MATERIALS AND METHODS

SELECTION OF SPECIMENS

Animals representing different classes of vertebrates were selected for investigation with a view to trace, if possible, the evolution of alimentary canal in the vertebrate series. Emphasis was given on the study of modification of gastro intestinal tract and an attempt was made to correlate with feeding habits of the animal concerned. Availability of specimens round the year is a very important consideration in all investigations and the selection had necessarily to be restricted to the animals belonging to different groups of vertebrates, most common in and around Calcutta.

Healthy and mature specimens only were selected for study. For morphological and experimental studies live fishes were dissected. The other specimens, toad and glass lizard were anaesthetised by plugging the external narial apertures with cotton wool soaked in chloroform.

Systematