the non- steroidal anti-inflammatory drugs on erythrocytes may be due to a stabilizing effect of the drugs on certain proteins in the cell membranes. ***12a** The association of these drugs with biological membrane of cells and cell organelles is likely to produce a change in selective permeability attributing to biochemical activities like inhibition of bio-synthesis of mucopolysaccharides and antibodies and also normal function of cell-organelles.

Hemolytic effect of penicillic acid and changes of erythrocyte membrane glycoproteins and lipid components during toxicosis are reported. The decreased membrane glycoproteins and lipid components indicate membrane damage during penicillic acid toxicosis. ***13** Penicillic acid affects erythrocyte membrane leading to membrane damage resulting in the liberation of membrane components from the membranes.

Toxic dose treatment of acetaminophen induces metabolic and membranal alterations making red cells prone to hemolysis, while Vitamin E which is an anti- oxidant shows its ameliorating role to these changes. ***14** Acetaminophen is a metabolite of acetophenetidine and it may cause hemolytic anemia due to metabolites that oxidize glutathione and components of red cell membrane.***15** Vitamin E behaves as a biological antioxidant and preserves membrane integrity.***16**

It also protects membrane from oxidative injuries. ***17** Prevention of hemolysis of red cell due to oxidative damage by Vitamin E has been reported.***18** The membrane stabilizing effects of Vitamin E has been studied by Wassall et al.***19** Disruption by Polyene antibiotics of the cholesterol rich membrane erythrocytes ***20** and lysosomes ***21** may be contrasted with failure of polyenes to interact with cholesterol-poor mitochondrial membrane.

Retinol destabilizes biological membranes causing hemolysis of erythrocytes while Vitamin E decreases membrane permeability and protects it from the disrupting effect of Retinol. Its membrane stabilizer action is through an interaction with the polyunsaturated fatty acid residues of phospholipid molecules.***22** Taurine, Zinc and Tocopherol have been found to possess membrane stabilizer action, proposed as the mechanism underlying the protective effect. ***23**

The composition of erythrocyte membranes in different species of animals may differ as reflected in ratio of protein to lipid. The ratio of lipid to protein etc., in animals of different species may be different leading to difference in stability of membranes.

In this part of the project, an attempt has been made to study the effect of selected metabolites on the stability of erythrocyte membranes in three different species of vertebrates - a fish, Tilapia (*Oreochromis mossambicus*), a bird, Chick (*Gallus domesticus*) and a mammal, Rabbit (*Oryctolagus cuniculus*) to establish the relative stability of erythrocyte membrane in these cases.

Different membranes within the cell and between cells have different compositions as reflected in their ratio of protein to lipid and hence the difference in their functions. These compositional differences

may lead to difference in effect of metabolites on erythrocyte membrane in different species of animals.

In vitro studies of the effects of different compounds on the stability of erythrocyte membranes of *Oreochromis, Oryctolagus* and *Gallus* during heat induced and hypotonic hemolysis were carried out.

The experiment was carried out in two steps – (1) Preliminary screening of physiological concentrations (10^{-3}M) of the selected metabolites and amino acids were studied to find out whether the metabolite has stabilizing or destabilizing effect during hypotonic hemolysis of RBC membrane. (2) In the next step, series of different concentrations (10^{-1}M - 10^{-4}M) of the stabilizers identified from the first experiment were used to study the effect on stability of erythrocyte membrane in the three different species.

Materials & Methods

Erythrocytes were collected from fresh blood of *Oreochromis* (of average size collected from Rice Research Station, Vyttila); *Gallus* (broiler chicken reared for meat) and *Oryctolagus* (bred for the studies). The stock suspension of erythrocytes was prepared from fresh blood collected in Alseiver's solution by centrifugation at 4°C for 20 minutes. The erythrocytes were then washed thrice with isotonic salt solution (154 mM in 10 mM sodium phosphate buffer pH 7.4). ***24** 10^{-3}M solution of sodium glycotaurocholate, L-glutamic acid, alpha ketoglutaric acid, sodium succinate, sodium pyruvate, glycine, taurine, sodium acetate, cysteine, ornithine and DOPA were prepared in sodium phosphate buffer pH 7.4.

Blood was collected from *Oreochromis* by cardinal vein puncture using plastic syringe as per the rapid method for repetitive bleeding in fish.***25**

Fresh blood was collected from the vein in the neck of *Gallus.*

In *Oryctolagus*, bleeding was carried out by cutting the marginal vein of ear or puncture of the central artery of the ear. Blood was drawn from ear vein of *Oryctolagus* using glass syringe containing Alseiver's solution. (Isotonic as well as anticoagulant). ***26**

Erythrocyte lysis in hypotonic solution was determined by release of hemoglobin as per procedure of Seiman & Weinstein with slight modifications to suit the working conditions. ***27**

The experiment was carried out as follows: -

To 0.2 ml of stock erythrocyte suspension, added 4 ml of hypotonic solution and 0.2 ml of the metabolite whose effect is to be studied (of known concentration). After incubation at room temperature for 30 minutes, the tubes were centrifuged at 1000g for 15 minutes. The hemoglobin content of the clear supernatant was measured in an uv-visible Spectrophotometer at 540 nm.

The effect of metabolite was studied by the above method in two steps - preliminary screening to identify erythrocyte membrane stabilizers. Secondly, different (10^{-1}M - 10^{-4}M) concentrations of the stabilizers identified were again screened to find out their effect on the erythrocyte membrane.

The hemoglobin released in each step measured colorimetrically was expressed as a percentage of total hemoglobin released (hemoglobin release by known concentration of Triton x-100 detergent at the initial stage of incubation and at the end of the incubation).

The experimental results obtained from the three species were analyzed statistically using 3 way ANOVA of the raw data to find out if the results were statistically significant. The verification and analysis was carried out to find out the level of significance of effect of difference in species and the action of metabolite on erythrocyte membrane.

Results

a. Preliminary screening of selected biochemicals: -

Preliminary screening carried out helped to reveal the membrane stabilizers and destabilizers of erythrocyte membrane in *Oreochromis, Gallus* and *Oryctolagus*. The results of the experiment were analyzed statistically too.

The membrane stabilizers observed in *Oreochromis* were glycine, taurine, sodium acetate, cysteine and ornithine. On statistical analysis the effects of glycine and taurine on the erythrocyte membrane is not significant.

The membrane labilizers observed in the fish were sodium glycotaurocholate, L-glutamic acid, alpha ketoglutaric acid, sodium succinate, sodium pyruvate and DOPA. The labilizing effect of DOPA on erythrocyte membrane in fish was found to be statistically significant, while the results of sodium pyruvate, alpha ketoglutaric acid, L-glutamic acid and sodium- glycotaurocholate are not significant statistically.

In *Gallus*, the observed erythrocyte membrane stabilizers are glycine, taurine, sodium acetate, cysteine and ornithine. Erythrocyte membrane labilizers observed in *Gallus* - sodium glycotaurocholate, L-glutamic acid, alpha ketoglutaric acid, sodium succinate, sodium pyruvate and DOPA.

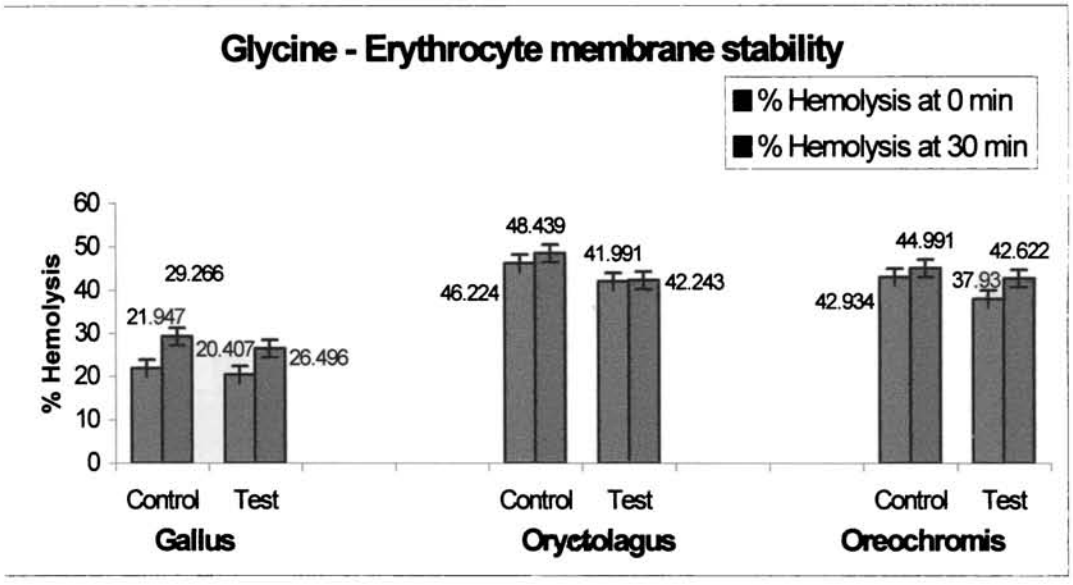
Statistical analysis carried out has revealed the following significant membrane stabilizers and destabilizers in *Gallus*. Statistically significant erythrocyte membrane stabilizers - sodium acetate, cysteine and ornithine. Statistically significant membrane labilizers - DOPA.

The experimentally observed membrane stabilizers identified in *Oryctolagus* - glycine, taurine, sodium acetate, alpha ketoglutaric acid, sodium succinate, sodium pyruvate, cysteine and ornithine. Statistically significant observations of erythrocyte membrane stabilizers in *Oryctolagus* - sodium acetate, cysteine and ornithine.

Statistically significant membrane destabilizers in *Oryctolagus* - DOPA.

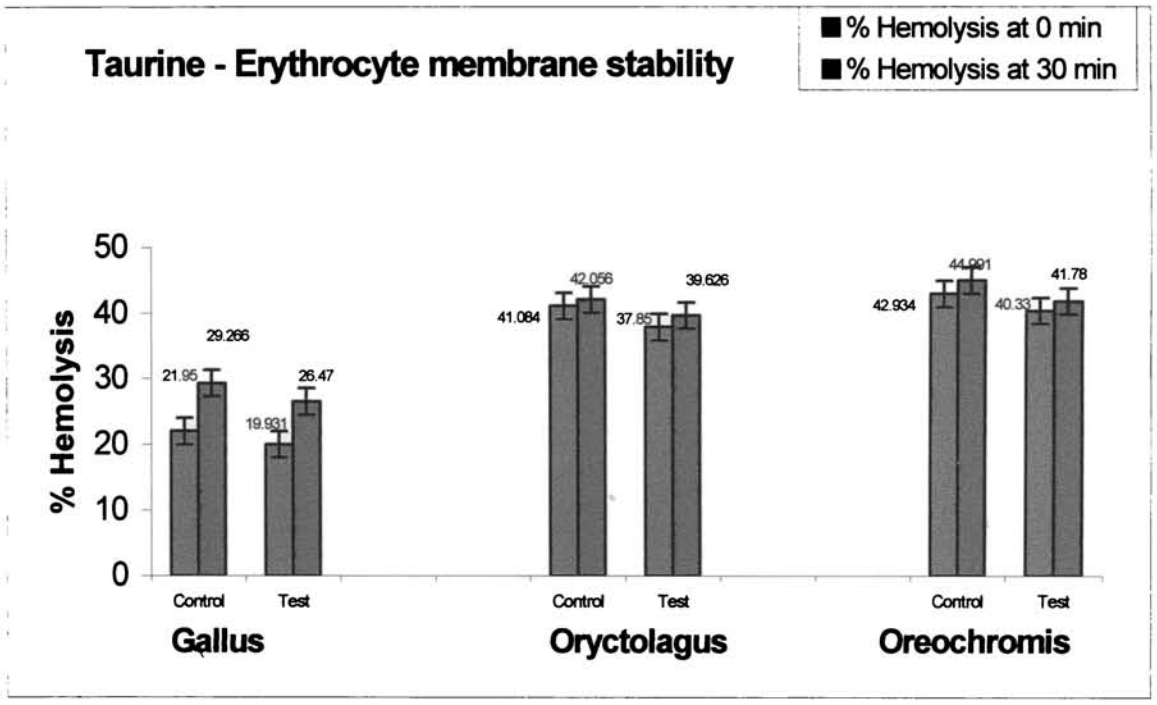
Glycine

Species	% of Hb released from RBC at 0 minute	% of Hb released from RBC at 30minutes
	(Room Temperature)	
Gallus		
Control	21.947 ± 0.291	29.266 ± 1.566
Test	20.407 ±0.589	26.496 ± 0.692
Oryctolagus		
Control	46.224 ± 0.279	48.439 ± 0.275
Test	41.991 ± 0.435	42.243 ±0.458
Oreochromis		
Control	42.934 ± 0.259	44.991 ±0.256
Test	37.934 ± 0.609	42.622 ± 0.213



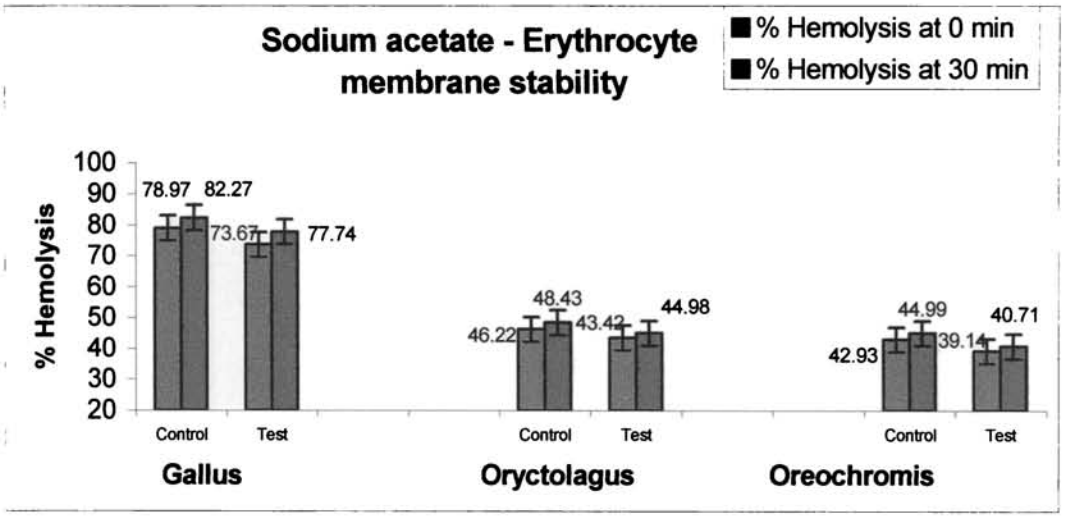
Taurine

Species	% of Hb released from RBC at 0 minute	% of Hb released from RBC at 30minutes
	(Room Temperature)	
Gallus		
Control	21.947 ± 0.290	29.266 ± 1.567
Test	19.931 ± 0.477	26.47 ± 0.725
Oryctolagus		
Control	41.084 ± 0.217	42.056 ± 0.354
Test	37.85 ± 0.307	39.626 ±0.289
Oreochromis		
Control	42.934 ± 0.258	*44.991 ± 0.256*
Test	40.33 ± 0.418	41.78 ± 0.209



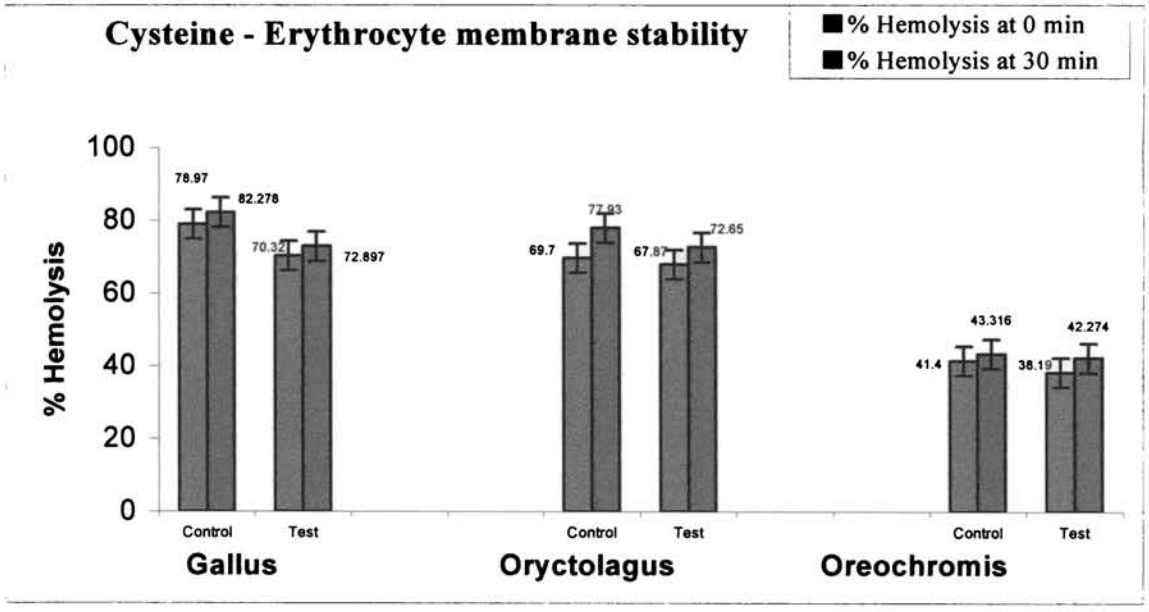
Sodium Acetate

Species	% of Hb released from RBC at 0 minute	% of Hb released from RBC at 30minutes
	(Room Temperature)	
Gallus		
Control	78.97 ± 1.047	82.27 ± 0.533
Test	73.67 ± 2.007	77.74 ± 1.049
Oryctolagus		
Control	46.22 ± 0.279	48.43 ± 0.275
Test	43.42 ± 0.451	44.98 ± 0.225
Oreochromis		
Control	42.93 ± 0.258	44.99 ± 0.256
Test	39.14 ± 0.512	40.71 ± 0.213



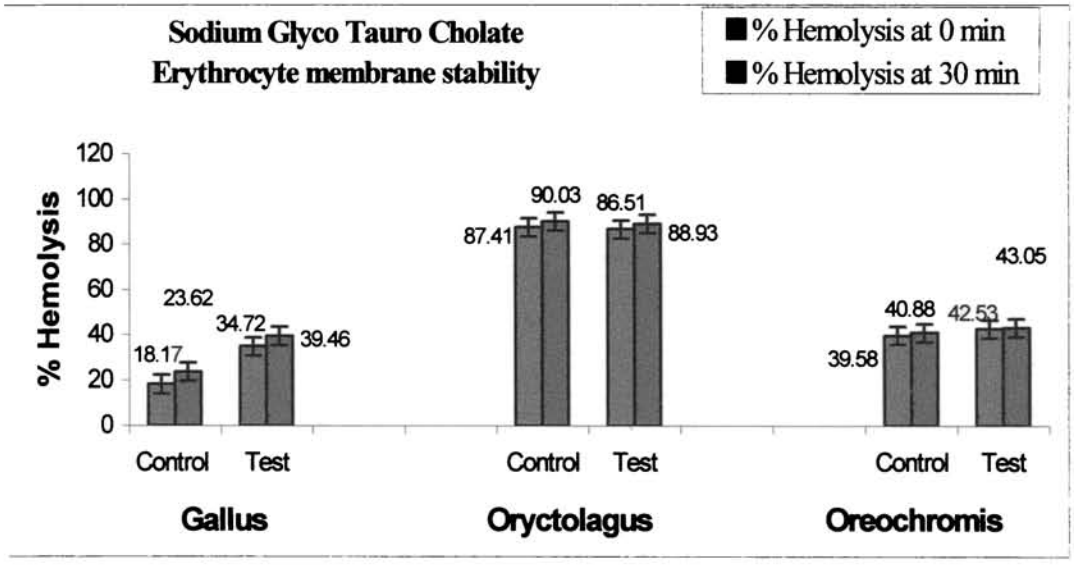
Cysteine

Species	% of Hb released from RBC at 0 minute	% of Hb released from RBC at 30minutes
	(Room Temperature)	
Gallus		
Control	78.97± 1.048	82.278± 0.534
Test	70.32± 1.955	72.897± 0.902
Oryctolagus		
Control	69.7± 1.48	77.93± 2.97
Test	67.87± 2.523	72.65± 0.615
Oreochromis		
Control	41.4± 0.546	43.316± 0.212
Test	38.19± 0.269	42.274± 0.392



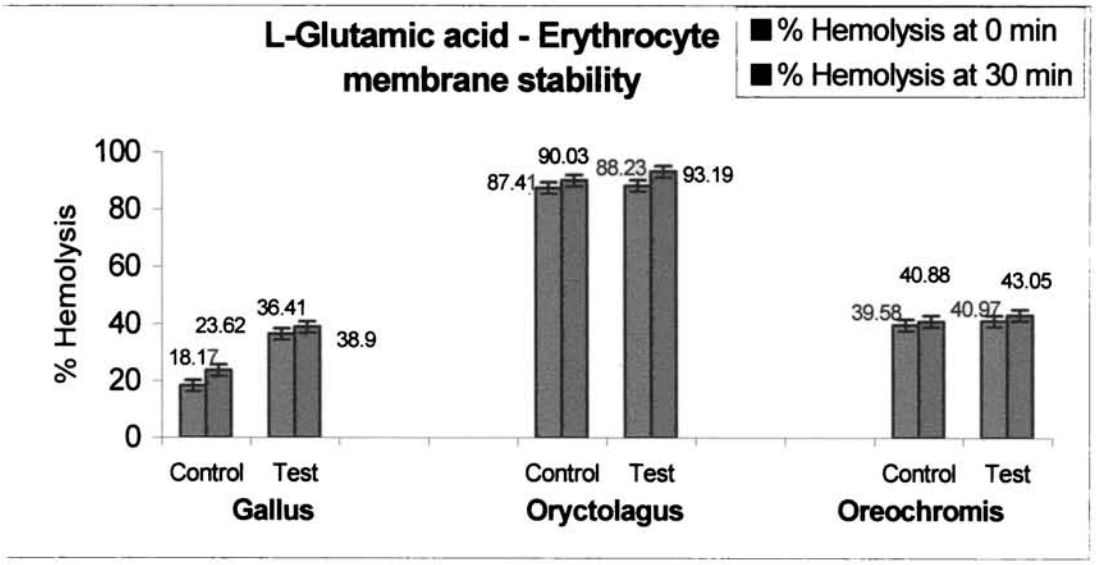
Sodium Glyco Tauro Cholate

Species	% of Hb released from RBC at 0 minute	% of Hb released from RBC at 30minutes
	(Room Temperature)	
Gallus		
Control	18.17± 0.458	23.62± 0.279
Test	34.72± 0.375	39.46± 0.357
Oryctolagus		
Control	87.41± 0.768	90.03± 0.431
Test	86.51± 0.963	88.93± 0.58
Oreochromis		
Control	39.58± 0.658	40.88± 0.435
Test	42.53± 0.630	43.05± 0.630



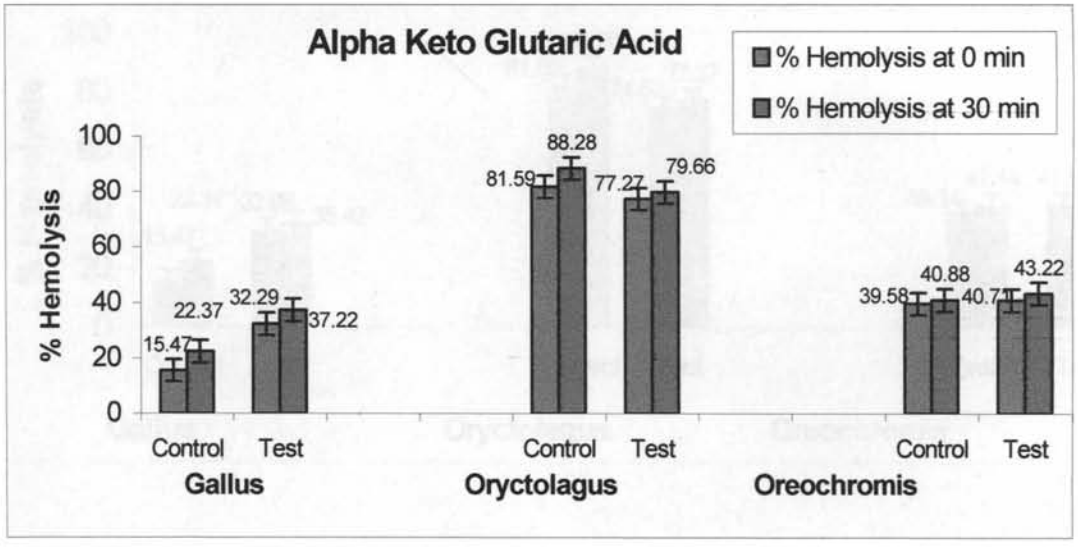
L-Glutamic Acid

Species	% of Hb released from RBC at 0 minute	% of Hb released from RBC at 30minutes
	(Room Temperature)	
Gallus		
Control	18.17± 0.458	23.62± 0.279
Test	34.72± 0.375	39.46± 0.357
Oryctolagus		
Control	87.41± 0.768	90.03± 0.431
Test	86.51± 0.963	88.93± 0.58
Oreochromis		
Control	39.58± 0.658	40.88± 0.435
Test	42.53± 0.630	43.05± 0.630



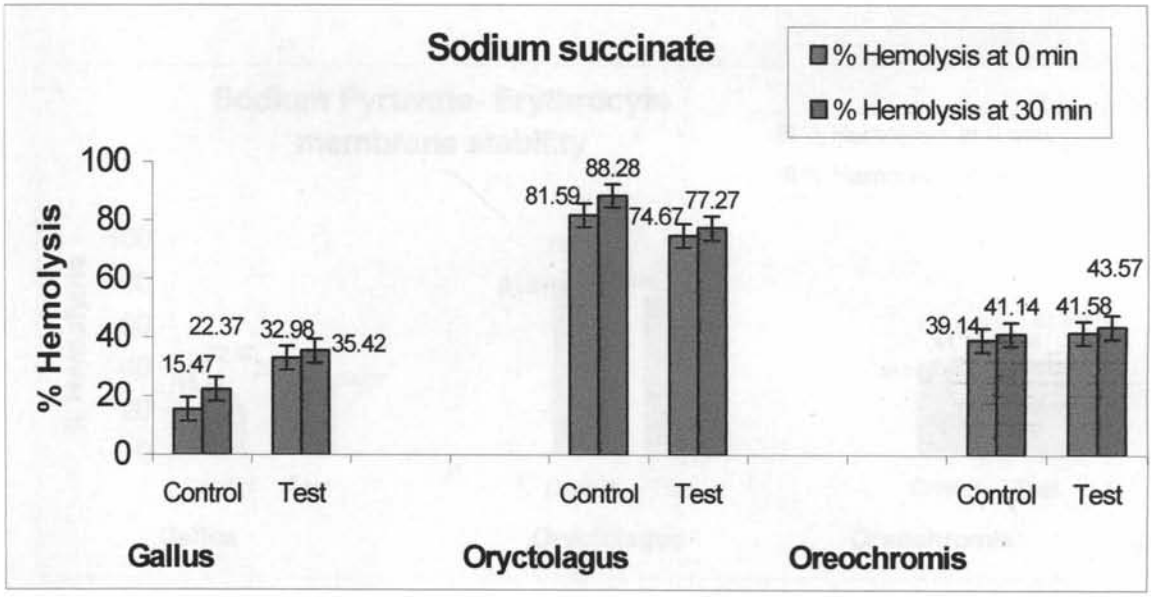
Alpha Keto Glutaric Acid

Species	% of Hb released from RBC at 0 minute	% of Hb released from RBC at 30minutes
	(Room Temperature)	
Gallus		
Control	15.47 ± 0.076	22.37 ± 0.396
Test	32.29 ± 0.449	37.22 ± 0.501
Oryctolagus		
Control	81.59 ± 1.159	88.28 ± 0.888
Test	77.27 ± 0.646	79.66 ± 0.916
Oreochromis		
Control	39.58 ± 0.658	40.88 ± 0.435
Test	40.71 ± 0.212	43.22 ± 0.329



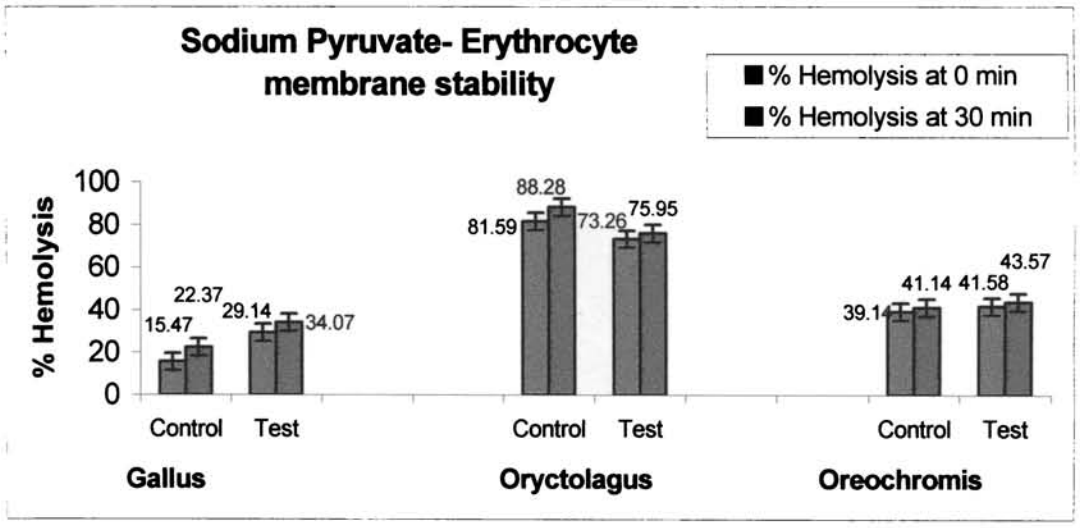
Sodium Succinate

Species	% of Hb released from RBC at 0 minute	% of Hb released from RBC at 30minutes
	(Room Temperature)	
Gallus		
Control	15.47 ± 0.076	22.37 ± 0.369
Test	32.98 ± 0.062	35.42 ± 0.296
Oryctolagus		
Control	81.59 ± 1.159	88.28 ± 0.888
Test	74.67 ± 0.388	77.27 ± 0.646
Oreochromis		
Control	39.14 ± 0.212	41.14 ± 0.465
Test	41.58 ± 0.897	43.57 ± 0.784



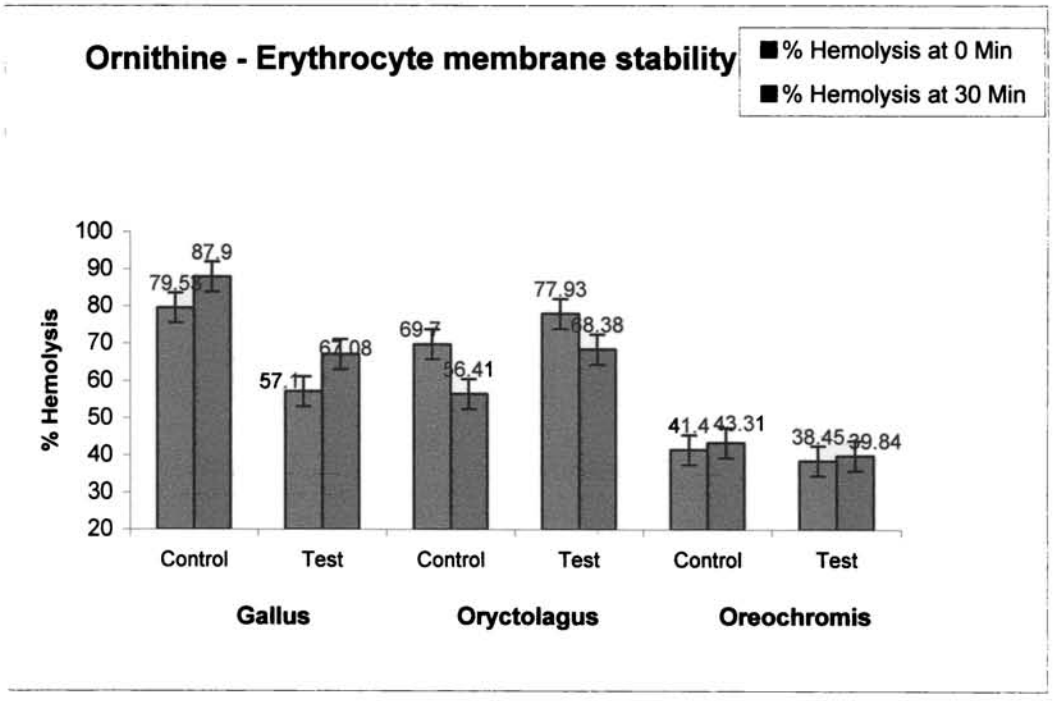
Sodium Pyruvate

Species	% of Hb released from RBC at 0 minute	% of Hb released from RBC at 30minutes
	(Room Temperature)	
Gallus		
Control	15.47 ± 0.076	22.37 ± 0.369
Test	29.14 ± 0.255	34.07 ± 0.124
Oryctolagus		
Control	81.59 ± 1.159	88.28 ± 0.888
Test	73.26 ± 0.288	75.95 ± 0.579
Oreochromis		
Control	39.14 ± 0.212	41.14 ± 0.465
Test	41.58 ± 0.897	43.57 ± 0.784



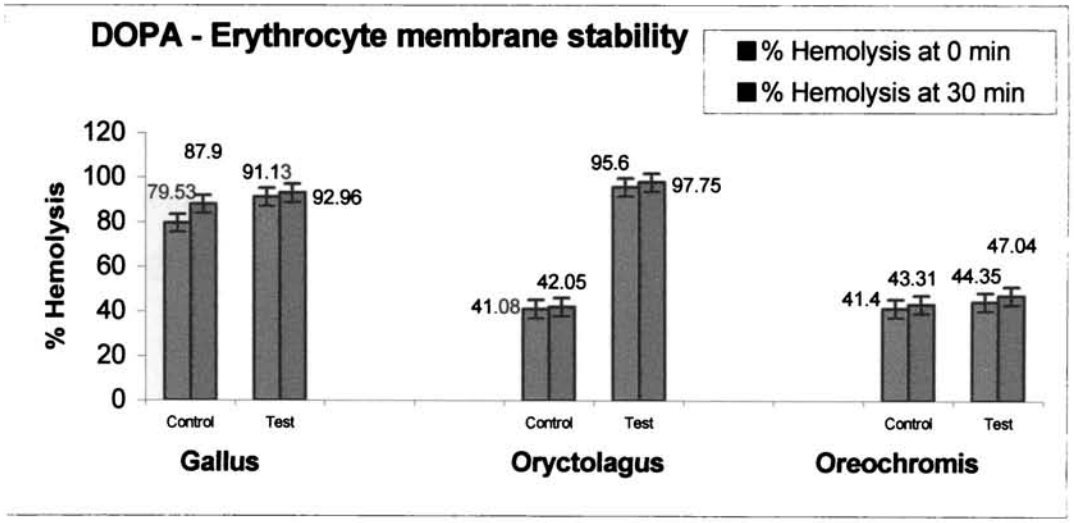
Ornithine

Species	% of Hb released from RBC at 0 minute	% of Hb released from RBC at 30minutes
	(Room Temperature)	
Gallus		
Control	79.53± 1.300	87.9± 2.411
Test	57.1± 67.089	67.08± 1.666
Oryctolagus		
Control	69.7± 1.479	56.41± 1.51
Test	77.93± 2.97	68.38± 3.395
Oreochromis		
Control	41.4± 0.546	43.31± 0.212
Test	38.45± 0.608	39.84± 0.285



DOPA

Species	% of Hb released from RBC at 0 minute	% of Hb released from RBC at 30minutes
	(Room Temperature)	
Gallus		
Control	79.53± 1.300	87.9± 2.411
Test	91.13± 0.800	92.96± 0.217
Oryctolagus		
Control	41.08± 0.217	42.05± 0.354
Test	95.6± 0.307	97.75± 0.289
Oreochromis		
Control	41.4± 0.546	43.31± 0.212
Test	44.35± 0.766	47.04± 1.024



ANOVA TABLE (Three way ANOVA)

Glycine

Source	Sum of Square	Degrees of Freedom	Mean Square	F
Total	0.01977	11		
Between Species	0.01745	2	0.008723	54.5689***
Between Control & Test	0.00029	1	0.00029	1.81478^{NS}
Between time Of Incubation	0.00092	1	0.000919	5.74775*
Error	0.00112	7	0.00016	

Species	Means of Species	Time	Means of time of incubation	Least Significant Difference for Species	Least Significant Difference for Time of Incubation
Gallus	0.161	0 Min	0.09833	0.0211979	0.01731
Oryctolagus	0.0795	30 Min	0.11583		
Oreochromis	0.08075				

* $p < 0.05$
*** $p < 0.001$
NS Not Significant

Taurine

Source	Sum of Square	Degrees of Freedom	Mean Square	F
Total	0.02127	11		
Between Species	0.01888	2	0.009422	51.4202***
Between Control & Test	0.00022	1	0.000217	1.18036^{NS}
Between time Of Incubation	0.00088	1	0.000884	4.81446^{NS}
Error	0.00129	7	0.000184	

Species	Means of Species	Time	Means of time of incubation	Least Significant Difference for Species
Gallus	0.16025	0 Min	0.09583	0.0227
Oryctolagus	0.07175	30 Min	0.113	
Oreochromis	0.08125			

*** $p<0.001$
NS Not Significant.

Sodium Acetate

Source	Sum of Square	Degrees of Freedom	Mean Square	F
Total	0.02944	11		
Between Species	0.02912	2	0.014561	2824.83***
Between Control & Test	0.0002	1	0.0002	38.8152**
Between time Of Incubation	0.00008	1	0.00008	15.5358**
Error	0.000036	7	0.000005	

Species	Means of Species	Time	Means of time of incubation	Least Significant Difference for Species	Least Significant Difference for Control & Test	Least Significan Difference for Time of Incubatio
Gallus	0.18525	0 Min	0.113	0.0037	0.0031	0.0031
Oryctolagus	0.8125	30 Min	0.11817			
Oreochromis	0.08025					

** $p < 0.01$
*** $p < 0.001$

Cysteine

Source	Sum of Square	Degrees of Freedom	Mean Square	F
Total	0.03906	11		
Between Species	0.03849	2	0.019245	598.366***
Between Control & Test	0.00025	1	0.000248	7.69589*
Between time Of Incubation	0.000096	1	0.000096	2.97759^{NS}
Error	0.00023	7	0.000032	

Species	Means of Species		Means of Control and Test	Least Significant Difference for Species	Least Significant Difference for Control & Test
Gallus	0.1805	Control	0.3677	0.00948	0.00774
Oryctolagus	0.4773	Test	0.3357		
Oreochromis	0.0793				

* $p < 0.05$
*** $p < 0.001$
NS Not Significant

Sodium Glyco Tauro Cholate

Source	Sum of Square	Degrees of Freedom	Mean Square	F
Total	0.04662	11		
Between Species	0.03411	2	0.017056	14.9856**
Between Control & Test	0.004707	1	0.00407	3.57611^{NS}
Between time Of Incubation	0.00047	1	0.000469	0.41186^{NS}
Error	0.00797	7	0.001138	

Species	Means of Species	Least Significant Difference for Species
Gallus	0.19025	0.0565
Oryctolagus	0.0745	
Oreochromis	0.08	

** $p < 0.01$

NS Not Significant

L-Glutamic Acid

Source	Sum of Square	Degrees of Freedom	Mean Square	F
Total	0.04873	11		
Between Species	0.03572	2	0.017859	14.7576**
Between Control & Test	0.00407	1	0.00407	3.36334^{NS}
Between time Of Incubation	0.00047	1	0.000469	0.38735^{NS}
Error	0.00847	7	0.00121	

Species	Means of Species	Least Significant Difference for Species
Gallus	0.192	0.0582942
Oryctolagus	0.0735	
Oreochromis	0.07925	

** $p < 0.01$

NS Not Significant

Alpha Keto Glutaric Acid

Source	Sum of Square	Degrees of Freedom	Mean Square	F
Total	0.04025	11		
Between Species	0.02779	2	0.013894	11.7853**
Between Control & Test	0.00347	1	0.003468	2.94159^{NS}
Between time Of Incubation	0.00074	1	0.000736	0.62457^{NS}
Error	0.00825	7	0.00179	

Species	Means of Species	Least Significant Difference for Species
Gallus	0.176	0.575427
Oryctolagus	0.0695	
Oreochromis	0.079	

** $p < 0.01$

NS Not Significant

Sodium Succinate

Source	Sum of Square	Degrees of Freedom	Mean Square	F
Total	0.0383	11		
Between Species	0.027	2	0.013502	12.3722**
Between Control & Test	0.00315	1	0.003146	2.88287^{NS}
Between time Of Incubation	0.00052	1	0.000516	0.47296^{NS}
Error	0.00764	7	0.001091	

Species	Means of Species	Least Significant Difference for Species
Gallus	0.174	0.553535
Oryctolagus	0.06825	
Oreochromis	0.07943	

** $p < 0.01$

NS Not Significant

Sodium Pyruvate

Source	Sum of Square	Degrees of Freedom	Mean Square	F
Total	0.03051	11		
Between Species	0.02189	2	0.010944	13.4043**
Between Control & Test	0.00218	1	0.002182	2.67207^{NS}
Between time Of Incubation	0.00072	1	0.000724	0.88659^{NS}
Error	0.00572	7	0.000816	

Species	Means of Species	Least Significant Difference for Species
Gallus	0.1655	0.4787
Oryctolagus	0.0675	
Oreochromis	0.0848	

** $p < 0.01$
NS Not Significant

Ornithine

Source	Sum of Square	Degrees of Freedom	Mean Square	F
Total	0.03828	11		
Between Species	0.03501	2	0.017506	79.4481***
Between Control & Test	0.0014	1	0.001395	6.33251*
Between time Of Incubation	0.00033	1	0.000329	1.49151^{NS}
Error	0.00154	7	0.00022	

Species	Means of Species		Means of Control and Test	Least Significant Difference for Species	Least Significant Difference for Control & Test
Gallus	0.17275	Control	0.3719	0.0248567	0.0203
Oryctolagus	0.0453	Test	0.2931		
Oreochromis	0.07825				

* $p < 0.05$
*** $p < 0.001$
^{NS} Not Significant

DOPA

Source	Sum of Square	Degrees of Freedom	Mean Square	F
Total	0.04237	11		
Between Species	0.03203	2	0.016016	22.1262**
Between Control & Test	0.00516	1	0.005158	7.12634*
Between time Of Incubation	0.00011	1	0.000113	0.15591^{NS}
Error	0.00507	7	0.000724	

Species	Means of Species		Means of Control And Test	Least Significant Difference for Species	Least Significant Difference for Control&Test
Gallus	0.20825	Control	0.4025	0.0450923	0.03682
Oryctolagus	0.12325	Test	0.1003		
Oreochromis	0.08455				

* $p < 0.05$
** $p < 0.01$
NS Not Significant

b.Results of screening of different concentrations of the erythrocyte membrane stabilizers observed in the three species above:-

i.Different concentrations of membrane stabilizers and erythrocyte membrane stability in *Oreochromis*

All concentrations (10^{-1}M - 10^{-5}M) of sodium acetate, taurine and cysteine were observed to stabilize erythrocyte membrane in *Oreochromis.*

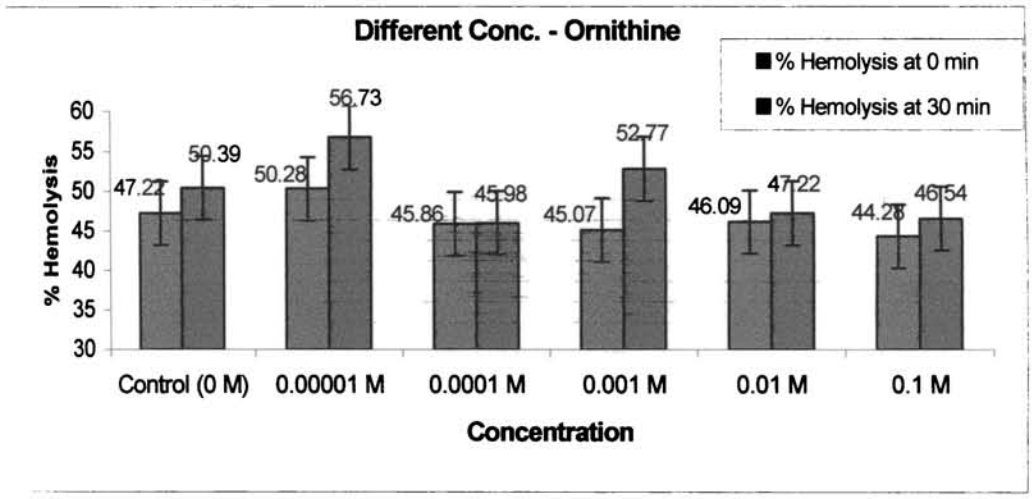
The lower concentrations of ornithine and glycine were observed to destabilize erythrocyte membrane in *Oreochromis* while higher concentrations were found to be stabilizing.

The results of statistical analysis using three way ANOVA with repeated number of observations were carried out on the raw data obtained from experimental values.

Statistically significant results of effect on erythrocyte membrane were obtained in the case of glycine and sodium acetate. The results in the case of cysteine, ornithine and taurine were not statistically significant.

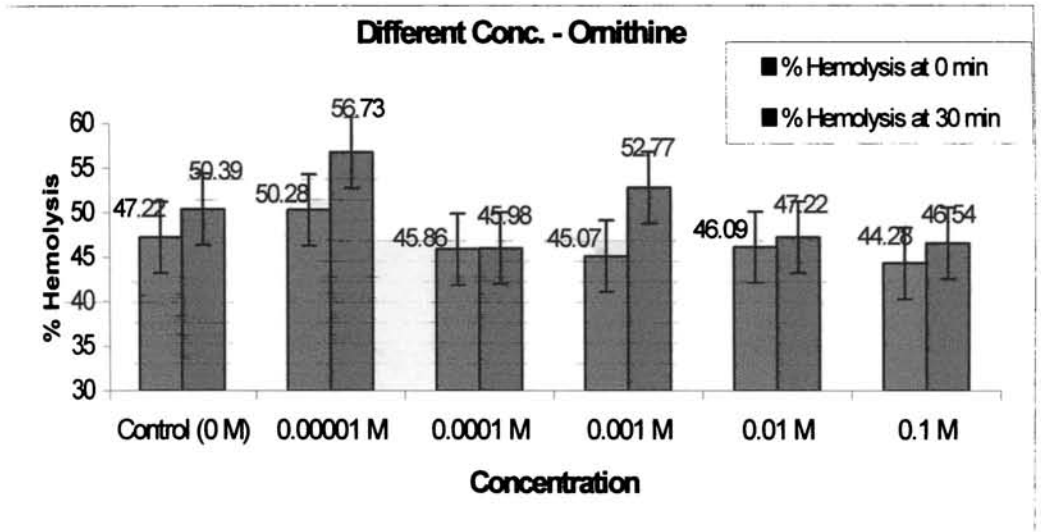
Sodium Acetate

Concentration of Biochemical	% of Hb released from RBC at 0 Min	% of Hb released from RBC at 30 Min
	(At Room Temperature)	
Control (0 M)	44.309 ± 0.315	50.203 ± 0.498
0.00001 M	42.378 ± 0.51	43.598 ± 0.334
0.0001 M	42.276 ± 0.315	42.886 ± 0.498
0.001 M	42.378 ± 0.51	42.785 ± 0.249
0.01 M	42.581 ± 0.713	42.988 ± 0.51
0.1 M	41.159 ± 0.334	43.496 ± 0.629



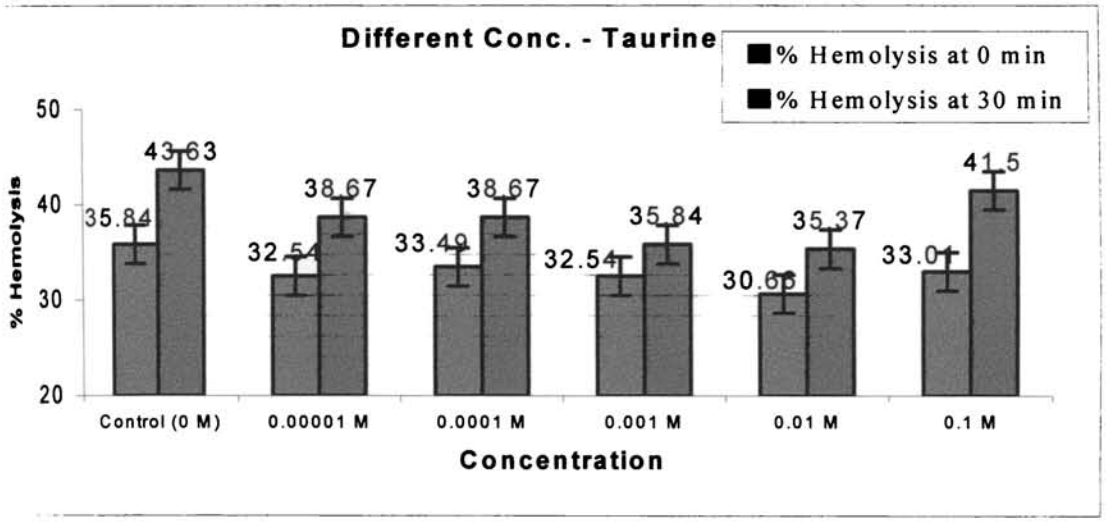
Ornithine

Concentration of Biochemical	% of Hb released from RBC at 0 Min	% of Hb released from RBC at 30 Min
	(At Room Temperature)	
Control (0 M)	47.22 ± 0.372	50.39 ± 0.668
0.00001 M	50.28 ± 2.105	56.73 ± 1.117
0.0001 M	45.86 ± 0.372	45.98 ± 0.555
0.001 M	45.07 ± 0.555	52.77 ± 0.351
0.01 M	46.09 ± 0.277	47.22 ± 0.372
0.1 M	44.28 ± 2.832	46.54 ± 0.372



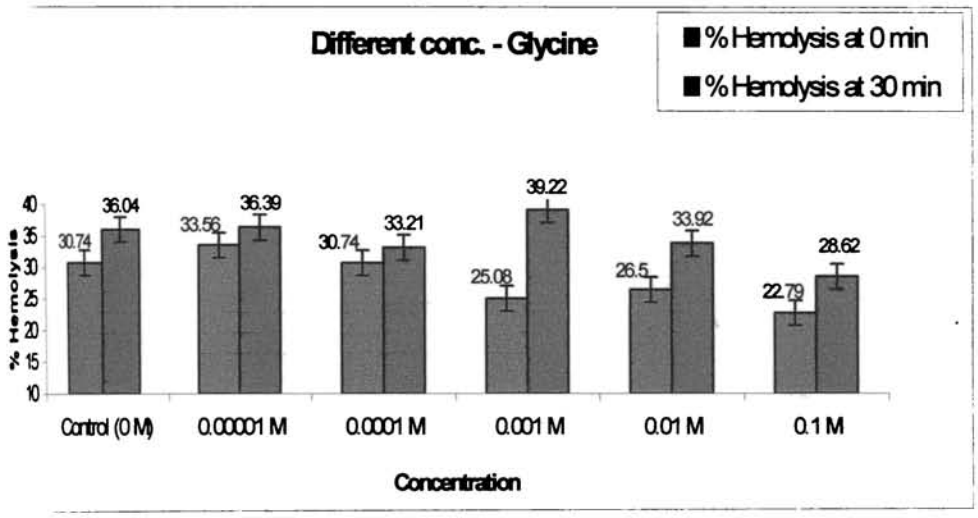
Taurine

Concentration of Biochemical	% of Hb released from RBC at 0 Min	% of Hb released from RBC at 30 Min
	(At Room Temperature)	
Control (0 M)	35.84 ± 1.155	43.63 ± 1.391
0.00001 M	32.54 ± 1.55	38.67 ± 1.462
0.0001 M	33.49 ± 2.131	38.67 ± 1.462
0.001 M	32.54 ± 1.266	35.84 ± 1.462
0.01 M	30.66 ± 1.155	35.37 ± 1.266
0.1 M	33.01 ± 1.462	41.5 ± 1.462



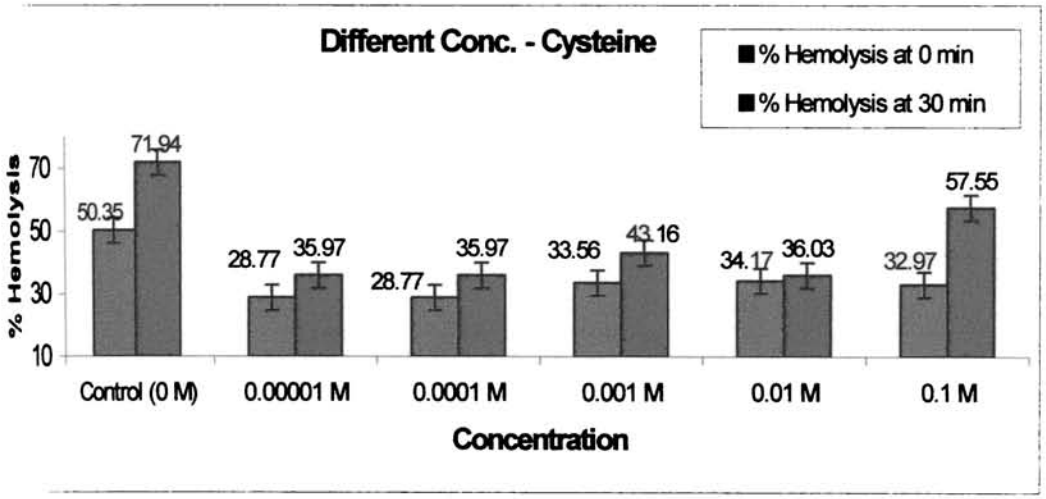
Glycine

Concentration of Biochemical	% of Hb released from RBC at 0 Min	% of Hb released from RBC at 30 Min
	(At Room Temperature)	
Control (0 M)	30.74 ± 0.948	36.04 ± 1.896
0.00001 M	33.56 ± 0.866	36.39 ± 0.866
0.0001 M	30.74 ± 1.161	33.21 ± 1.095
0.001 M	25.08 ± 1.596	39.22 ± 2.844
0.01 M	26.5 ± 0.948	33.92 ± 1.341
0.1 M	22.79 ± 0.887	28.62 ± 1.161



Cysteine

Concentration of Biochemical	% of Hb released from RBC at 0 Min	% of Hb released from RBC at 30 Min
	(At Room Temperature)	
Control (0 M)	50.35 ± 0.42	71.94 ± 1.26
0.00001 M	28.77 ± 0.42	35.97 ± 1.2
0.0001 M	28.77 ± 1.672	35.97 ± 0.56
0.001 M	33.56 ± 0.65	43.16 ± 0.42
0.01 M	34.17 ± 1.012	36.03 ± 0.42
0.1 M	32.97 ± 0.84	57.55 ± 0.86

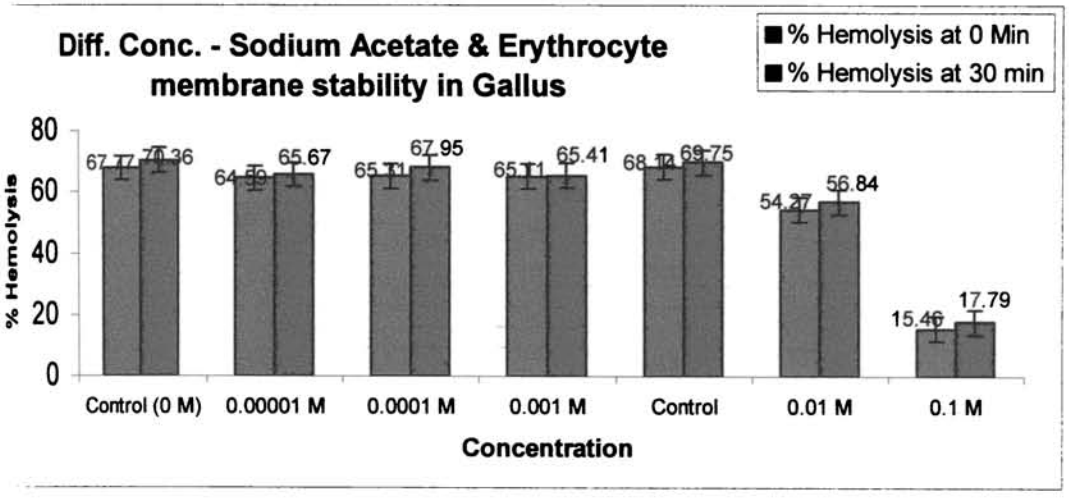


2. DIFFERENT CONCENTRATIONS OF MEMBRANE STABILIZERS AND ERYTHROCYTE MEMBRANE STABILITY IN *GALLUS*

All concentrations of sodium acetate, taurine, glycine, cysteine and ornithine were observed to have stabilizing effect on erythrocyte membrane in *Gallus*. Statistical analysis using three way ANOVA with repeated number of observations carried out using the raw data in the above case revealed that only glycine and sodium acetate had significant effects on the erythrocyte membrane. In the case of cysteine, ornithine and taurine, the results were not statistically significant

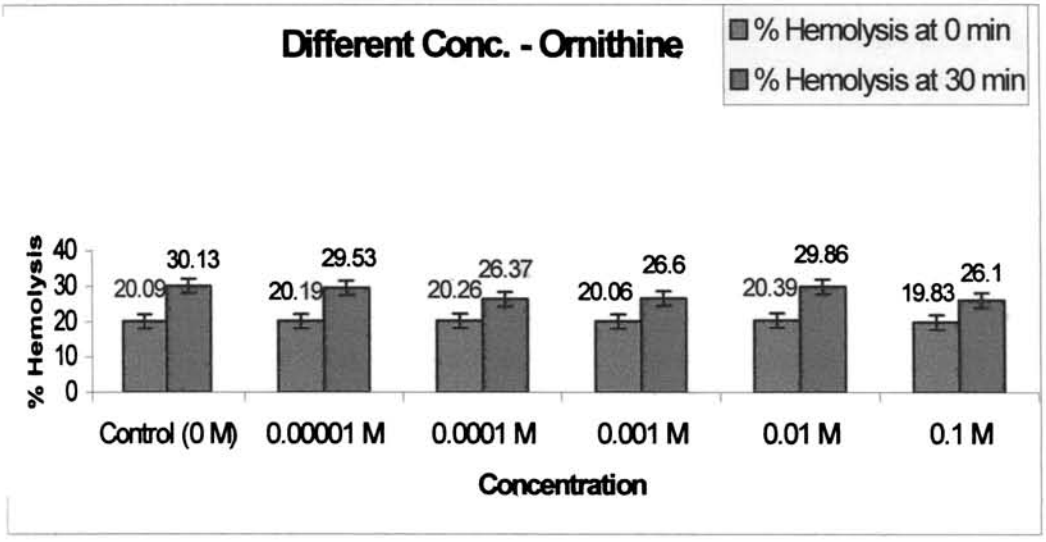
Sodium Acetate

Concentration of Biochemical	% of Hb released from RBC at 0 Min	% of Hb released from RBC at 30 Min
	(At Room Temperature)	
Control (0 M)	67.77 ± 0.592	70.36 ± 0.257
0.0001 M	65.31 ± 0.105	67.95 ± 0.517
0.001 M	65.11 ± 0.214	65.41 ± 0.186
Control	68.14 ± 0.072	69.75 ± 0.38
0.01 M	54.27 ± 0.393	56.84 ± 1.009
0.1 M	15.46 ± 0.276	17.79 ± 0.46



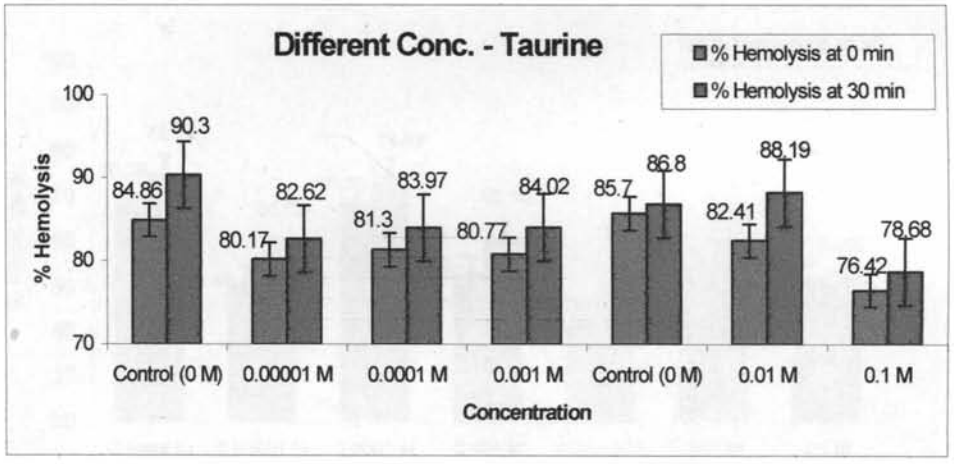
Ornithine

Concentration of Biochemical	% of Hb released from RBC at 0 Min	% of Hb released from RBC at 30 Min
	(At Room Temperature)	
Control (0 M)	20.09± 0.109	30.13± 0.195
0.00001 M	20.19± 0.217	29.53± 0.745
0.0001 M	20.26± 0.205	26.37± 0.523
0.001 M	20.06± 0.205	26.6± 0.647
0.01 M	20.39± 0.125	29.86± 0.149
0.1 M	19.83± 0.149	26.1± 0.766



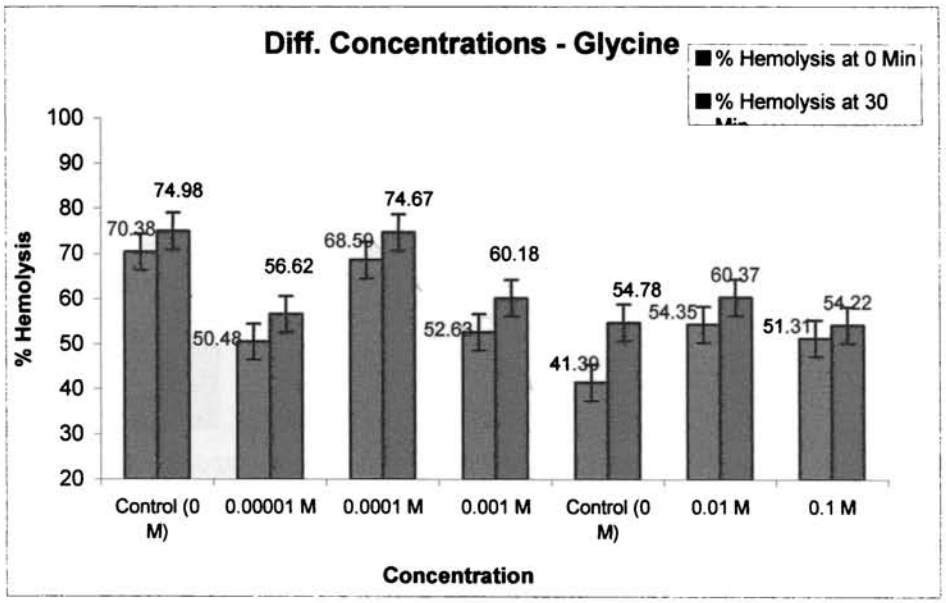
Taurine

Concentration of Biochemical	% of Hb released from RBC at 0 Min (At Room	% of Hb released from RBC at 30 Min Temperature)
Control (0 M)	84.86 ± 0.755	90.30 ± 1.410
0.00001 M	80.17 ± 0.047	82.62 ± 0.168
0.0001 M	81.30 ± 0.344	83.97 ± 0.520
0.001 M	80.77 ± 0.000	84.02 ± 0.321
Control (0 M)	85.70 ± 0.112	86.80 ± 0.133
0.01 M	82.41 ± 0.728	88.19 ± 0.451
0.1 M	76.42 ± 0.133	78.68 ± 0.687



Glycine

Concentration of Biochemical	% of Hb released from RBC at 0 Min	% of Hb released from RBC at 30 Min
	(At Room Temperature)	
Control (0 M)	70.38± 0.233	74.98± 1.062
0.00001 M	50.48± 0.233	56.62± 0.761
0.0001 M	68.59± 0.277	74.67± 0.301
0.001 M	52.63± 0.489	60.18± 0.301
Control (0 M)	41.39±0.1504	54.78± 1.269
0.01 M	54.35± 0.451	60.37± 1.000
0.1 M	51.31±1.162	54.22± 0.362



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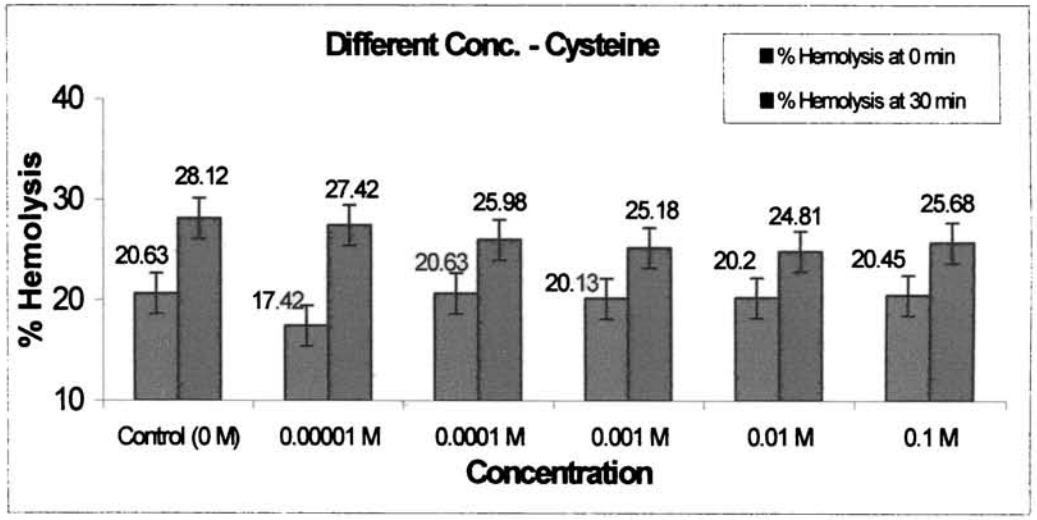
Cysteine

Concentration of Biochemical	% of Hb released from RBC at 0 Min	% of Hb released from RBC at 30 Min
(At Room Temperature)		
Control (0 M)	20.63± 0.082	28.12± 0.322
0.00001 M	17.42± 7.563	27.42± 0.668
0.0001 M	20.63± 0.082	25.98± 0.304
0.001 M	20.13± 0.164	25.18± 0.989
0.01 M	20.2± 0.104	24.81± 0.63
0.1 M	20.45± 0.159	25.68± 0.381



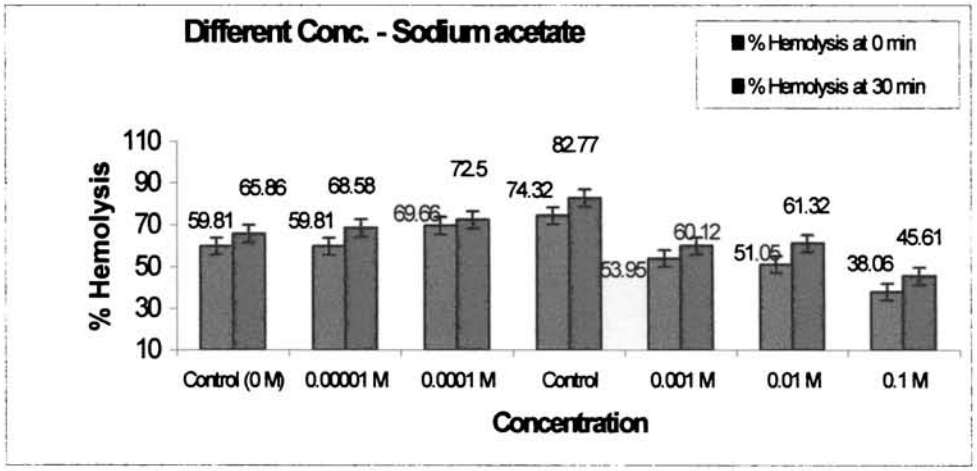
iii) DIFFERENT CONCENTRATIONS OF MEMBRANE STABILISERS AND ERYTHROCYTE MEMBRANE STABILITY IN *ORYCTOLAGUS*

All different concentrations of sodium acetate, glycine and cysteine were observed to stabilize erythrocyte membrane.

Taurine and ornithine were observed to be membrane stabilizing only at certain concentrations. In the case of taurine, only 10^{-1}M solution was found to be stabilizing. The higher concentrations of ornithine ((10^{-1}M - 10^{-3}M) solutions were found to stabilize erythrocyte membrane but lower concentrations (10^{-4}M - 10^{-5}M) were found to labilize red blood cell membranes. Statistical significance has been noted only in the case of glycine and sodium acetate.

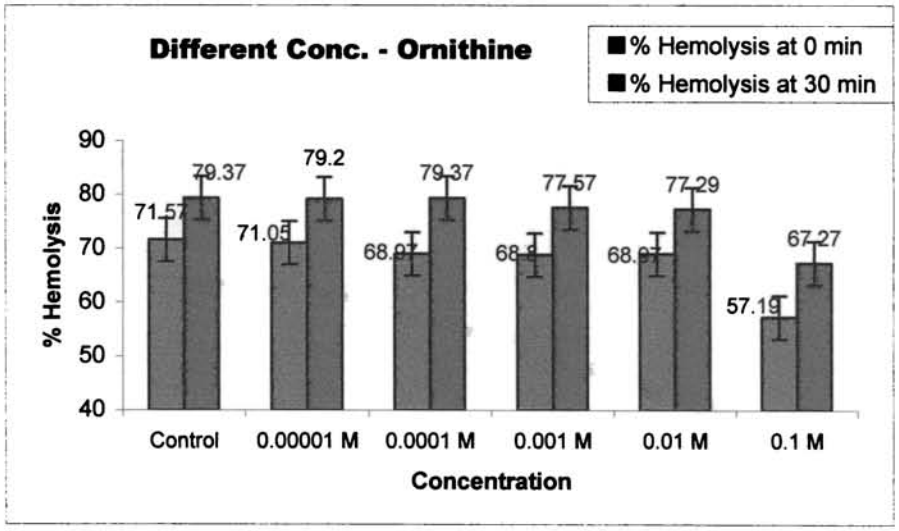
Sodium Acetate

Concentration of Biochemical	% of Hb released from RBC at 0 Min	% of Hb released from RBC at 30 Min
	(At Room Temperature)	
Control (0 M)	59.81± 1.146	65.86± 1.48
0.00001 M	59.81± 1.146	68.58± 1.782
0.0001 M	69.66± 0.9	72.50± 1.146
Control	74.32± 1.146	82.77± 1.48
0.001 M	53.95± 2.904	60.12± 0.74
0.01 M	51.05± 0.74	61.32± 0.74
0.1 M	38.06± 1.146	45.61± 2.409



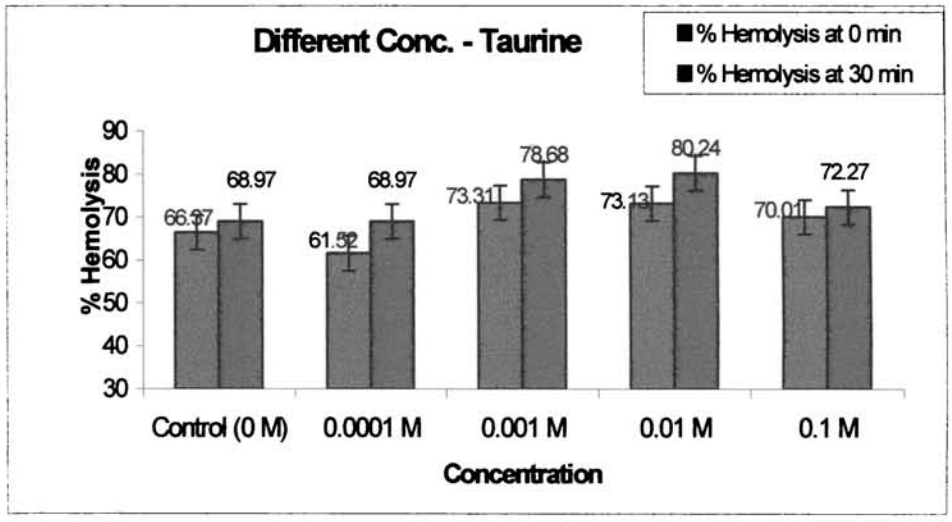
Ornithine

Concentration of Biochemical	% of Hb released from RBC at 0 Min	% of Hb released from RBC at 30 Min
	(At Room Temperature)	
Control (0 M)	71.57 ± 0.783	79.37 ± 1.074
0.00001 M	71.05 ± 0.849	79.20 ± 0.425
0.0001 M	68.97 ± 1.074	79.37 ± 0.849
0.001 M	68.80 ± 0.783	77.57 ± 0.509
0.01 M	68.97 ± 0.537	77.29 ± 1.566
0.1 M	57.19 ± 1.315	67.27 ± 1.017



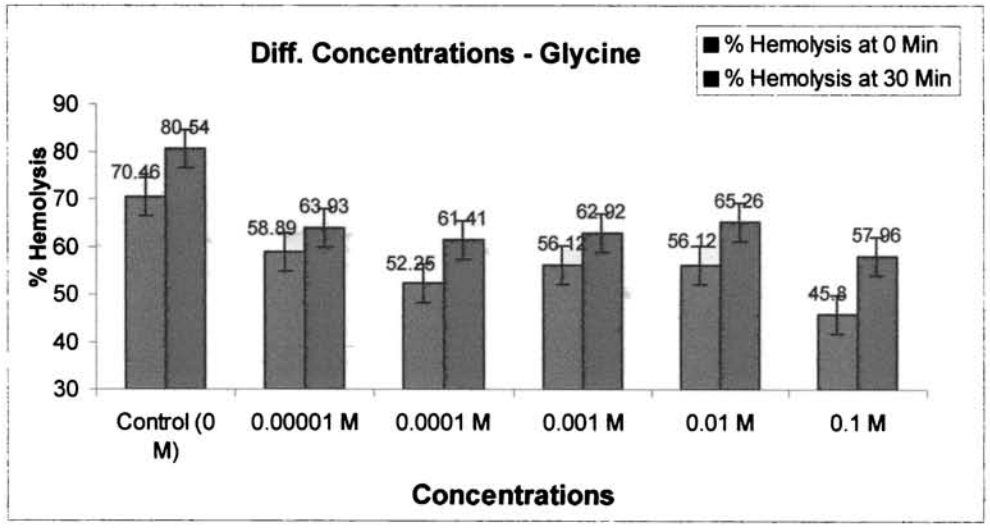
Taurine

Concentration of Biochemical	% of Hb released from RBC at 0 Min	% of Hb released from RBC at 30 Min
	(At Room Temperature)	
Control (0 M)	66.37 ± 0.425	68.97 ± 0.537
0.0001 M	61.52 ± 0.425	68.97 ± 0.537
0.001 M	73.31 ± 1.091	78.68 ± 0.537
0.01 M	73.13 ± 0.849	80.24 ± 0.425
0.1 M	70.01 ± 0.537	72.27 ± 0.569



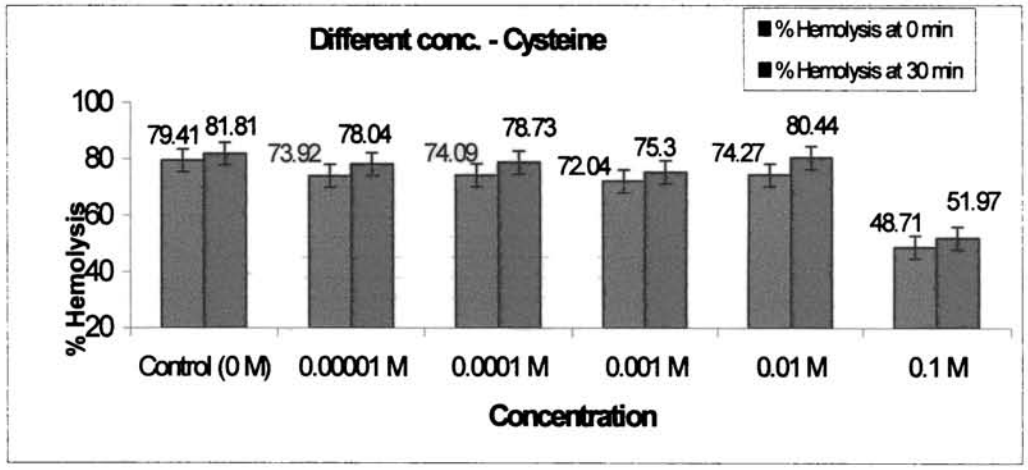
Glycine

Concentration of Biochemical	% of Hb released from RBC at 0 Min	% of Hb released from RBC at 30 Min
	(At Room Temperature)	
Control (0 M)	70.46± 1.559	80.54± 1.829
0.00001 M	58.89± 2.527	63.93± 2.274
0.0001 M	52.25± 0.739	61.41± 1.559
0.001 M	56.12± 0.617	62.92± 3.119
0.01 M	56.12± 1.485	65.26± 1.132
0.1 M	45.8± 0.779	57.96 ± 0.742



Cysteine

Concentration of Biochemical	% of Hb released from RBC at 0 Min	% of Hb released from RBC at 30 Min
(At Room Temperature)		
Control (0 M)	79.41 ± 0.42	81.81 ± 1.261
0.00001 M	73.92 ±0.42	78.04 ± 1.203
0.0001 M	74.09 ± 1.722	78.73 ± 0.564
0.001 M	72.04 ± 0.651	75.3 ± 0.42
0.01 M	74.27 ± 1.012	80.44 ± 0.42
0.1 M	48.71 ± 0.84	51.97 ± 0.861



ANOVA TABLE (Three way Anova)

Sodium Acetate

Source	Sum of Square	Degrees of Freedom	Mean Square	F
Total	1.207	35		
Between Species	1.002	2	0.5009	102.1***
Between Concentration	0.072	5	0.0145	2.9529*
Between Time of Incubation	0.0004	1	0.0004	0.0875 NS
Error	0.132	27	0.0049	

Means of Species		Means of Concentration		Least Significant Difference for Species	Least Significant Difference of Concentration
Gallus	0.4024	Control	0.693	0.0586	0.0829
Oryctolagus	0.0296	0.1	0.236		
Oreochromis	0.0712	0.01	0.569		
		0.001	0.659		
		0.0001	0.663		
		0.00001	0.649		

* $p<0.05$.
*** $p<0.001$.
NS Not Significant.

Cysteine

Source	Sum of Square	Degrees of Freedom	Mean Square	F
Total	0.03443	35		
Between Species	0.0154	2	0.0077	20.512***
Between Concentration	0.0042	5	0.0008	2.2165 NS
Between Time of Incubation	0.0047	1	0.0047	12.499*
Error	0.0101	27	0.0049	

Means of Species		Means of Time of Incubation		Least Significant Difference for Species	Least Significant Difference of Time of Incubation
Gallus	0.1151	0 Min	0.0882	0.0168	0.0137
Oryctolagus	0.0703	30 Min	0.111		
Oreochromis	0.1133				

* p<0.05.
*** p<0.001.
NS Not Significant.

Ornithine

Source	Sum of Square	Degrees of Freedom	Mean Square	F
Total	0.0275	35		
Between Species	0.0213	2	0.0107	102.31***
Between Concentration	0.0005	5	0.8724	0.0872 NS
Between Time of Incubation	0.0029	1	27.46	27.46**
Error	0.0028	27	0.0001	

Means of Species		Means of Time of Incubation		Least Significant Difference for Species	Least Significant Difference of Time of Incubation
Gallus	0.1219	0 Min	0.0786	0.0084	0.0069
Oryctolagus	0.0694	30 Min	0.0964		
Oreochromis	0.0713				

** p<0.01.
*** p<0.001.
NS Not Significant.

Glycine

Source	Sum of Square	Degrees of Freedom	Mean Square	F
Total	0.1111	35		
Between Species	0.0982	2	0.0491	216.57***
Between Concentration	0.0040	5	0.0008	3.5511*
Between Time of Incubation	0.0028	1	0.0028	12.441**
Error	0.0061	27	0.0002	

Means of Species		Means of Concentration		Means of time of Incubation		Least Significant Difference for species	Least Significant Difference of Concentration	Least Significant Difference of Time of Incubation
Gallus	0.1543	Control	0.453	0 Min	0.105	0.0119	0.0168	0.0097
Oryctolagus	0.0404	0.1	0.329	30 Min	0.123			
Oreochromis	0.1479	0.01	0.367					
		0.001	0.371					
		0.0001	0.377					
		0.00001	0.414					

* $p<0.05$.
** $p<0.01$.
*** $p<0.001$.

Taurine

Source	Sum of Square	Degrees of Freedom	Mean Square	F
Total	3.4570	35		
Between Species	3.4438	2	1.7219	5786.4***
Between Concentration	0.0035	5	0.0007	2.3437 NS
Between Time of Incubation	0.0016	1	0.0016	5.5142*
Error	0.008035	27	0.0003	

Means of Species		Means of Time of Incubation		Least Significant Difference for Species	Least Significant Difference of Time of Incubation
Gallus	0.7260	0 Min	0.2818	0.0146	0.0119
Oryctolagus	0.0685	30 Min	0.2953		
Oreochromis	0.0713				

* $p<0.05$.
*** $p<0.001$.
NS **Not Significant.**

Discussion

The mechanism of protective action of taurine on membrane stability is unclear. ***23** A possible clue in respect of this mechanism is provided by a recent observation that an increase in the number of poly unsaturated fatty acids in the membrane of cultured human retinoblastoma cells increases affinity of taurine for its carrier transport. This effect is specific for taurine and indicates an important interaction of amino acid (taurine) with poly-unsaturated sites in the membrane. These interactions might be responsible for the stabilizing properties of taurine in membranes containing large number of polyunsaturated fatty acids.

Different resistance of Mammalian RBC to hemolysis by bile salts was studied by Salvioli et al., ***28** No correlation was detected between TDC50 and Phospholipid composition.

The lower concentrations of ornithine and glycine (10^{-5} M and 10^{-4} M) were found to destabilize erythrocyte membrane in *Oreochromis* while higher concentrations were found to be stabilizing. The critical micellar concentration of these metabolites may be high.

Polyene antibiotics disrupt limiting membrane by interacting with lipids present in them. They preferentially react with sterol of model membranes rather than with phospholipids or other membrane constituents. This was supported by works of Demel et al,. ***29** Filipin and Nystatin penetrated monolayers of cholesterol or ergosterol but failed to penetrate monolayers of natural or synthetic phospholipids.

In bilayer experiments carried out by Van Zutphen, Van Deenen and Kinsky, ***30** Filipin did not show any interaction with black lipid films formed from lecithin but they were rendered unstable by filipin when bilayers were prepared from sterol and lecithin (1:1).

Studies reported by Demel et al,.***31** suggest that filipin can also interact with cetyl alchohol and oleic acid. Therefore, until sufficient studies are performed in model systems with these lipids it cannot be excluded that the disruption of erythrocytes or lysosomes, the membranes of which are rich in sphingomyelin results from the interaction of filipins with such receptors.

It is by no means certain that mammalian membranes are disrupted by the interaction of polyenes with cholesterol alone. Presently it is reasonable to suspect that polyenes do indeed owe their biological effects to a common but quantitatively different affinity for sterols.

Decreased lipid parameters observed in the study of action of penicillic acid on erythrocytes ***13** indicate direct interaction of penicillic acid with erythrocytes leading to shedding of cholesterol and phospholipids from the membrane. ***32** reported that leakage of hemoglobin, cholesterol and phospholipids from erythrocytes was due to membrane damage done by methyl salicylate.

Selected clinically important non-steroidal anti-inflammatory drugs react with erythrocyte membrane causing membrane stabilization while at higher concentrations resulted in erythrocyte lysis. The biphasic behaviour of drugs is due to their potentiality to form micellar aggregates at higher concentrations (called the critical micellar concentrations). So, when drugs are present as micelles above the critical micellar concentration the interaction with erythrocyte membrane results in hemolysis.

Since, erythrocytes are free from intracellular membranes and organelles any effect of a drug on osmotic hemolysis can be interpreted as an effect on the plasma membrane. The stabilizing effect of non-steroidal anti-inflammatory drugs on erythrocytes may be due to a stabilizing effect of the drugs on certain proteins in the cell membranes. Erythrocyte membrane stabilization study is simple, rapid, though non-specific and is useful as a preliminary screening test for the potential anti- inflammatory compounds. ***10** Oxidative stress induces numerous types of alterations in membrane.***33** The structural role of Vitamin E in preventing hemolysis ***34** and protecting the red cell membrane lipid -protein complexes against oxidative damage ***35** has been well established. ***36** Multivalent cations (e.g., calcium) not only enhances the stability of red cell membranes but also stabilize cell membranes against inverted structures.

Chapter – 3

Effect of selected biochemicals on the stability of liver lysosome membrane in *Oreochromis mossambicus*

Introduction

Lysosomes, the intracellular unit membrane bound organelles enclosing hydrolytic enzymes (having an acidic optimum pH) have been implicated in the defence mechanism of the cell and in the pathogenesis and progression of different disorders.*37

Under normal conditions, the various hydrolytic enzymes remain latent and bound by the single unit membrane. Lysosomes are present in the endoplasmic reticulum enclosed by a membrane and contain several kinds of hydrolases such as acid phosphatase and ß glucuronidase. When the membrane of a lysosome is destabilized by chemical action resident enzymes are released. *38

The effect of chemicals on a lysosome membrane thus can be evaluated by measuring the activity of released enzymes. The activity of these hydrolytic enzymes are found to increase as a result of thawing, freezing and use of detergents or hypotonic media. Certain agents have been found to have a labilizing effect on lysosome membranes. eg., Progesterone, endotoxin, testosterone, vitamin A and vitamin E, protease, digitonin, UV and irrdiation and bile salts. Lysosomal activity has been studied not only in relation to normal physiology but also in relation to pathology. eg. fever, congestive heart failure, hepatitis, pyelonephritis, hyper- tension, joint and tissue injury.

Lysosomes play a role in certain diseases. eg., silicosis, a disease found among miners and contracted from over exposure to silica dust causes the silica particles to collect around lysosomes - leading to their eventual breaking in the lungs. Several congenital diseases in which *some lysosomal enzymes are defective lead to eventual enormous accumulation of undigested products (eg. lipidosis, glycogen* accumulation), which begin to interfere with other cellular functions. eg., In Tay Sachs disease, the lack of functional lipases within the

lysosomes of nerve cell leads to massive lipid accumulation in these defective lysosomes and eventual impaired nerve function.

Labilizers are substances that can cause rupture of lysosome membrane, leading to release of lysosome's acid hydrolases in the cell. This eventually leads to cellular destruction or autolysis. Known labilizers include uric acid (causes gout), asbestos (cause lung disease) and excessive alcohol (causes liver damage). Stabilizers are substances that may strengthen the lysosomal membrane. eg., this is thought to be one mechanism of action for cortisone(a well known anti-inflammatory drug) *37

Several agents capable of releasing hydrolytic enzymes from lysosomes are also known to affect membranes of erythrocytes.

Lysosomes are organelles bounded by a unit membrane containing hydrolytic enzymes having active pH capable of breaking down or digesting worn out cell parts of ingested foreign matter. Lysosomes are round or oval and may contain bits of other organelles or variably dense granular material - primary lysosome which has been newly formed by the Rough Endoplasmic Reticulum and Golgi Pathway *39

Substances like vitamin A when used over excessively can cause the lysosome membranes to become unstable. On the other hand, cortisone and certain other drugs have been proven to stabilize the lysosome membranes. The anti-inflammatory agents in several cases are stabilizers of lysosome membranes.

Phospholipases are a potent package which could completely digest the cell, hence their confinement to the lysosome. However, if they should leak into the cell, they are unlikely to do any damage because the cellular pH is about 7.2 at which the enzyme activity gets

neutralized. The function therefore of the organelles is digestion of unwanted cell debris or digestion of the contents of endosomal vesicles.

Linked biochemical and cytochemical investigations have demonstrated that increased fragility of lysosomes induced by phenanthrene corresponds directly with increased catabolism of cytosolic proteins. ***40**

Because of the importance of lysosomes in the catabolic processes occurring in the cell, a detailed study has been made of the enzyme composition and stability of isolated kidney lysosomes. ***41**

Preliminary screening of selected biochemicals to study their effect on the lysosome membrane was to investigate and quantify using biochemicals to identify lysosome membrane stabilizers. Different concentrations of the biochemicals tried also helped to reveal whether concentration had any effect on stability of membrane. Arresting the labilization of membrane by suitable agents has got far reaching implications in clinical pathology . Such studies can assure methods of protection against pollutant induced damages and harmful effects of physical, chemical and natural factors.

Materials and methods

Male specimen of *Oreochromis mossambicus* having an average length of 10 cm were collected from Rice Research Institute, Vyttila, Cochin, Kerala and brought to the lab immediately. They were acclimated in large acquarium tanks for 3 weeks and fed adlibitum. The water in the tank was changed daily after feeding.

$10^{-3}M$ suspensions of selected biochemicals in isotonic sucrose solution were used for preliminary screening. $10^{-3}M$ concentrations of glycine, taurine, sodium acetate, cysteine, ornithine, DOPA, sodium

succinate, alpha ketoglutaric acid, sodium pyruvate and L-glutamic acid were screened to identify lysosome membrane stabilizers. A series of concentrations 10^{-1} - 10^{-4} molarities of the stabilizers identified were prepared in isotonic sucrose solution and further tested for their lysosome membrane stabilizing action.

Experimental studies were carried out using isolated lysosome from liver tissue of *Oreochromis.*

The stability of lysosome membrane in *Oreochromis* was determined following the procedure of Philip and Kurup***37** and Rao and Sisodia***42** with slight modification. The lysosome lability index of lysosome membrane was determined by measuring the amount of acid phosphatase enzyme liberated.

For the experiment, live *Oreochromis* was collected and killed by pithing. The process was carried out in the minimum time and care was taken to inflict minimum stress while handling. Liver was collected in cold sterile isotonic sucrose solution. Liver tissue was weighed and homogenised in a mortar and pestle. Then the homogenate was centrifuged at 600g for 10 minutes at 0°C. The nuclear fraction was collected and supernatant again centrifuged at 15000 X g for 30 minutes at 0°C. The lysosome rich fraction obtained was suspended in isotonic sucrose solution.

The lysosome rich fraction obtained from liver of acclimated fish was suspended in isotonic sucrose solution. The biochemicals whose effects on lysosome membrane are to be studied were added to fixed quantities of the lysosomal suspension. Then the mixtures were incubated at 30°C and samples retrieved after fixed time intervals, including 0 minute sample and centrifuged at 15000g for 30 minutes at 0°C in a refrigerated centrifuge.

Acid phosphatase enzyme (the marker enzyme of lysosomes) released was assayed following the procedure of Anon*43 with slight modification.

To a mixture of 0.5 ml of citrate buffer (pH 4.8) and 0.5 ml of para nitrophenyl phosphate, 0.3 ml of the sample to be assayed were added and incubated for 30 minutes at room temperature. After 30 minutes the reaction was stopped by the addition of 4 ml of 0.1N NaoH.

The yellow colour of p-nitrophenol developed as a result of the reaction wass measured at 410 nm in a uv-visible spectrophotometer. The amount of p-nitrophenol liberated was calculated from a calibration curve obtained by plotting the absorbance of the standard para nitrophenol against concentration.

Specific activity of acid phosphatase was expressed as millimoles of para nitrophenol phosphate formed per hour/gm protein using bovine serum albumin as standard by Lowry's method.*44

The effects of these biochemicals on the native enzyme of the lysosome sample were also determined. Lysosome rich fraction obtained from liver of acclimated fish as explained earlier were suspended in isotonic sucrose solution and then carefully processed in citrate buffer until native enzyme was released. The results of these were compared with results from previous experiment and necessary corrections made in values to make the interpretations more reliable.

This step has helped to rule out the possibility of the effect (inhibitory or activating effect) of the biochemical on the native enzyme from interfering with studies on the effect on lysosome membrane.

A control was run simultaneously in all cases for comparing the effect of biochemicals used.

The lysosome membrane activity in the presence of selected biochemicals was expressed as a percentage of total activity obtained in an equal quantity of lysosome suspension treated with a detergent.

Results

EFFECT OF SELECTED BIOCHEMICALS ON LYSOSOME MEMBRANE STABILITY IN *OREOCHROMIS*

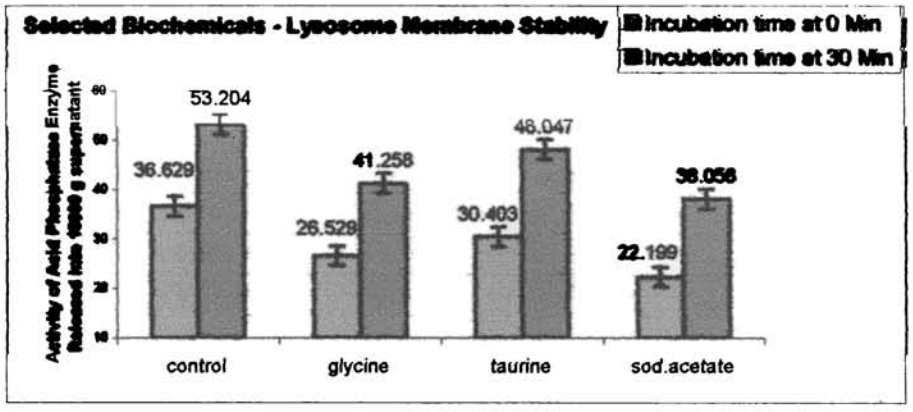
(a) Preliminary Screening

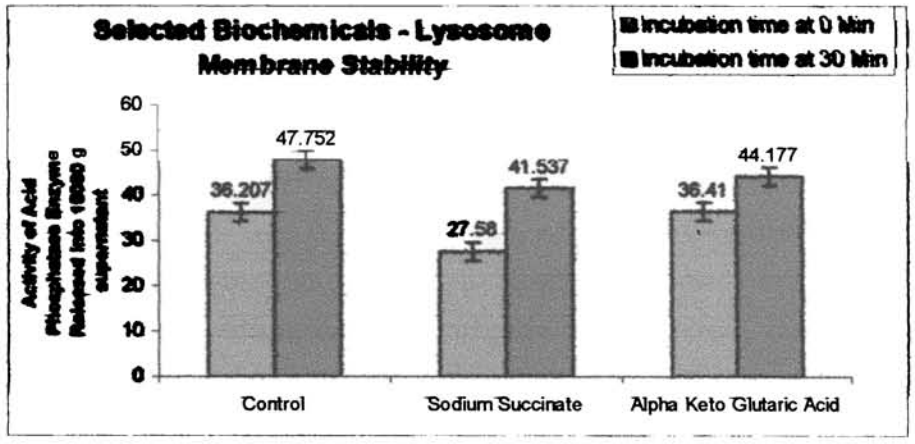
Preliminary screening of 10^{-3}M concentrations of glycine, taurine, sodium acetate, cysteine, ornithine, DOPA, sodium succinate, alpha ketoglutaric acid, sodium pyruvate and L-glutamic acid has revealed that glycine, taurine, sodium acetate, sodium succinate, sodium pyruvate, cysteine and ornithine possess lysosome membrane stabilizing property. On the other hand, 10^{-3}M suspension of DL-DOPA, L-glutamic acid and alpha ketoglutaric acid were observed to labilize lysosome membrane in *Oreochromis* . The effect of those biochemicals were found to increase with increase in period of incubation.

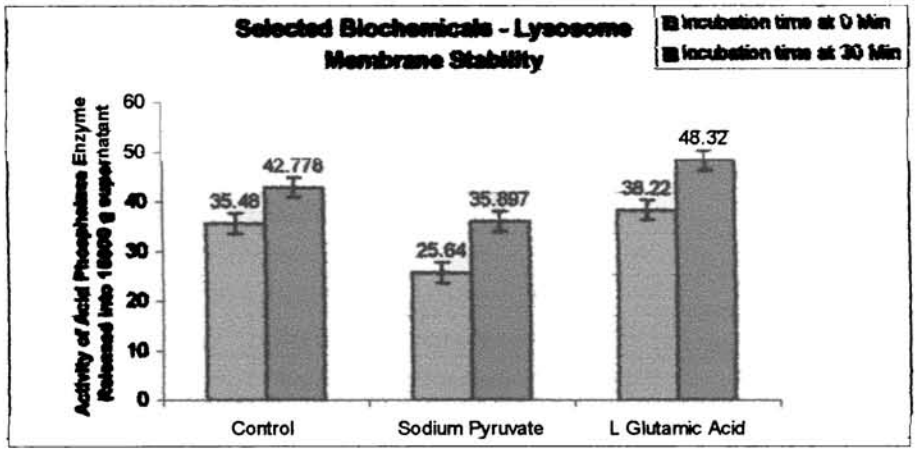
Acid Phosphatase Enzyme Activity released into 15000 g supernatant

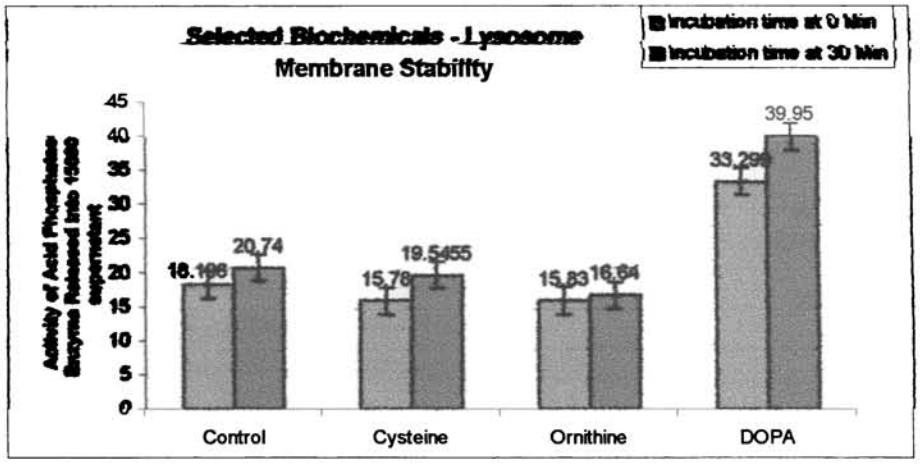
Name of Biochemical	Time of Incubation in minutes (Room Temperature)	Release (mgpnp/hr/kg wet wt)	% of Total Lysosomal Activity released
Control	0	36.629 ± 0.0033	37.93
	30	53.2048 ± 0.0001	55.09
Glycine	0	26.5299 ± 0.00023	27.47
	30	41.258 ± 0.00129	42.72
Taurine	0	30.4025 ± 0.00387	31.48
	30	48.047 ± 0.001498	49.75
Sodium Acetate	0	22.1996 ± 0.001359	22.988
	30	38.0567 ± 0.00503	39.408
Control	0	36.207 ± 0.00022	52.036
	30	47.752 ± 0.00095	68.62
Sodium Succinate	0	27.58 ± 0.00048	39.63
	30	41.537 ± 0.0005	59.6
Alpha Keto	0	36.41 ± 0.032	52.32
Glutaric Acid	30	44.177 ± 0.0014	63.49
Control	0	35.48 ± 0.0045	56.59
	30	42.778 ± 0.00138	68.23
Sodium Pyruvate	0	25.64 ± 0.00214	40.89
	30	35.897 ± 0.00138	57.26
L Glutamic	0	38.22 ± 0.0024	60.96
Acid	30	48.32 ± 0.00035	77.07

Name of Biochemical	Time of Incubation in minutes (Room Temperature)	Release (mgpnp/hr/kg wet wt)	% of Total Lysosomal Activity released
Control	0	18.196 ± 0.0089	28.759
	30	20.74 ± 0.00332	33.08
Cysteine	0	15.78 ± 0.000778	25.17
	30	19.5455 ± 0.00510	31.17
Ornithine	0	15.83 ± 0.0034	25.25
	30	16.64 ± 0.0023	26.54
DOPA	0	33.299 ± 0.0041	53.116
	30	39.95 ± 0.0032	63.726









The results were statistically analyzed performing two-way Anova using repeated number of observations from the raw data. The effects of various biochemicals studied above were found to be significant statistically. The means of selected biochemicals studied and the means of the time of incubation were compared.

The least significant difference at 5% interval was calculated for each biochemical and the means were separated. Significantly higher level of absorbance is noticed in control followed by that in test chemicals. Regarding the effect of time of incubation, the absorbance level is found to be significantly higher after 30 minutes of incubation than at 0 minute of incubation.

ANOVA TABLE (Two way Anova)

Taurine, Glycine, Sodium Acetate

Source	Sum of Square	Degrees of Freedom	Mean Square	Rounded to	F
Total	0.1195	39			
Activity	0.0833	3	0.02776	0.0278	111.232***
Time of Incubation	0.0276	1	0.02756	0.0276	110.4316***
Error	0.00875	35	0.00025		

Means of Biochemicals		Means of time of Incubation		Least Significant Difference for Activity	Least Significant Difference for time of Incubation
Control	0.279	0 Min	0.117	0.01414	0.01155
Taurine	0.025	30 Min	0.333		
Glycine	0.050				
Sodium Acetate	0.1513				

*** $p<0.001$

Cysteine, Ornithine, DOPA

Source	Sum of Square	Degrees of Freedom	Mean Square	Rounded to	F
Total	0.129	28			
Activity	0.111	3	0.0369	0.037	288.629***
Time of Incubation	0.0219	1	0.0219	0.0219	170.837***
Error	0.0031	24	0.000128		

Means of Biochemicals		Means of time of Incubation		Least Significant Difference for Activity	Least Significant Difference for time of Incubation
Control	0.663	0 Min	0.7722	0.0294	0.01471
Cysteine	0.6216	30 Min	0.9831		
Ornithine	0.4513				
Dopa	0.108				

*** p<0.001

Sodium Succinate, Alpha Keto Glutaric Acid.

Source	Sum of Square	Degrees of Freedom	Mean Square	Rounded to	F
Total	0.0277	19			
Activity	0.00428	2	0.002138	0.0021	20.688***
Time of Incubation	0.0218	1	0.02178	0.0218	214.756***
Error	0.00162	16	0.000102		

Means of Biochemicals		Means of time of Incubation		Least Significant Difference for Activity	Least Significant Difference for time of Incubation
Control	0.286	0 Min	0.2924	0.00958	0.00782
Sodium Succinate	0.277	30 Min	0.5617		
Alpha Keto Glutaric Acid	0.2731				

*** p<0.001

Sodium Pyruvate, L-glutamic Acid.

Source	Sum of Square	Degrees of Freedom	Mean Square	Rounded to	F
Total	0.0835	27			
Activity	0.0167	2	0.00838	0.0084	24.279***
Time of Incubation	0.0585	1	0.05851	0.0585	169.0911***
Error	0.00830	24	0.00035		

Means of Biochemicals		Means of time of Incubation		Least Significant Difference for Activity	Least Significant Difference for time of Incubation
Control	0.4368	0 Min	0.2418	0.0171	0.0139
Sodium Pyruvate	0.1830	30 Min	0.695		
L Glutamic Acid	0.276				

*** $p<0.001$

(b) EFFECT OF DIFFERENT CONCENTRATIONS OF MEMBRANE STABILIZERS IDENTIFIED :-

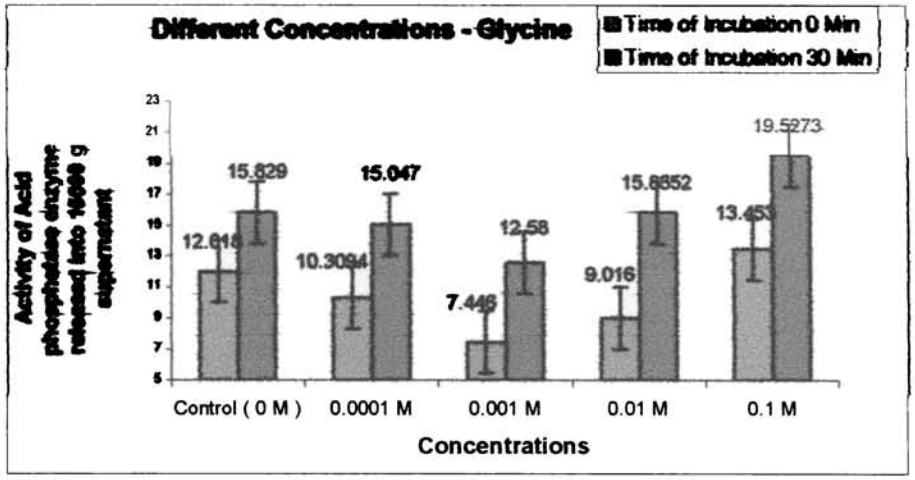
Different concentrations (10^{-5} to 10^{-1} M) of lysosome membrane stabilizers identified were again screened for determining their lysosome membrane stabilizing action.

All concentrations of taurine (10^{-4}- 10^{-1} M), sodium acetate, cysteine, ornithine and alpha ketoglutaric acid were observed to stabilize lysosome membrane in *Oreochromis.* Lower concentrations of glycine and sodium pyruvate (10^{-3} and 10^{-4} M concentrations) were observed to stabilize lysosome membrane while higher concentrations (10^{-1} and 10^{-2}) were found to labilize the lysosome membrane.

Acid phosphatase Enzyme Activity released into 15000 g supernatant (mgpnp/hr/kg wet wt)

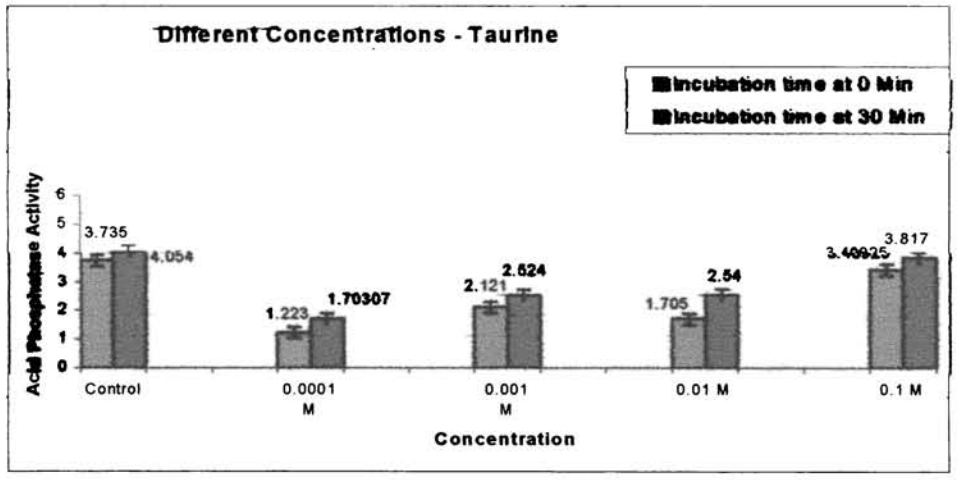
Glycine

Concentration of Biochemical	Time of Incubation in minutes (Room Temperature)	Release	% of Total Lysosomal activity released
Control (0 M)	0	12.018±0.0038	28.3
	30	15.829±0.00037	37.2
0.0001 M	0	10.3094±0.00029	24.2
	30	15.047±0.00044	35.4
0.001 M	0	7.446±0.0019	17.5
	30	12.58±0.0004	29.6
0.01 M	0	9.016±0.0025	21.2
	30	15.8352±0.0067	37.2
0.1 M	0	13.453±0.0022	31.6
	30	19.5273±0.00201	45.9



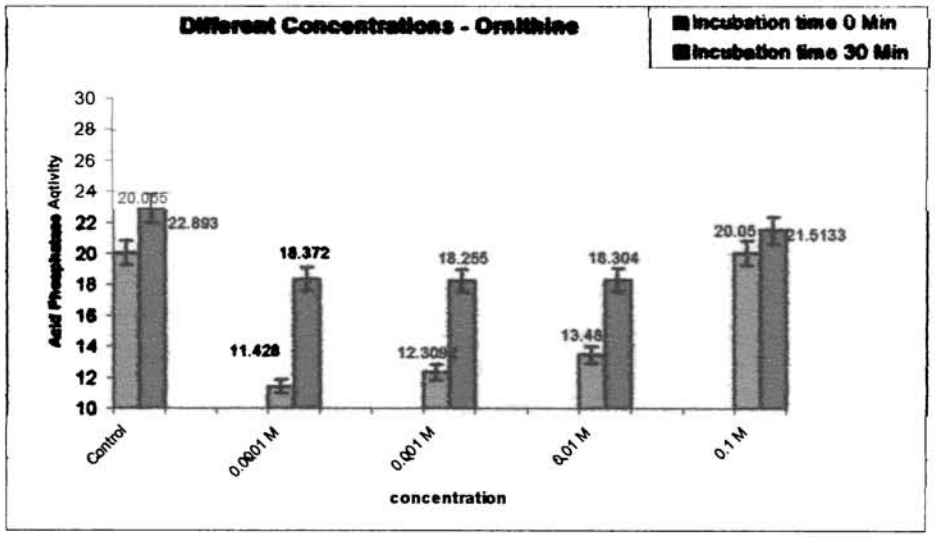
Taurine

Concentration of Biochemical	Time of Incubation in *minutes* (*Room* Temperature)	Release (mgpnp/hr/kg wet wt)	% of Total Lysosomal *activity* released
Control	0	3.7349±0.00096	40
	30	4.0540±.00078	43.4
0.0001 M	0	1.223±0.00395	13.1
	30	1.70307±0.00046	18.2
0.001 M	0	2.121±0.00175	22.7
	30	2.524±0.00033	27
0.01 M	0	1.70542±0.00047	18.3
	30	2.54±0.00033	27.2
0.1 M	0	3.40925±0.001	36.5
	30	3.817±0.00023	40.9



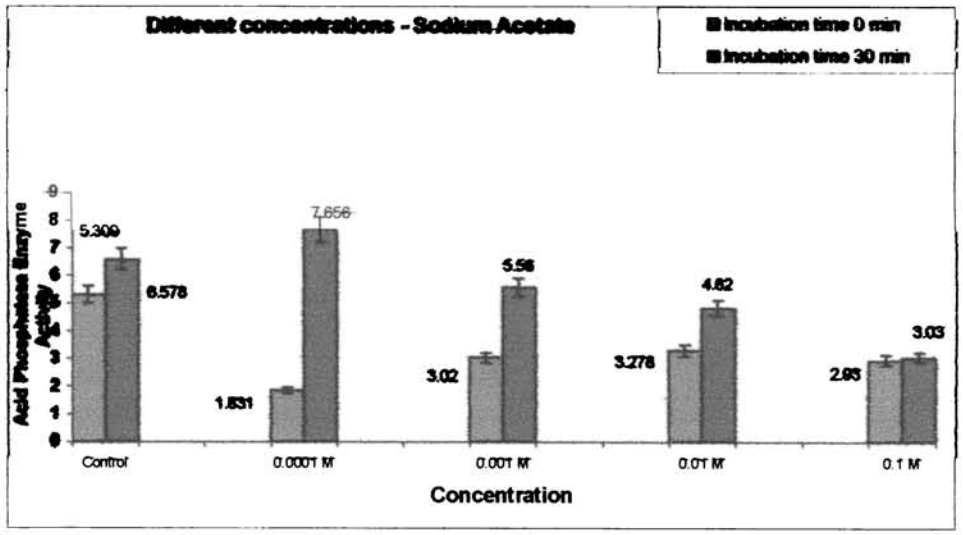
Ornithine

Concentration of Biochemical	Time of Incubation in minutes (Room Temperature)	Release (mgpnp/hr/kg wet wt)	% of Total Lysosomal activity released
Control	0	20.055±0.00049	56.3
	30	22.893±0.000266	64.3
0.0001 M	0	11.429±0.000224	32.1
	30	18.37±0.00077	51.4
0.001 M	0.	12.309±0.00033	34.6
	30	18.255±0.000125	51.2
0.01 M	0	13.48±0.00036	37.8
	30	18.305±0.000069	51.4
0.1 M	0	20.057±0.0032	56.3
	30	21.513±0.0028	60.4



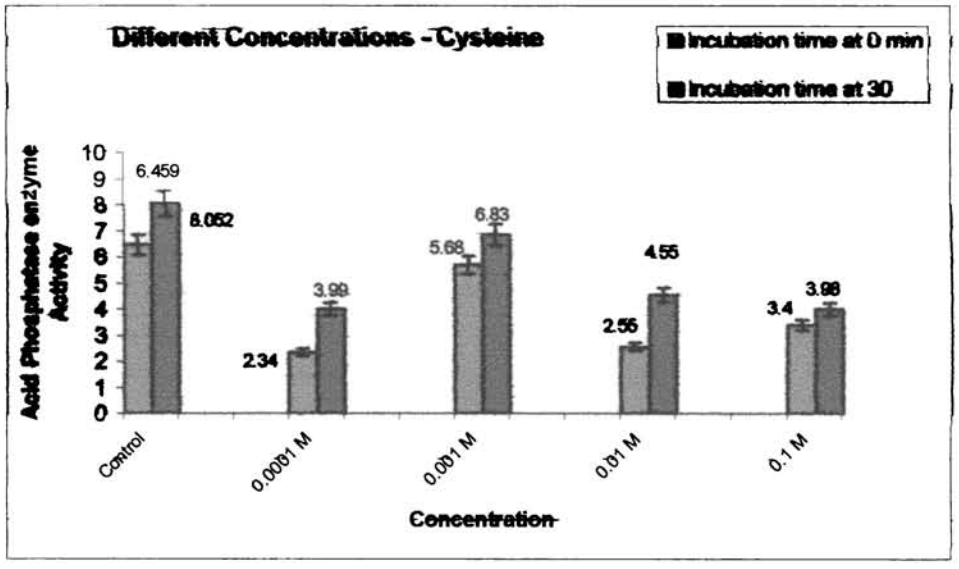
Sodium Acetate

Concentration of Biochemical	Time of Incubation in minutes (Room Temperature)	Release (mgpnp/hr/kg wet wt)	% of Total Lysosomal activity released
Control	0	5.309±0.00098	40.4
	30	6.578±0.00344	50.1
0.0001 M	0	1.831±0.0034	13.9
	30	7.656±0.00047	58.3
0.001 M	0	3.019±0.000116	23
	30	5.556±0.000454	42.3
0.01 M	0	3.278±0.00038	24.9
	30	4.821±0.0005	36.7
0.1 M	0	2.923±0.0014	22.3
	30	3.035±0.00426	25.5



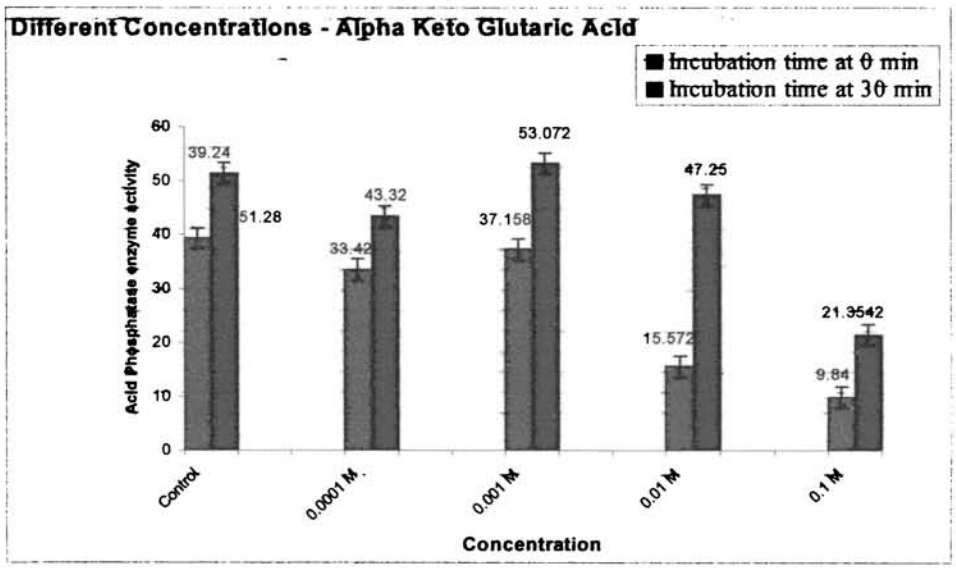
Cysteine

Concentration of Biochemical	Time of Incubation in minutes (Room Temperature)	Release (mgpnp/hr/kg wet wt)	% of Total Lysosomal activity released
Control	0	6.4589±0.00203	59.7
	30	8.052±0.0048	74.4
0.0001 M	0	2.344±0.000195	21.7
	30	3.988±0.002	36.8
0.001 M	0	5.681±0.00035	52.5
	30	6.837±0.0035	63.2
0.01 M	0	2.554±0.00024	23.6
	30	4.553±0.0043	42.1
0.1 M	0	3.403±0.0009	31.5
	30	3.983±0.004	36.8



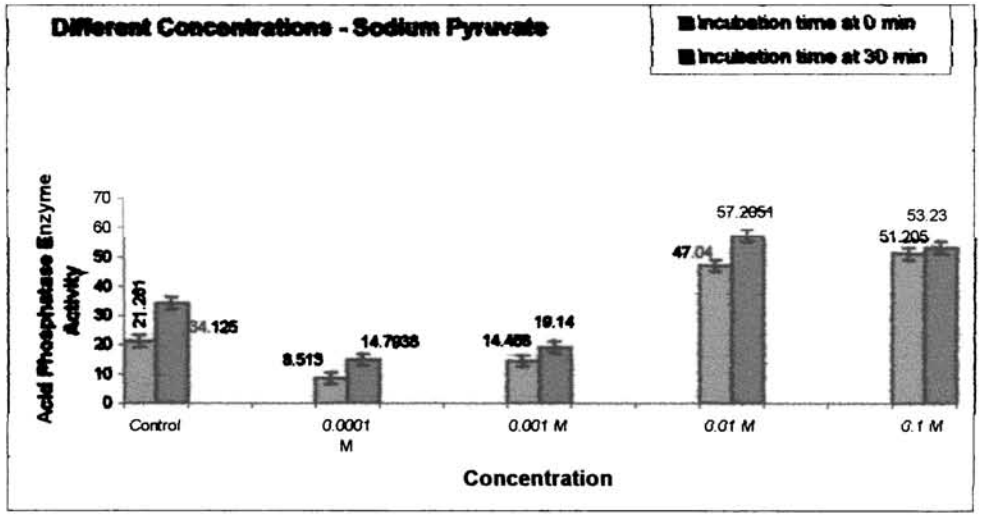
Alpha Keto Glutaric Acid

Concentration of Biochemical	Time of Incubation in minutes (Room Temperature)	Release (mgpnp/hr/kg wet wt)	% of Total Lysosomal activity released
Control	0	39.24±0.0002	17.2
	30	51.28±0.0015	22.4
0.0001 M	0	33.42±0.002	14.6
	30	43.322±0.00421	18.9
0.001 M	0	37.158±0.00245	16.2
	30	53.072±0.000123	23.2
0.01 M	0	15.572±0.0035	6.81
	30	47.25±0.00041	20.7
0.1 M	0	9.84±0.003	4.3
	30	21.354±0.00065	9.34



Sodium Pyruvate

Concentration of Biochemical	Time of Incubation in minutes (Room Temperature)	Release (mgpnp/hr/kg wet wt)	% of Total Lysosomal activity released
Control	0	21.261±0.00013	7.77
	30	34.125±0.00074	12.5
0.0001 M	0	8.513±0.00227	3.11
	30	14.794±0.0035	5.41
0.001 M	0	14.468±0.0002	5.29
	30	19.14±0.0034	7
0.01 M	0	47.04±0.0004	17.2
	30	57.205±0.0017	18.7
0.1 M	0	51.205±0.0001	18.7
	30	53.23±0.002	19.5



Statistical analysis of the observations performing 2 - way Anova on the raw data (Least Significant Difference at 5% level was calculated for each and Means separated) has revealed that all the observations in terms of activity and time of incubation were statistically significant except in the case of ornithine.

ANOVA TABLE (Two way Anova)

Different Concentration – Glycine

Source	Sum of Square	Degrees of Freedom	Mean Square	Rounded to	F
Total	0.0241	39			
Concentration	0.0077	4	0.002	0.0019	131.3275***
Time of Incubation	0.0159	1	0.02	0.0159	1099.004***
Error	0.00049	34	0.00002		

Means of Biochemicals		Means of time of Incubation		Least Significant Difference for Concentration	Least Significant Difference for time of Incubation
Control	0.1135	0 Min	0.1687	0.00341	0.00278
.1 M	0.1391	30 Min	0.3468		
.01 M	0.0986				
.001 M	0.0644				
.0001 M	0.0918				

*** $p < 0.001$

Different Concentration – Taurine

Source	Sum of Square	Degrees of Freedom	Mean Square	Rounded to	F
Total	0.0221	39			
Concentration	0.0186	4	0.0047	0.0047	200.3511***
Time of Incubation	0.00265	1	0.0027	0.0027	115.095***
Error	0.000798	34	0.000024		

Means of Biochemicals		Means of time of Incubation		Least Significant Difference for Concentration	Least Significant Difference for time of Incubation
Control	0.01045	0 Min	0.121	0.00434	0.00354
.1 M	0.093	30 Min	0.176		
.01 M	0.043				
.001 M	0.045				
.0001 M	0.0273				

*** $p < 0.001$

Different Concentration – Cysteine

Source	Sum of Square	Degrees of Freedom	Mean Square	Rounded to	F
Total	0.414	37			
Concentration	0.382	4	0.0955	0.0956	508.615***
Time of Incubation	0.026	1	0.026	0.026	138.326***
Error	0.006	32	0.000188		

Means of Biochemicals		Means of time of Incubation		Least Significant Difference for Concentration	Least Significant Difference for time of Incubation
Control	0.599	0 Min	0.742	0.12263	0.010013
.1 M	0.2051	30 Min	1.186		
.01 M	0.1922				
.001 M	1.1491				
.0001 M	0.138				

*** $p < 0.001$

Different Concentration – Ornithine

Source	Sum of Square	Degrees of Freedom	Mean Square	Rounded to	F
Total	1.5123	39			
Concentration	0.2805	4	0.07013	0.07013	2.1287NS
Time of Incubation	0.1117	1	0.1117	0.11171	3.391NS
Error	1.12	34	0.0329		

Means of Biochemicals		Means of time of Incubation		Least Significant Difference for Concentration	Least Significant Difference for time of Incubation
Control	0.1046	0 Min	0.168	nil	nil
.1 M	0.098	30 Min	0.0779		
.01 M	0.071				
.001 M	0.067				
.0001 M	0.774				

NS - Not Significant

Different Concentration - Alpha Keto Glutaric Acid

Source	Sum of Square	Degrees of Freedom	Mean Square	Rounded to	F
Total	3.4616	39			
Concentration	0.9587	4	0.2396	0.2397	4.977**
Time of Incubation	0.8655	1	0.8655	0.8655	17.97**
Error	1.637	34	0.0481		

Means of Biochemicals		Means of time of Incubation		Least Significant Difference for Concentration	Least Significant Difference for time of Incubation
Control	0.6305	0 Min	0.617	0.1962	0.1603
.1 M	3.13	30 Min	4.41		
.01 M	0.303				
.001 M	0.627				
.0001 M	0.432				

** $p < 0.01$

Different Concentration:- Sodium Pyruvate

Source	Sum of Square	Degrees of Freedom	Mean Square	Rounded to	F
Total	0.3272	39			
Concentration	0.311	4	0.0778	0.0778	553.986***
Time of Incubation	0.011	1	0.011	0.0111	79.039***
Error	0.0047	34	0.00014		

Means of Biochemicals		Means of time of Incubation		Least Significant Difference for Concentration	Least Significant Difference for time of Incubation
Control	0.2028	0 Min	0.533	0.01058	0.0086
.1 M	0.6826	30 Min	0.772		
.01 M	0.5936				
.001 M	0.0836				
.0001 M	0.043				

*** $p < 0.001$

Discussion

The membrane stabilizing action of cysteine, taurine, and sodium succinate has already been proved. Bile salts are accepted membrane labilizers. No previous reports regarding the action of other biochemicals screened for their membrane stabilizing action has been obtained. Lower concentration of glycine and sodium pyruvate (10^{-4} M and 10^{-3} M) were found to be lysosome membrane stabilizing, while higher concentrations (10^{-2} M and 10^{-1} M) were found to be lysosome membrane labilizing. The difference in stabilizing property of glycine and sodium pyruvate at different concentrations can be explained by their potentiality to form micellar aggregates at Critical Micellar Concentrations. When biochemicals are present as micelles above the Critical Micellar Concentrations, the interaction with lysosome membrane result in lysis of membrane.

Drugs or biochemicals by stabilizing the lysosome membrane can prevent the rupture of lysosomes and inhibit the release of lysosomal enzymes. Most of the steroidal and non-steroidal anti-inflammatory drugs stabilize in vitro lysosomal membranes. ***45**

Agents (toxins) which disrupt cell bio-membranes release cellular constituents including lysosomal enzymes into the surrounding tissue or cytoplasm which may further provoke inflammation and tissue injury ***46** Lysosomal enzymes can competely degrade the components of connective time such as collagen, protein mucopolysaccharide complexes, glycoproteins and elastin ***47**

Since lysosomal enzymes possess the capacity to completely degrade the components of connective tissue, use of anti-inflammatory agents that stabilize the lysosomal membrane seems rational in treatment of crippling inflammatory disorders. ***48**

A membrane stabilizer action is proposed as the mechanism underlying the protective effect of taurine and zinc. Taurine has been established as a membrane stabilizer ***49**

Sodium succinate has also been reported to exert some stabilizing effect on lysosome membrane in vitro in hepatic lysosomes from aniline treated rats.***37**

Lysosomes contain more than 50 enzymes mostly hydrolytic - a number of cationic proteins and probably one anionic protein which may be involved in the inflammatory response.***50**

Stabilization may be a specific mechanism of action of some clinically useful anti- inflammatory drugs***48**.

DL DOPA (Amino acid L Hydroxy Phenyl Alanine) is used for curing Parkinson's disease and also as a fracture healing promoter and an antidepressant drug. Studies have revealed that amino acids like glutathione, cysteine, methionine and vitamin C minimizes the toxic effects of aluminium.

Vincent. P.Hollander reported that cysteine, L glutamic acid, pyruvic acid and succinic acid when tested for 50% inhibition of prostatic acid phosphatase was found to have no effect. ***51**

The protective effect of taurine on stability is related to the increase in number of polyunsaturated fatty acid units in the membrane of cultured human retino blastoma cells increasing the affinity of taurine for its carrier transport.***52**

Retinol destabilizes biological membranes causing hemolysis of erythrocytes and increased secretion of enzymes from lysosomes. ***53**

Vitamin E behaves as biological antioxidant and preserves membrane integrity***16**

It also protects membrane from oxidative injuries ***54**. Its structural role ***55** and membrane stabilizing effects ***56** have also been shown. It has been proposed that tocopherol could also act as a membrane stabilizer through an interaction with polyunsaturated fatty acid residues of phospholipid molecules. ***57**

Chapter 4

Effect of natural products on lysosome membrane stability in *Oreochromis mossambicus*

Introduction

The substances from plant and animal sources are being used as food since antiquity. Later on, these substances were differentiated as foodstuffs and therapeutic agents as man tried to explore and utilize these natural products for treating the ailments. The term drug was used for the products used to remove disorders. The science dealing with the study of crude drugs of the natural sources processed scientifically is called pharmacognosy.

Knowledge of chemistry and plant and its products has led scientists to an array of new chemicals in fruits and vegetables. Many of these obscure chemicals exhibit unique ability to disrupt and prevent the formation of tumours.

In recent years, investigation of natural products has produced large numbers of potential drugs and many of them are used for several other purposes in various industries -- drugs from natural origin are being used suitably in confectionaries, in food industries, in beverages, as spices, as condiments, and for still other purposes as technical products.

Spices are major ingredients in the culinary art in most parts of the world, although use of particular spices may vary. They impart the characteristic aroma and taste of food and some of them give specific colour for the preparation. Spices have been shown to have anti-oxidant activity and are being used as food preservatives. ***58**

In this chapter, 1% crude extracts of selected spices in boiling water were used to assess their action on the liver lysosome membrane of *Oreochromis mossambicus* in vitro.

Materials and Methods

1% extract of selected spices - *Garcinia gambogis* (Malabar Tamarind); *Zingiber zingiberus* (Ginger); *Brassica nigra* (Mustard); *Curcuma longa Linn.* (Turmeric); *Allium sativum* (Garlic); *Corriandrum sativum* (corriander); *Cuminum- cyminum* (Cumin seed); Fenugreek; *Capsicum frutescens* (Red chilly).

0.5 Gms of the spice in 25 ml-distilled water was extracted in boiling water for 10 minutes. pH was adjusted to 7. Molarity of the solution was adjusted by addition of sucrose to 0.35 M. The solution was made isotonic with that of lysosome.

Liver lysosome suspension was prepared from live *Oreochromis mossambicus* as described in the previous chapter.

Lysosomal suspension was divided into equal quantities in three test tubes. To one, added equal volume of 0.35 molar sucrose and maintained as control, to the second one added equal volume of the spice extract to be studied and to the third one an equal volume of the detergent 0.2% Brij in sucrose was added to determine the total lysosomal activity in the sample used. The effect of drug on the lysosome membrane studied was expressed as a % of the total lysosomal activity by comparing with the control where no drug was added.

The samples were incubated at room temperature for 30 minutes. Then it was centrifuged at 15000g for 30 minutes and supernatant assayed for acid phosphatase enzyme using para nitrophenylphosphate as the substrate.

The effect of these spice extracts on native enzyme was also determined following the same procedure. For this purpose the spice extract was prepared in buffer pH 4.8 in boiling water for 10 minutes. Later it was cooled and filtered and pH adjusted to 4.8. Native enzyme of lysosome

carefully processed and suspended in citrate buffer was treated with spice extract and the activity of acid phosphatase enzyme assayed as described earlier.

The results thus obtained of the effect of spice extract on lysosome membrane was corrected with the help of results where native enzyme was used to avoid the influence of other interfering factors.

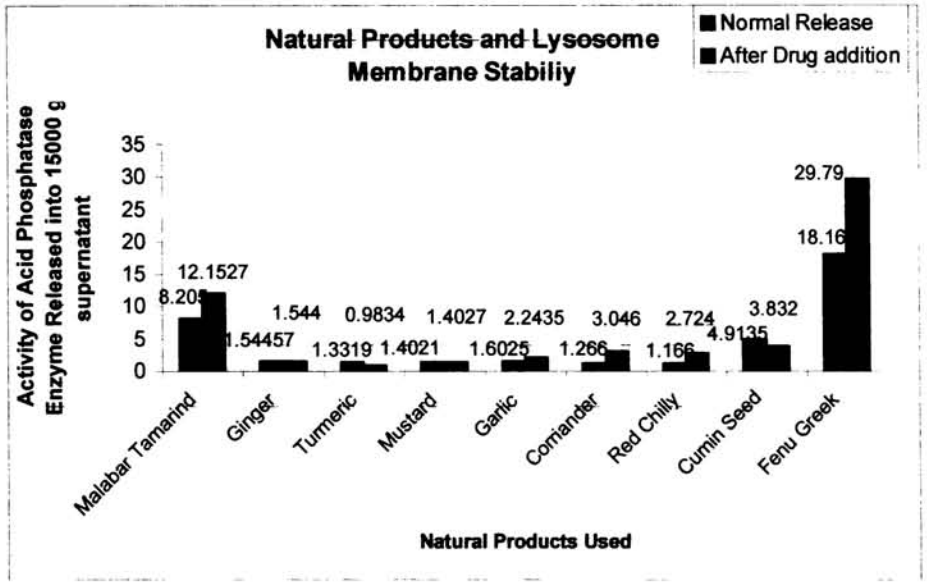
Results

In vitro studies on the effect of 1% extract of Malabar tamarind, ginger, turmeric, mustard, garlic, corriander, red chilly, cumin seed and fenugreek on the liver lysosome membrane of *Oreochromis* revealed that turmeric and cumin seed extracts stabilized the lysosome membrane, while ginger and mustard did not have any effect on the lysosome membrane.

1% extract of Malabar tamarind, red chilly, garlic, corriander and fenugreek were observed to destabilize the *Oreochromis* lysosome membrane in vitro.

Activity of Acid phosphatase enzyme released into 15000 g supernatant

	Release by Natural Products	
Natural Product	Normal Release	Release After addition of test material
Malabar Tamarind	8.205	12.153
Ginger	1.545	1.544
Turmeric	1.332	0.983
Mustard	1.402	1.403
Garlic	1.603	2.244
Corriander	1.27	3.046
Red Chilly	1.166	2.724
Cumin Seed	4.914	3.832
Fenu Greek	18.16	29.79



The results were analyzed statistically using two-way anova. The effect of 1% spice extracts on lysosome membrane in *Oreochromis* was found to be statistically significant at $p < 0.01$ level. The experimental results obtained are acceptable.

ANOVA TABLE - (Natural Products)

Source	Sum of Square	Degrees of Freedom	Mean Square	Rounded to	F
Total	1.9395	53			
Natural products	0.1745	8	0.0218	0.0218	0.649 NS
Control & Test	0.2615	1	0.2615	0.2616	7.69 **
Error	1.503	44	0.034		

Means of Natural Products		Means of Control & Test		Least Significant Difference for Control & Test
Garlic	0.0463	Control	1.743	0.1967
Coriander	0.0448	Test	0.055	
Cumin	0.371			
Fenugreek	0.106			
Red Chilly	0.0337			
Tamarind	0.029			
Ginger	0.0283			
Mustard	0.0226			
Turmeric	0.021			

^{NS} - Not Significant

** - $p < 0.01$

Discussion

The identification of natural lysosome membrane stabilizing agents has great potential for development in clinical pathology in the development of drugs. Spices being food additives are readily available, non-toxic and a potential benefit for developing countries like India, since their use will be more economical.

Spices which are liberally used in the preparation of Indian diets are reported to possess medicinal properties. Certain natural products have been proved to possess membrane stabilizing action and anti-inflammatory action e.g, Turmeric. ***59** Curcumin the active principle from turmeric is used as a modern drug . ***60**

The anti-inflammatory action of tea leaf saponin a mixture of saponins separated from leaves of *Camellia sinensis Var. sinesis* was investigated by Sagesake Yuko et al,. ***61**

The anti-inflammatory effect of the Ayurvedic preparation Brahmi Rasayan has been noticed in rodents mediating its anti-inflammatory activity by interfering with the action or synthesis of prostaglandins and also perhaps by stabilization of lysosomal membranes. Its anti-inflammatory activity is comparable to that of Indo- methacin ***62**

Red-hot chillies taken in low doses can help combat pain, inflammation and hyper reactive disorders, besides protecting cell membranes from pollution-induced damage. Capsaicin, the pungent chemical in Indian Chilly *Capsicum frutescens* is reported to reduce pain and inflammation caused by pollutants ***63**.

Garcinia anomala (pr clusiaceae) were found to possess very low anti-inflammatory activity ***64**

Kola viron, bioflavanoid extract of *Garcinia kola* (found to inhibit turpentine - induced joint edema) was found to possess anti-inflammatory activity comparable to that of phenyl butazone and acetyl salicylic acid ***65**

Onion (*Allium Cepa Linn.*) and garlic (*Allium Sativum Linn.*) are two important *Allium* species with several medicinal properties. Thiosulphinates (allicins) and Cepaenes (onion principles) have been shown to possess anti-inflammatory properties. ***66 & *67**

Excessive intake of *Allium* species may interfere with hemoglobin production and may lead to lysis of red blood cells. Abdominal hemorrhage and death of laboratory animals fed on excessive quantity of garlic has been reported. Therefore, only customary amounts of these species may be used as part of diet, salads and pickles.

Curcumin found to inhibit Long terminal repeat sequence of HIV might keep the virus inactive indefinitely not only in acutely infected cells

but also in chronically infected cells along with anti-oxidant nutrients such as beta carotene, vitamin C, or vitamin E ***68.**

Curcumin may be particularly important because it is a food additive readily available, non-toxic and is of a potential benefit for developing countries. It has also been tested for potential pharmaceutical uses and has been used for various purposes in traditional Indian, Chinese and Western herbal medicine.

Chapter - 5

Effect of Environmental factors on Lysosome membrane stability in *Oreochromis*

Introduction

Every species has its own peculiar and perhaps unique set of environmental requirements for survival. These requirements are not totally inflexible, for they may vary throughout the lifetime of the individual and the history of the species. The environmental factors which influence the growth, distribution, abundance, behaviour and ultimate survival of organisms are of two basic types - (1) Non living or abiotic environmental factors or abiotic environment; (2) Living or biotic environmental factors ***69**.

The abiotic environmental factors are of two basic types - (1) Physical abiotic factors - including temperature, light, pressure, geomagnetism, gravity etc., and (2) Chemical abiotic factors - including pH, salinity, nutrient availability, oxygen availability etc,. The environmental factors are not constant. They fluctuate temporally and geographically and the stresses on animals resulting from these environmental alterations affect the complex biotic structure of the biosphere. Probably, the first response of any organism to a change in environment is physiological.***70** Different abiotic environmental factors influence organisms physiologically in various ways ***71** Thus, the same abiotic environmental factor may produce different effects at different times and under different conditions. eg.Temperature may be lethal if extreme masking as when cold reduces the demand of cold-blooded organisms for food, directive by inducing a search for more favourable locations or controlling as a modifier of the rate of metabolism.

The idea that factors could be limiting at their maximum and minimum quantities was incorporated into the law of tolerance formulated by V.E. Shelford in 1913 ***72.** Each ecological factor to which an organism

responds has a maximum and minimum limiting effect between which lies a range or gradient called limits of tolerance.

The above facts from an ecological point of view has to be borne in mind before attempting to study the effect of environmental factors on the lysosome membrane stability in O*reochromis mossambicus*, which is euryhaline.

The use of lysosome stability has been proposed as providing a useful index of cellular condition and correlates significantly with physiological conditions. ***73** The lysosome is considered widely responsive to environmental stresses. The successful application of "Lysosome Enzyme Release Assay" techniques to samples from natural populations has already been described for *Mytilus edulis*. ***74** Variability in biochemical and physiological responses may be due to many causes, most significant being seasonal changes, both endogenous and exogenous, the multivariate nature of these responses (i.e., their sensitivity to many environmental stimulations).

Different responses will vary in their sensitivity to particular environmental variables. Most physiological effects have a low specificity in the context, providing instead a general measure of animal health. Injury resulting in the destabilization of lysosome membrane bears a quantitative relationship to the magnitude of the stress response. ***75** This presumably contributes to the intensity of catabolic or degradative effects as well as to the level of pathological change that results.

Various chemico-physical alterations in the environment were found to damage lysosomal integrity by different mechanisms causing comparable changes in the organization of the membrane. Lysosomal membranes showed increased fragility correlated with the physiological conditions of treated animals. ***76** Hyperthermia subsequently causes an increased

fragility of lysosomes by activation of lysosomal lipases and production of non esterified fatty acids or detergent phospholipids ***77** whereas several polluting cations induce swelling and possibly consequent burst by their accumulation inside the organelles ***78.**

The pattern of lysosomal response observed is essentially very generalized and can be induced by non chemical stressors such as hypoxia, hyperthermia, osmotic shock and dietary depletion ***79**

Thus, it would appear that many adverse conditions are capable of inducing autophagic type changes. This non-specificity of lysosomal reactions is therefore of value as a general indicator of deterioration in the health of the animal. ***80**

Natural physiological stresses affect the stability of membrane systems. The effects of a diverse range of environmental factors on permeability characteristics of membranes of intra cellular lysosomes (so called lysosomal latency) are well documented for a variety of marine vertebrates ***81** and invertebrate species ***82**.

Lysosomal membrane damage as a consequence of exposure to contaminants both in the form of environmental pollutant 'cocktails' or as individual compounds under experimental conditions is well documented in scientific literature. ***83** The most widely used test has been that for lysosome membrane fragility; this has been applied to both molluscan and fish species and is based on the demonstration of latency of lysosomal hydrolyses. Particularly ß hexosaminidases, ß glucuronidase and aryl sulphatase. More recently damage to lysosomes as a result of exposure to stress has been demonstrated invitro in fish hepatocyte ***84**.

Several studies have used biochemical techniques for quantitative evaluation of the effects of environmental stresses on aquatic organisms. ***85**

However free organisms can be subjected to numerous stresses simultaneously making the biochemical indicator of stress non-specific. ***86** The lysosome enzyme release assay is a sensitive indicator of numerous environmental stresses in molluscs. ***87**

A few studies have been conducted on aquatic vertebrates. ***88** It has been suggested that altering the cellular physiology resulting from exposure to toxic substances can modify the function of the lysosome. Chvapil etal,. Showed that lysosome membrane lability assay permits its use as a health-monitoring tool. ***89**

In this chapter, an attempt has been made to study liver lysosome membrane stability in *Oreochromis mossambicus* under the influence of physiological stress caused by variations in temperature and salinity.

a) **Effect of temperature on liver lysosome membrane stability in *Oreochromis***

Oreochromis mossambicus, mainly a brackish water species can tolerate temperatures as low as 10°C and as high as 40°C. However, breeding occurs only at 21°C. (Jingran).

Acclimatization of the fish *Tilapia zilli* to high temperature of 30°C leads to higher values of hematological parameters (viz., RBC count, WBC count, Hemoglobin concentration and hematocrit etc), while low temperature causes a depression of these values. ***90** Similar changes in blood parameters due to temperature have been observed in many other fishes such as *Heteropneustes fossilis* (Pande 1977). ***91**

In some animals a relationship between the total intake of food and resistance to thermal resistance has been demonstrated. Some species are more sensitive to elevated temperature, when they are starved even for short

periods of time. Gold fish showed increased resistance to high temperature when placed on a high fat diet. Starvation can also bring about change in lysosome membrane stability ***92**

Effect of short exposure to cold stress at an abrupt decrease of water temperature from 25°C to 10°C on the basic hematological parameters studied within 24-hour period. Role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. ***93**

In the present study, the change in stability of the lysosome membrane of *Oreochromis* exposed for 96 hours to three different temperature ranges (Room temperature; higher temperature of 40°C and lower temperature of 20°C) has been observed. The experimental setup at room temperature is maintained as control experiment.

Materials and Methods

Oreochromis mossambicus collected from a fresh water pond near Kumbalam and maintained in large aquarium tanks were used for the purpose. Fish of approximately 8cm were selected and introduced in to separate tubs maintained at three different temperatures - viz., room temperature, high temperature of 40°C and a lower temperature of 20°C for 96 hours. The tub maintained at room temperature was treated as control set up. The fishes were fed ad libitum and water was changed daily (Starvation can also bring about changes in lysosome membrane stability ***94**). At the end of 96 hours of exposure to high and low temperatures, the fishes were taken out and dissected to isolate lysosomes as described in earlier chapters to study the stability of lysosome membranes due to exposure to different temperature ranges.

The release of acid phosphatase enzyme from isolated lysosomes of *Oreochromis* maintained at different temperature were determined using the procedure of Anon 1963. ***43**

Results

The activity of acid phosphatase enzyme in the sub cellular fractions of the hepatic cells of *Oreochromis* revealed that the lysosome stability index expressed as ratio of lysosomal to soluble fraction in fishes maintained at 20°C was found to be nearer to that in fish maintained at 30°C(control). The lysosome membrane was found to be more stable at 20°C than at the temperature of 40°C.

The acid phosphatase activity in isolated lysosomes from the experimental groups above maintained at different temperatures show that the % of total lysosomal activity released into the supernatant at 15000 g at the end of incubation for 30 minutes at room temperature in the case of sample at 20°C was nearer to that in control group. The lysosome membrane lability index expressed as % of total lysosomal activity released into 15000g supernatant of fishes maintained at 20°C gave results almost equal to that of the control. The lower temperature of 20°C was found to have more stabilizing effect on liver lysosome in *Oreochromis* than on the liver lysosome in *Oreochromis* maintained at higher temperature of 40°C. The lability index of lysosomes was seen to increase with time of incubation. The lysosome lability index was high at the end of incubation for 30 minutes in the higher temperature group(40°C) than lysosome lability index of fish exposed to lower temperature(20°C).

The results were statistically analyzed using Two-way Anova and found to be highly significant.

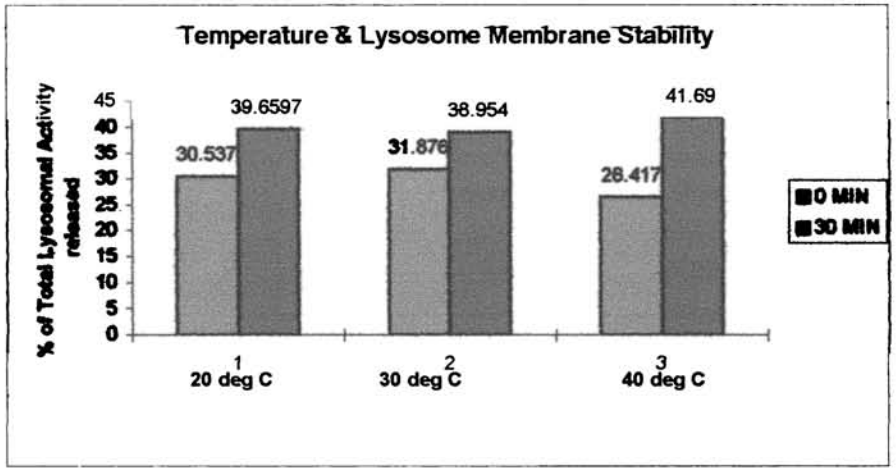
Sub cellular Activity of Acid Phosphatase Enzyme

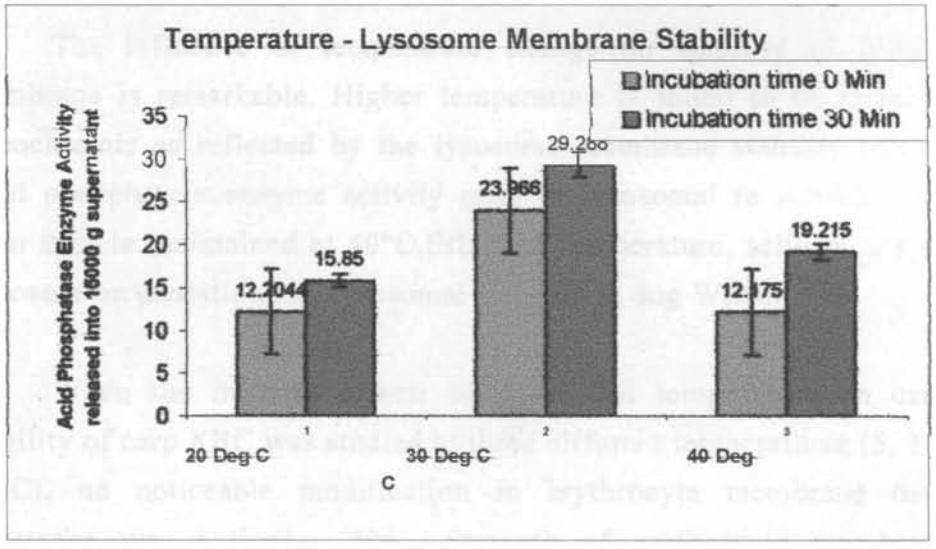
GROUP	Acid Phosphatase Enzyme Activity in Nuclear Fraction (mg pnp / hr/gm protein)	Acid Phosphatase Enzyme Activity in Lysosomal Fraction (mg pnp / hr/gm protein)	Acid Phosphatase Enzyme Activity in Soluble Fraction (mg pnp / hr/gm protein)	Ratio of Lysosomal to Soluble Fraction
Low Temp (20 Deg)	9.22 ± 0.0002	39.965 ± 0.113	47.475 ± 0.076	0.842
Control (30 deg)	39.5 ± 0.013	75.185 ± 0.015	72.05 ± 0.0148	1.044
High Temp (40 Deg)	26.68 ± 0.098	46.088 ± 0.001	91.1985 ± 0.020	0.505

ACID PHOSPHATASE ENZYME ACTIVITY IN ISOLATED LYSOSOMES RELEASED INTO 15000 g SUPERNATANT

Time of Incubation	Low Temperature (20 deg C) Acid Phosphatase Enzyme Activity *	% of Total Lysosomal Activity	Control (30 deg C) Acid Phosphatase Enzyme Activity *	% of Total Lysosomal Activity	High Temperature (40 deg C) Acid Phosphatase Enzyme Activity *	% of Total Lysosomal Activity
0 MIN	12.204±0.002	30.537	23.966 ± 0.016	31.876	12.175± 0.006	26.417
30 MIN	15.85± 0.005	39.659	29.288± 0.010	38.954	19.215± 0.011	41.69

* Acid Phosphatase Enzyme Activity in (mg pnp / hr/kg wet wt)





ANOVA TABLE (Diff. Temp)

Source	Sum of Squares	Degrees of freedom	Mean Square	Rounded to	F
Total	0.1752	13			
Temperature	0.1541	2	0.077	0.077	300.98***
Time	0.0185	1	0.0185	0.0185	72.314***
Error	0.0026	10	0.0003		

Means of Temperature		Means of time		Least Significant Difference for Temperature	Least Significant difference for time
Control	0.119	0	1.128	0.0226	0.0159
High Tem	0.029	30	1.717		
Low Temp	0.067				

*** $p < 0.001$

Discussion

The influence of temperature change on stability of lysosomal membrane is remarkable. Higher temperature is found to be stressful to Oreochromis as reflected by the lysosome membrane stability index,i.e., Acid phosphatase enzyme activity ratio in lysosomal to soluble fraction from sample maintained at 40°C.Effect of temperature, salinity and aerial exposure on predation and lysosomal stability in dog Whalk. ***95**

When the in vitro effects of gases and temperature on osmotic fragility of carp RBC was studied at three different temperatures (5, 11 and 20°C), no noticeable modification in erythrocyte membrane osmotic resistance was noticed. ***96** Strength of erythrocyte membrane is influenced by unsaturation index of the polar lipid component. Fatty acids in diet are particularly important for the structural integrity of cell membranes. This has been authenticated by examining osmotic fragility of erythrocytes in rainbow trout. ***97**

The effects of temperature and pressure upon hydrocarbon order and phase state are reviewed to indicate the magnitude of disturbances experienced by animals in their environments over seasonal and evolutionary time scale. ***98**

(b) Effect of salinity on liver lysosome membrane stability in *Oreochromis*

Introduction

Oreochromis mossambicus is widely accepted as a euryhaline fish, though basically a brackish water inhabitant. Tilapia is an introduced Cichlid (mouth-brooder) from Africa. It may be a hybrid but is probably close to *Tilapia mossambica* (Glen Black 1988). Fishes in saline waters must maintain the proper concentration of salts in the body fluid and prevent excessive loss of water (Parry 1966,Conte 1969). This requires various adaptive mechanisms and expenditure of energy since the osmotic concentration in fishes is less than that of seawater. Osmoregulatory mechanisms include drinking water and excretion or secretion of accumulating salts. Their mechanisms are aided by limited skin permeability of marine adapted fish.

Fish vary in their ability to osmoregulate in high salinity waters. The highest salinity at which living fish have been reported is the occurrence of *Cyprinodon Variegatus* at 142.4 0/00(Simpson and Gunter 1956). Some species can tolerate a wide range of salinities (euryhaline) by tolerating a certain degree of change in the body fluids or well-developed osmoregulatory mechanisms.

Thus, the optimum tolerance curve of the population plotted against environmental factor (salinity) is very broad.

Fish can acclimate to salinity changes through behavioural and physiological means. The time required for acclimation ranges from hours to days and varies by species. (Parry 1966). The upper limits are set by physiological constraints of the individual organism. Some fish which generally have a narrow tolerance range for salinity can be acclimated by

very gradual changes to salinity well outside the range. *Tilapia mossambica*, a fresh water species has been acclimated to salinity as high as 69 0/00 (Parry 1966).

Salinity is defined as the total amount of solid material in grams contained in 1kg of water when all the carbonate has been converted into oxide, bromine and iodine replaced by chlorine and all organic matter completely oxidized. All types of natural waters contain various amounts of different salts (ions) such as Sodium ions, Potassium ions, Magnesium ions, Chloride ions, Sulphate ions, Phosphate ions, Carbonate ions, Hydrogen Carbonate ions, Nitrate ions etc., and all these salts are responsible for the saltiness, salinity or salt content of water. The salinity of marine water is rather constant being about 3.5%. The salinity of fresh water varies greatly.

Salinity of water acts as an important limiting factor for the distribution of a number of species of plants and animals. Stability of lysosome membrane in different concentrations of NaCl and KCl was determined following the method of Philip and Kurup (1977, 1978) Rao & Sisodia (1986) with slight modification.

Oreochromis, though widely accepted as a euryhaline form, a sudden change of salinity is fatal to the fish. It is cultured in large scale in fresh water ponds and has a high survival rate. *Oreochromis* can tolerate salinity in the range of 0ppt-48ppt,though 5ppt-20ppt is optimum (Jingran).

In this experiment the change in stability of lysosome membrane in liver lysosome of *Oreochromis mossambicus* exposed to different salinity was determined following the procedure of Bhaskar Rao & Sisodia mentioned in previous chapters.

Materials and Methods

Oreochromis mossambicus, collected from fresh water ponds in Kumbalam and acclimatized in large aquarium tanks for over a month was used in the experiment. *Oreochromis* in the size range of 7 - 8 cms were used.

A series of large tubs containing water with salinities ranging from 0 ppt - 30 ppt (Parts per thousand) were prepared by dissolving appropriate quantities of common salt in 80 ltrs of tap water. Salinometer was used to ascertain the salinity of the medium.

Stability of lysosome membrane in different salinities was determined following the method of Philip and Kurup, Rao & Sisodia with slight modification.

Fishes in the 0 ppt tub was maintained as the control. The fishes were maintained in each salinity for 96 hours and at the end of it dissected and stability of lysosome membrane determined. The transfer of fishes from control to 10 ppt, 20 ppt, and 30 ppt were performed gradually by acclimating the fishes in intermediate salinity ranges in between transfers to prevent stress due to sudden introduction.

Lysosome membrane stability in isolated lysosomes from the liver of fishes maintained in different salinities were prepared following the method of Bhaskar Rao & Sisodia mentioned in previous chapters.

The total lysosomal activity was determined using 0.2% Brij - 35 solution prepared in citrate buffer. The acid phosphatase activity of nuclear and soluble fractions were also determined following the procedure of Anon 1963. ***43**

The protein value of these fractions were determined following the

Lowry's method of protein determination. ***44** The activity of acid phosphatase enzyme released in the different sub cellular fractions in medium treated with different salinities-0ppt to 30ppt was determined and comparisons made.

Results

The lysosome membrane stability index represented by the ratio of acid phosphatase activity in lysosomal to soluble fraction was found to decrease gradually from 10 ppt to 30 ppt, the maximum being in the control group (0 ppt) followed by group exposed to 10 ppt and minimum in group exposed to 30 ppt.

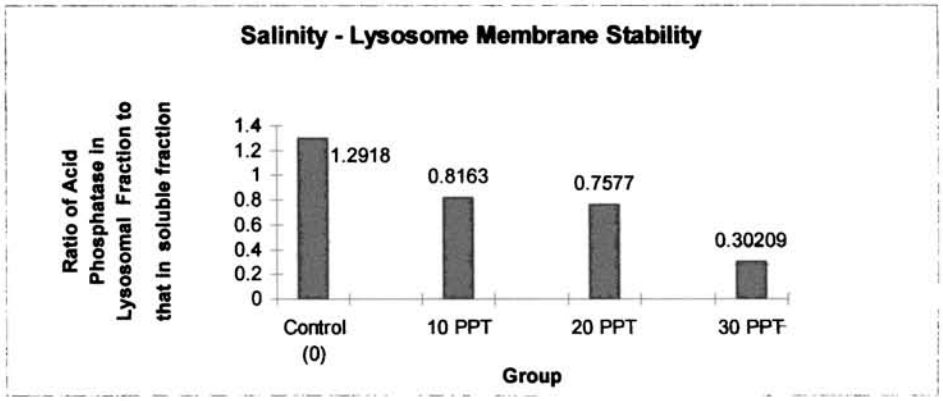
The percentage of total lysosomal activity of acid phosphatase enzyme released into 15000 g supernatant in fishes exposed to 20 ppt was found to be the lowest when compared to that in other groups. On the other hand the percentage of total lysosomal activity in fishes exposed to 30 ppt was found to be maximum.

Statistical analysis of the results using Two-way Anova revealed the effect of salinity to be significant. The activity at 30 minutes of incubation was found to be more significant than activity at 0 time of incubation.

STABILITY OF LYSOSOME MEMBRANE IN *OREOCHROMIS* ON EXPOSURE TO DIFFERENT SALINITIES FOR 96 HOURS

Sub cellular Activity of Acid Phosphatase Enzyme (mg pnp / hr/gm protein)

GROUP	Activity in Nuclear Fraction	Activity in Lysosomal Fraction	Activity in Soluble Fraction	Ratio of Lysosomal to Soluble Fraction
Control (0 ppt)	1.383 ± 0.006	5.821 ± 0.067	4.506 ± 0.003	1.292
10 ppt	1.921 ± 0.037	4.706 ± 0.004	5.764 ± 0.038	0.816
20 ppt	1.707 ± 0.078	3.188 ± 0.095	4.207 ± 0.106	0.758
30 ppt	2.445 ± 0.085	2.271 ± 0.250	7.516 ± 0.013	0.302

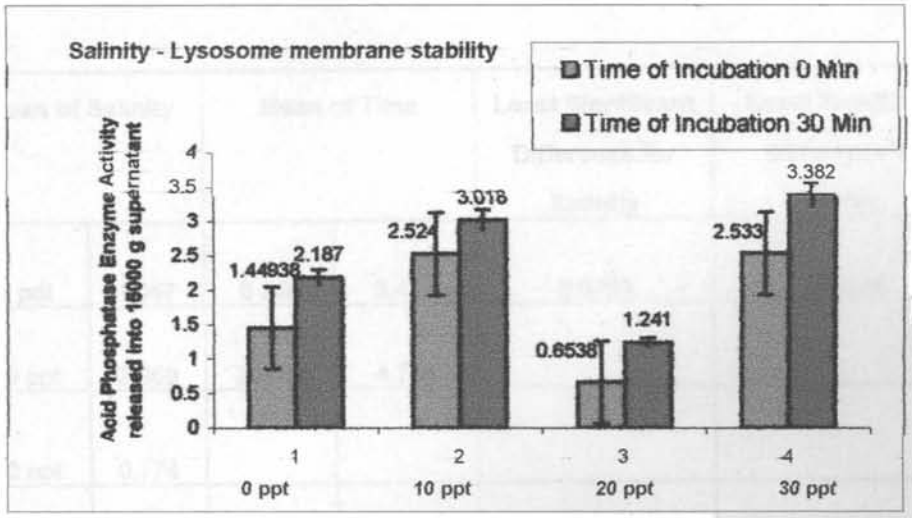


ACID PHOSPHATASE ENZYME ACTIVITY IN ISOLATED LYSOSOMES ON INCUBATION AT ROOM TEMPERATURE FOR 30 MINUTES

Time of Incubation	Control (0 ppt) Acid Phosphatase Enzyme Activity	% of Total Lysosomal Activity released	10 ppt Acid Phosphatase Enzyme Activity	% of Total Lysosomal Activity released
0 MIN	1.45 ± 0.016	24.89	2.52 ± 0.03	53.638
30 MIN	2.187± 0.0162	37.517	3.018 ± 0.136	64.136

20 ppt Acid Phosphatase Enzyme Activity	% of Total Lysosomal Activity released	30 ppt Acid Phosphatase Enzyme Activity	% of Total Lysosomal Activity released
0.654 ± 0.004	20.51	2.533 ± 0.235	111.556
1.241 ± 0.003	38.93	3.382 ± 0.309	148.94

* Activity of Acid Phosphatase enzyme released into 15000 g supernatant as mg pnp / hr/gm protein.



ANOVA TABLE (Diff. Salinity)

SOURCE	Sum of Square	Degree of Freedom	Mean Square	Rounded to	F
TOTAL	0.655	23			
SALINITY	0.543	3	0.1801	0.1809	228.975***
TIME	0.0970	1	0.0970	0.0969	122.653***
ERROR	0.0151	19	0.0008		

Mean of Salinity		Mean of Time		Least Significant Difference for Salinity	Least Significant Difference for Time
0 ppt	1.057	0 min	3.473	0.0263	0.0186
10 ppt	2.059	30 min	4.704		
20 ppt	0.774				
30 ppt	0.961				

*** $p < 0.001$

Discussion

Analyzing the acid phosphatase enzyme activity in the lysosome fraction and soluble fraction clearly shows that in the different sub cellular fractions the enzyme activity ratio of lysosomal to soluble fraction is gradually seen decreasing, the highest value obtained in the control group (0 ppt) followed by 10 ppt and 20 ppt and least in 30 ppt group. It indicates maximum lysosome stabilization on exposure to 10 ppt and minimum at the 30 ppt exposure on comparing with the value of the control (0 ppt). The experimental specimen was collected from fresh water ponds which might be the reason for maximum lysosomal stability at 0 ppt.

Acid phosphatase enzyme activity in the isolated lysosomes from different experimental groups above on incubation at room temperature for 30 minutes revealed that maximum lysosomal lability (% of total lysosomal activity released into 15000 g

supernatant) is noted in samples exposed to 30 ppt. The lysosome lability index in 20 ppt sample is seen to be nearer to lysosome lability index in control group. The lysosome lability index after 30 minutes of incubation is found to increase considerably than at 0 time of incubation.

Chapter 6

Effect of sub lethal dose of natural fish toxin – Mahua oil cake on lysosome membrane stability in *Oreochromis*

Introduction

The use of lysosomal membrane stability has previously been proposed as providing a useful index of cellular conditions which correlates significantly with physiological conditions ***99.** It is considered widely responsive to environmental stresses, while stimulation or induction of NADPH - neotetrazolium reductase is considered to be responsive to xenobiotics, which induce the microsomal detoxication system.. ***73**

Hyperthermia subsequently causes an increased fragility of lysosomes by activation of lysosomal lipases and production of non-esterified fatty acids or detergent phospholipids ***77,** where as several polluting cations induce swelling and possibly consequent burst by their accumulation inside the organelles. ***78**

Possible biochemical mechanism by which an organism might respond to changes in physical properties of its gill membranes as a result of contamination was studied by comparing the composition of fatty acids of *Gammarus duebeni* gills in relation to degree of contamination by lipophilic materials. The results indicate that at higher levels of gill contamination a greater proportion of polyunsaturated 20: 5 fatty acid occurs in the gill phospholipids. ***100**

Linked biochemical and cytochemical investigations have demonstrated that increased fragility of lysosomes induced by phenanthrene corresponds directly with increased catabolism of cytosolic proteins ***101.**

The lysosome enzyme release assay is a sensitive indicator of numerous environmental stresses in molluscs. However, only a few studies have been conducted on aquatic vertebrates. ***102**

The damage to lysosomes as a result of contaminant exposure has been demonstrated in vitro in fish hepatocytes using the capacity of lysosomes to take up and retain overtime, the cationic probe neutral red as an indicator of damage. ***103**

It has been suggested that altering the cellular physiology resulting from exposure to toxic substances can modify the function of the lysosomes. Chvapil et al,. showed that lysosome membrane lability assay permits its use as a health-monitoring tool. ***104**

Though organisms appear to be relatively tolerant to many metals and organic xeno biotics, it does not mean that the animals are unresponsive; in fact there is considerable evidence for pathological reactions to even low concentrations of contaminants. Such pathological reactions have been described at all levels of biological organisation ranging from the molecular level to the physiology of the whole animal. ***105**

At the cellular level, the lysosomal system has been identified as a particular target for the toxic effects of many contaminants. Pathological alterations in lysosomes have been especially useful in the identification of adverse environmental impact. Lysosomal reactions fall into essentially 3 categories: - changes in lysosomal contents, changes in fusion events and changes in membrane permeability (Hawkins 1970). The major response of lysosomes appears to involve enhanced autophagy and current evidence suggests that this is a generalized response to stress. These autophagic changes involve an increase in the volume of the lysosomal compartment together with frequent swelling of the lysosomes and increases in hydrolase activities. (Moore et al,. 1986, Lowe 1988, Moore 1988)

The characteristic pathological alterations in marine molluscs include swelling of digestive cell lysosomes, accumulation of

unsaturated neutral lipid in the lysosomes, increased fragility of the lysosome membrane and excessive build up of lipofuscin in lysosomal compartment. These changes are accompanied by atrophy of the digestive epithelium, apparently involving augmented autophagic processes, although there is also evidence of increased cell deletions (probably analogous to apoptosis in mammals) and the relation between the two processes if any is unclear (Lowe 1988). E.g., Do autophagic type changes predispose the cells to deletion? Exposure to a variety of contaminant effluents such as sewage, sludge, oil spillage, pulp mill waste and mixed waste from industry have all been found to increase the fragility of molluscan digestive cell lysosomes as well as fish hepatocyte lysosomes. ***106**

Lysosome membrane damage as a consequence of exposure to contaminants both in the form of environmental pollutant 'cock tails' or as individual compounds under experimental conditions is well documented in the scientific literature. ***107** Lysosomes are remarkable that they have the capacity to accumulate a wide range of toxicants, however this role can be potentially hazardous for the cells.

The presence of elevated levels of toxicants in lysosomes can result in the break down of their limiting membrane and leakage of the acid hydrolases and toxic contents into the cytosol resulting in cell injury or death.

The speed at which contaminant exposure will result in lysosomal damage depends on a complex set of factors which relate not only to the nature and concentration of the contaminant, but also the physiological state of the animals.***108** e.g., When mussels are spawning the test most widely used to assess damage in the lysosomes - the lysosomal latency test which determines the permeability of the lysosomal membrane to substrates is unreliable due to the effects of natural physiological stresses on the membrane system which may affect its stability.

The failure of lysosomal membranes to repair following chronic long term exposure is indicative of a disturbance to their structure and in all probability the associated Mg^{2+}ATP ase dependant proton pump (Holzman 1989) by a cytotoxic agent. On the basis of field studies ***84** and experimental studies the cause of the disturbance to lysosomal function is chronic petroleum hydrocarbon seepage or spillage. The failure of lysosomal membranes to recover following a period of depuration supports the observations of Regoli (1992) for metal contaminants and indicates that contaminant exposure is not transient and as such may have long term consequences for individuals and population of animals.

A reduction in the lysosomal latency reflects deleterious structural changes in the lysosomal membrane (Schneider et al,. 1994). Evidence of structural change has ranged from breaks and gaps in digestive cell lysosome membrane in nut clam, *Nucula sulcata* (Owen 1973) to a membrane composed of discontinuous overlaps in *Mytilus edulis* digestive cell lysosomes (Nott & Moore 1987) Whilst the precise description of alterations in structure may differ, the net result is a membrane that is fragile and more permeable to substrates.

In the present investigation, the liver lysosome membrane stability in *Oreochromis* retained in medium containing 25-ppm mahua oil cake for 48 hours was studied.

Mahua oil cake, a derivative from the plant *Bassia latifolia* is used extensively in aquaculture practices in India both as a fish toxicant and as an organic manure in fishponds after its toxic effects are completely diminished. Most information regarding the effects of toxicity of mahua oil cake have been obtained through studies on the mortality rate ***109**

The bark of *Bassia latifolia* is used as fish poison. ***110** The effect of a medicine dosed with 25-ppm mahua oil cake dosed medium on the lysosome membrane stability of *Oreochromis* was studied.

Materials and Methods

The study was carried out on juvenile males of *Oreochromis mossambicus* of average size 8 -10 cms and acclimated in large aquarium tanks for 2 months. The effect of mahua oil cake on *Oreochromis* was found to be fatal; therefore after repeated trials a sub lethal dose of 25-ppm mahua oil cake was selected as sub lethal concentration. 25-ppm mahua oil cake soaked overnight in water and made to a paste was applied to the water medium in the experimental tanks. A control experimental set up in which mahua oil cake was not dosed was run simultaneously to the experimental set up in duplicate where fishes were exposed to mahua oil cake (25 ppm) medium.

Oreochromis treated with mahua oil cake was seen to be severely affected. At the end of 48 hours of treatment, the live fishes, which were fully exhausted by the toxin, were used for the study. The lysosomes were isolated from the liver of such fishes as described in previous chapters. The lysosome membrane stability was ascertained using procedure described in chapter 3(a). The results were compared with that of a control experimental set up run simultaneously and in which mahua oil cake was not dosed.

Results

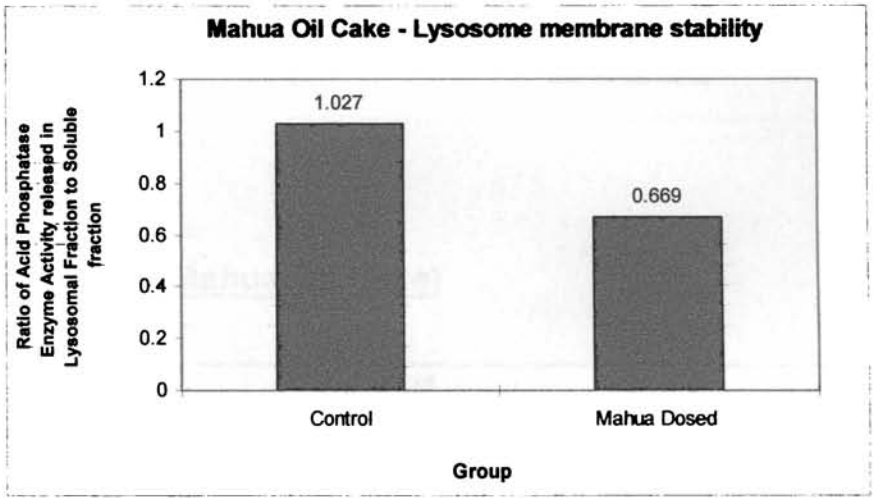
Mahua oil cake was observed to be highly toxic to fishes. Even in case of 25-ppm concentration, at the end of treatment for 48 hours fishes tend to become totally inactive and lose their balance.

The fishes in distinctly distressed conditions came to the surface frequently and ultimately lay at the bottom. Slime was found to ooze out from the mouth of dead fishes.

STABILITY OF LIVER LYSOSOME MEMBRANE IN *OREOCHROMIS* ON EXPOSURE TO 25 PPM NATURAL FISH TOXIN - MAHUA OIL CAKE

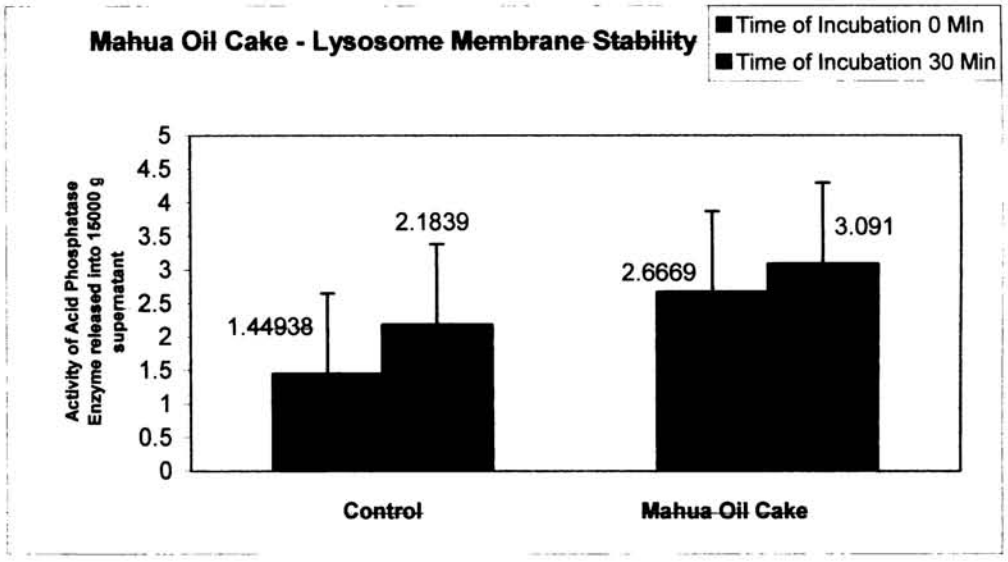
Sub cellular Activity of Acid Phosphatase Enzyme (mg pnp/hr/gm protein)

GROUP	Activity in Nuclear Fraction	Activity in Lysosomal Fraction	Activity in Soluble Fraction	Ratio of Lysosomal to Soluble Fraction
Control	2.645 ± 0.003	4.634 ± 0.083	4.512 ± 0.0014	1.027
Mahua Dosed (48 hrs)	3.342 ± 0.0014	3.523 ± 0.096	5.265 ± 0.131	0.669



Acid Phosphatase Enzyme Activity in isolated lysosomes on incubation at room temperature for 30 minutes

TIME OF INCUBATION	CONTROL		Mahua Dosed (48 hrs)	
	Activity of Acid Phosphatase Enzyme released into 15000 g supernatant (mg pnp / hr/gm protein)	% of total lysosomal activity released	Activity of Acid Phosphatase Enzyme released into 15000 g supernatant (mg pnp / hr/gm protein)	% of total lysosomal activity released
0 MIN	1.449 ± 0.044	31.277	2.667 ± 0.0922	75.699
30 MIN	2.184 ± 0.086	47.128	3.091 ± 0.092	87.73



ANOVA TABLE (Mahua Oil Cake)

Source	Sum of Square	Degrees of Freedom	Mean Square	Rounded to	F
TOTAL	0.050616	9			
CONTROL&TEST	0.0094	1	0.0094	0.0094	6.000593**
TIME	0.03025	1	0.03025	0.0302	19.2785**
ERROR	0.010966	7	0.001567		

	Mean of Control & Test	Time	Mean of Time	Least Significant Difference for Control & Test	Least Significant Difference for Time
Control	0.116	0	0.076	0.059335	0.0419563
Mahua	0.065	30	0.104		
20 ppt	0.774				
30 ppt	0.96				

** $p < 0.01$

	Mean of Control & Test	Time	Mean of Time	Least Significant Difference for Control & Test	Least Significant Difference for Time
Control	0.116	0	0.076	0.059335	0.0419563
Mahua	0.065	30	0.104		
20 ppt	0.774				
30 ppt	0.96				

** p< 0.01

Discussion

Mahua oil cake was found to be highly toxic to *Oreochromis*. 25 ppm was found to be a sub lethal dose.

The stability of lysosome membrane was found to decrease making it more labile.

The total lysosomal activity of acid phosphatase enzyme was found to decrease in *Oreochromis* exposed to mahua oil cake indicating decrease in lysosome content.

Evidence of strong hemolytic action of mahua oil cake on live fishes has been reported. ***111**

Saponin - one of the hemolytic components of mahua oil cake has a lethal effect on fishes and other organisms. Mahua oil cake contains 4 - 6% of saponin, a fish toxicant known to be hemolytic in reaction and enters the circulation through gills and buccal epithelium. The gradual shrinkage and ultimate hemolysis of RBC and subsequent alteration of blood values could be understood from this fact. The bark of *Bassia latifolia* is a fish poison. ***112**

The flowers of mahua contain sugar, cellulose, albuminous substances, ash, water, etc., Dried flowers contain 50 - 60% sugar, seeds, 50 - 55% fatty oil, fat, tanin, extractive matter, bitter principle probably saponin, albumen, gum, starch, mucilage and ash .

Chapter 7

Lysosome membrane stability as an index of freshness in fish on storage

Introduction

Fish is a highly perishable commodity. The fish dies within a few minutes after it is removed from water. As soon as fish dies, the cell proteins get coagulated bringing out rigor mortis, the sign of a perfectly fresh fish. Usually it lasts only for a short period of 4-5 hours under tropical conditions. Later the fish becomes limp and flabby producing changes in odour, flavour, colour and even in composition and the fish is said to be spoiled or stale.

The spoilage is the result of the whole series of complicated changes brought about in the dead fish tissue by its own enzymes, by bacteria and by chemical action. In addition to enzymic and bacterial changes, chemical changes involving oxygen from the air and the fat in the flesh produce rancid odours and flavours. The spoilage in fish is mainly due to autolysis catalysed by degradative enzymes released from lysosomes followed by bacterial degradation.

Autolytic spoilage of fish is brought about by the release of hydrolytic lysosomal enzymes. The two processes though independent of each other can take place only under optimum conditions of temperature and in presence of water. The stability of lysosomes depends on the integrity of its membrane structure.

As soon as fish dies, spoilage begins. Rigor mortis, the first change-taking place in flesh after death is an important sign of freshness and wholesomeness. It is caused by breaking down of glycogen of muscle cell into lactic acid leading to the water and protein of the cell setting into gel like consistency resulting in a firming of the flesh and the body becomes rigid when pressure is applied. Since rigor mortis is a sign of freshness, it is important to determine ways of prolonging this condition. The slower the onset, the longer the state will continue. The faster the onset, the sooner is its disappearance. * **113**

Factors like temperature of flesh, size and method of catch are factors directly related to rigor mortis (1) higher the temperature of air and water, sooner the onset of rigor mortis, faster the fish passes through towards deterioration (2) smaller the fish, more rapid the onset of rigor mortis. (3) Struggle of fish - any method causing fish to struggle hastens onset of rigor mortis. Spoilage changes are brought about by the enzymes of living fish, which become active after its death. Millions of bacteria and other microorganisms many of them potential spoilers are present in the surface slime on gills and in intestine of living fish. They do not harm living healthy fish because the natural resistance of a healthy fish keeps them at bay. It is believed that microorganisms enter through the gills and kidney along veins and arteries and directly through the skin and peritoneum lining the belly cavity. In addition to enzymic and bacterial changes, chemical changes involving oxygen from the air and the fat in the flesh produce rancid odours and flavours.

The initial steps of spoilage in the widest sense of the word are not entirely undesirable. The optimal taste and best quality will be reached only if the biochemical processes connected with rigor mortis are resolved. Any preservation prior to rigor mortis does not give satisfactory results.* **114**

Any preservation prior to rigor mortis does not give satisfactory results. The initial steps of spoilage are characterized by the appearance of substances partly essential to an optimal taste.

The activity of the lysosomal enzyme acid phosphatase in the pressed out muscle tissue fluid gives an indirect estimate of the effects of the freezing treatment on the membrane structure. ***115** During ice storage of up to 14 days, an increase was seen in the activity of acid phosphatase after 3 days on ice with a further marked increase after 14 days on varying the thawing conditions. Instead, the fastest thawing also resulted in the least effect on the membrane. Different steps during

thawing were shown to have various effects on the enzyme leakage. Fast tampering and short duration in the latent temperature zone resulted in the least membrane disintegration.

Autolysis is seen to be retarded under alkaline conditions. Apart from freezing, pickling and spicing are used for preservation of fish. Smearing setted dried slices of fish with paste of chillies, mustard, garlic and tamarind and further drying is followed by coastal people of Malabar.

The existing methods of assessment of fish freshness include sensory methods and biochemical methods like measurement of Tri methyl amine oxide, volatile oils etc.,

In this chapter, an attempt has been made to link the stability of lysosome membrane to freshness of fish stored under different temperature conditions. This has been done with an aim to develop lysosome membrane stability as an index of freshness in stored fish. To indicate degree of freshness, time - temperature indicators (TTI) have already been presented as tool for labelling products. Though lysosomes have been implicated in the autolytic damage of fish flesh, no studies have been made in this direction.

During the study, both liver and muscle tissues were used, but studies using liver tissue were found to be more suitable as a monitoring method.

The biochemical changes in lysosomes of fish tissues due to storage of fish tissue under three different temperature conditions for 24 hours, i.e., at room temperature, at freezer temperature of $^{-}$4°C and at deep freezer temperature of $^{-}$15°C were studied.

Materials and Methods

The experiment was carried out in the liver tissue of *Oreochromis Mozambicus*. *Oreochromis* of uniform size were selected from aquarium tanks and the liver dissected out causing least disturbance to the fish. The liver tissue dissected under uniform conditions were transferred into three different sterile containers. One set was maintained at room temperature, another in freezer (¯4°C) and the third in deep freezer (¯15°C). Equal weight of liver tissue under sterile conditions were used to isolate lysosomes and later to study the stability of membranes in them following the method of Bhaskar Rao and Sisodia (1986) ***42** as described in chapter 3. A set of animals were maintained as the control. The fresh liver tissue was dissected out and analysed as control along with the preserved specimens.

After retaining the tissue under different storage conditions for 24 hours, the stability of lysosomal membrane in them were determined by measuring the amount of acid phosphatase released following the procedure of Anon (1963) using the stability of lysosomes isolated from freshly dissected tissue as control.

Results

The acid phosphatase enzyme activity in the isolated lysosomes from animals maintained in different temperature conditions was observed at 0 minute and also after 30 minutes of incubation. The residual activity of the isolated lysosomes was calculated in each case to assess the change in stability of lysosome due to storage under different storage conditions. The change in activity of acid phosphatase enzyme released into the 15000 g supernatant in isolated lysosomes on being incubated at 30 degrees for 30 minutes was also calculated.

The residual activity of lysosomes in sample maintained at room temperature for 24 hours was very high, (greater than the value obtained in control, i.e., activity in fresh tissue) but percentage of total lysosomal activity was low.

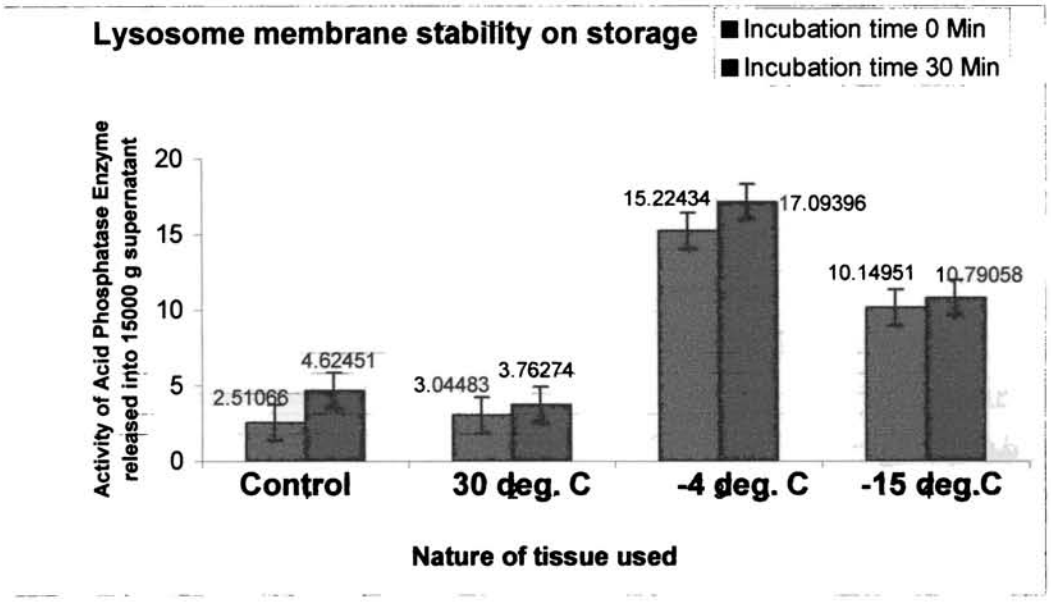
The residual activity of sample maintained in freezer (-4 °C) was least and the lysosomal activity due to incubation was higher than that at room temperature.

Due to storage in deep freezer (-15°C), the residual activity was high and nearer to that in control while the lysosomal activity due to incubation was least.

The results were statistically analyzed using two-way Anova and the results were found to be highly significant.

LYSOSOME MEMBRANE STABILITY AS AN INDEX OF FRESHNESS IN *OREOCHROMIS*

Nature of Tissue used	Time of of Incubation (Minute)	Activity of Acid Phosphatase Enzyme Released into 15000 g supernatant (mgpnp/hr/kg wet wt)	Residual Activity (Total Lysosomal Activity - 0 time activity)	Lysosomal Activity due to Incubation at 30 deg for 30 minutes	% of total lysosomal activity released in 30 Min
	(at Room temperature)				
Control (Fresh Liver Tissue)	0	2.511 ± 0.0024			
	30	4.625 ± 0.0017	6.46	2.11	32.71%
Total Lysosomal Activity		8.97 ± 0.0058			
Tissue preserved at room temp for 24 hrs (30 deg C)	0	3.045 ± 0.001			
	30	3.76 ± 0.0008	1.60258	0.71791	44.79%
Total Lysosomal Activity		4.645 ± 0.001			
Tissue preserved in freezer for 24 hrs (- 04 deg C)	0	15.224 ± 0.001			
	30	17.09 ± 0.008	4.00638	1.86962	46.66%
Total Lysosomal Activity		19.231 ± 0.006			
Tissue stored in Deep Freezer for 24 hrs (- 15 deg C)	0	10.150 ± 0.002			
	30	10.791 ± 0.002	7.69226	0.64107	8.33%
Total Lysosomal Activity		17.842 ± 0.002			



ANOVA TABLE (Room Temp, Freezer, Deep freezer)

SOURCE	Sum of Squares	Degrees of Freedom	Mean Square	Rounded to	F
TOTAL	1.004	23			
Activity	0.9863	3	0.3288	0.3288	1207.6***
TIME	0.0126	1	0.0126	0.0126	46.278***
ERROR	0.0052	19	0.0003		

Means of Activity		Means of time of Incubation		Least Significant Difference for Activity	Least Significant Difference for Time of Incubation
Control	0.041	0 Min	2.506	0.0208	0.0104
Rom temp	0.041	30 Min	2.983		
Freezer	0.187				
Deep freezer	0.119				

*** $p < 0.001$

Discussions

No definite pattern could be identified in the results of change in stability of lysosomal membrane due to storage at different temperature conditions. In the experiment, only the property of lysosome membrane was studied in relation to storage at different temperature conditions. The change in stability of lysosome membrane due to storage in deep freezer was low and near to that obtained in case of fresh tissue. In the case of tissue stored in freezer,the change in stability was found to be least. Storage of fish tissue in freezer and deep freezer temperature were found to be more effective than storage at room temperature for retaining the stability of lysosome membrane.

Lysosome membrane stability may be measured as the amount of acid phosphatase enzyme retained in the lysosomal fraction. The ratio of acid phosphatase enzyme activity in lysosomal fraction to that in soluble fraction used to measure the lysosome membrane stability may be developed as an index of fish freshness too.

Methods for evaluation of freshness in fish should be rapid, reliable and cheap for routine use. The experiments may be extended to evolve a model to predict the time after harvest or the remaining shelf life of an unknown sample by an evaluation of the stability of isolated lysosomes.

The stability of lysosome membrane, if successfully developed as a sensible and reliable index of fish freshness, the lysosome membrane stabilizers identified could be put to use as food additives to increase shelf life of fish tissue.

As mentioned earlier, the optimal taste and best quality will be reached only if the biochemical processes connected with rigor mortis are resolved. Therefore, any preservation prior to rigor mortis does not give satisfactory results. A thorough knowledge of degradation products of amino acids and the

processes governing their appearance is important both to an optimal taste and correct handling of the fresh fish. Thus the possibility of working out through research entirely new taste patterns emanating from fish seems to be an intriguing future objective.

Chapter – 8

Summary and Conclusions

Summary and Conclusions

Biological membranes forming closed boundaries between compartments of varying composition consist mainly of proteins and lipids. They are asymmetric, fluid structures that are thermodynamically stable and metabolically active. Normal cellular function begins with normal membrane structure and any variation in it may upset the normal functions. Biological membranes, though diverse in structure and function, share a number of common attributes. Different membranes within the cell and between cells differ in composition and hence in their functions. The degree of fluidity of a membrane depends on the chain length of its lipids and degree of unsaturation of constituent fatty acids.

Broad phase transitions are a general characteristic of cellular membranes due to heterogeneity of lipids in biological membranes and decreased mobility of lipids due to the presence of integral membrane proteins. Divalent cations like Ca^{2+} and Mg^{2+} are well known stabilizers of biological membranes and their removal often leads to lysis of cells. Temperature, ionic environments and fatty acid compositions of phospholipids and glycolipids and presence or absence of cholesterol can effect the general physical state of biological membrane. Presently many membrane stabilizers (enfenamic acid, phenyl butazone etc.,) and destabilizers (Vitamin A, bile salts etc.,) have been identified.

In response to environmental changes, many cells can regulate composition of their membranes to maintain the overall semifluid environment necessary for many membrane associated functions. The assembly and maintenance of membrane structures in cells is a dynamic process. The components are not only synthesized and inserted into a growing membrane but are also continuously degraded at a slower rate. This turnover process varies with each individual molecule.

Despite phylogenic differences, a unifying factor of all cells is that they contain many identical chemical constituents, metabolic pathways and mechanisms of cell recognition. This allows for a mode of biochemical deduction based on extrapolation of results in one species to another.

With all the above information in the background, it has been attempted in this project to study the various aspects of stability of erythrocyte and lysosome membrane biochemically. The statistical significance of the results also has been determined.

Erythrocytes were chosen for the purpose because of their ready availability and relative simplicity. Erythrocytes have been proved to be a model system to study the effect of toxic substances on its membrane by measuring hemoglobin leakage. The relative stability of erythrocyte membranes under the influence of physiological concentration of selected biochemicals, in vitro was assessed in three different species – (1) a fish *Oreochromis mossambicus*), (2) a bird (*Gallus domesticus*) and (3) a mammal (*Oryctolagus cuniculus*).

10^{-3} M concentration of sodium acetate, cysteine and ornithine were found to stabilize erythrocyte membrane in *Oreochromis, Gallus* and *Oryctolagus* while Dopa was found to destabilize the erythrocyte membrane.

The effect of a series of different concentrations of the erythrocyte membrane stabilizers identified 1(0^{-5} M to10^{-1} M) were again screened for their erythrocyte membrane stabilizing action. All concentrations of sodium acetate were found to stabilize erythrocyte membrane in *Oreochromis, Gallus, Oryctolagus*. The lower concentration of glycine was found to destabilize the (10^{-4} M and 10^{-5} M) erythrocyte membrane in *Oreochromis* while higher concentration (10^{-1} M and 10^{-2} M) was found to stabilize the erythrocyte membrane. The action of glycine was found to be biphasic in *Oreochromis*. Study of erythrocyte membrane stabilization is simple, rapid

though non-specific and is useful as a preliminary screening test for potential erythrocyte membrane stabilizers and potential anti-inflammatory drugs.

Lysosomes being indispensable for the catabolic processes occurring in the cell, a detailed study on various aspects of stability of the lysosome membrane has been carried out on isolated lysosomes of *Oreochromis mossambicus*. Lysosomes contain hydrolytic enzymes which remain latent and are stable under normal conditions. In certain pathological conditions, and also after death of a cell, the lysosomes rupture releasing the hydrolytic enzymes. This property has value as a built-in mechanism for self removal of dead cells. When the membrane of a lysosome is destabilized by chemical action, resident enzymes are released. The effect of chemicals on lysosome membrane thus can be evaluated by measuring the activity of released enzymes.

The lysosome enzyme release assay technique was employed to study the stability of lysosome membrane under the influence of selected biochemicals. The activity of acid phosphatase enzyme (a marker enzyme of lysosomes) released was measured colorimetrically to estimate the extent of damage to lysosome membrane and correlated with stability of the lysosome membrane. The specific activity of the enzyme and lysosome lability index was also calculated.

Preliminary screening of selected biochemicals and natural products were carried out to identify the lysosome membrane stabilizers and destabilizers. A series of different concentrations of the lysosome membrane stabilizers identified were further studied to reveal whether they possessed biphasic property. Lower concentrations (10^{-4} M and 10^{-3} M) of glycine and sodium pyruvate were observed tostabilize the lysosome membrane in *Oreochromis* , while higher concentrations (10^{-2} M and 10^{-1} M) were observed to labilize the lysosome membrane.

Drugs or biochemicals by stabilizing the lysosome membrane can prevent the rupture of lysosomes and inhibit the release of lysosomal enzymes. Most of the steroidal and non-steroidal anti-inflammatory drugs stabilize lysosome membranes in vitro. The stabilization of lysosome membrane with the help of stabilizing agents has got far reaching implications in clinical pathology. Membrane destabilizers could be utilized to get rid of undesirous cells like cancerous cells from the body.

The identification of lysosome membrane stabilizers of natural origin has a great potential in development of drugs. In the project, 1% crude extracts of selected spices in boiling water was used to assess their action on the liver lysosome membrane of *Oreochromis mossambicus* in vitro. 1% extract of turmeric and cumin seed were observed to exert a slight stabilizing effect on the lysosome membrane of *Oreochromis* in vitro while the same concentration of ginger and mustard did not have any effect on the *Oreochromis* lysosome membrane. 1% extract of malabar tarmarind, red chilly, garlic, coriander and fenugreek were observed to slightly reduce the stability of lysosome membrane in- vitro in *Oreochromis*. The observed results on the effect of extracts of spices on lysosomal membranes were statistically significant.

Spices being food additives are readily available, relatively cheap and non-toxic. The potential benefit of utilization of such spices (possessing lysosome membrane stabilizing action) as drugs in developing countries like India cannot be ruled out. Besides this, they have already been used for various purposes in traditional Indian, Chinese and Western herbal medicine.

The lysosomes were found to be responsive to environmental stresses. An attempt has been made to study the liver lysosome membrane stability in *Oreochromis mossambicus* under the influence of physiological stress caused due to variations in temperature and salinity. The lysosome membrane stability has been proved to be a useful index of cellular condition and correlates significantly with physiological conditions of the organism. Temperature and

salinity are natural physiological stresses that can affect lysosome membrane stability. The change in stability of lysosome membrane in *Oreochromis* on exposure to different temperatures and salinity ranges has been assessed using the lysosome enzyme release assay method. This technique was observed to be a sensitive indicator of environmental stresses in *Oreochromis*. Though *Oreochromis* was found to tolerate temperature as high as 40°C, the temperature of 20 °C was found have more stabilizing effect on the hepatic lysosomal membrane.

Oreochromis is widely accepted as a euryhaline species, and can tolerate salinity in the range 0 ppt. – 48 ppt. The results of the study in the subcellular Acid phosphatase enzyme activity indicates that exposure to 30 ppt. Salinity exerted maximum stabilizing effect on lysosome membrane followed by 20 ppt.

The stability of liver lysosome membrane of *Oreochomis* exposed to medium containing 25 ppm mahua oil cake for 48 hours was studied. The sub-lethal concentration of (25 ppm) mahua oil cake was found to have strong membrane labilizing action on the liver lysosome membrane in *Oreochromis*.

An endeavour in the direction of developing lysosome membrane stability as an index of fish freshness during storage has been undertaken, since spoilage in fish is mainly attributed to autolysis, catalyzed by degradative enzymes released from lysosomes followed by bacterial degradation, an attempt has been made to correlate lysosome membrane stability and fish freshness under different storage temperatures.

Lysosome membrane stability may be developed as a sensible and reliable index of fish freshness. The natural lysosome membrane stabilizers identified could be put to use as food additives to increase shelf-life of fish tissue. This can be utilized in combination with other factors like low temperature, etc., for the efficient preservation of fish in fish processing.

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*109 Lakshmanan 1983, Nath 1979). According to Lakshmanan 1983, susceptability of fishes differed with species of fish (Catla, Common Carp, Tilapia and Murrel were more susceptible to the cake than Rohu, Mugil, Magur and Singhi etc.,)

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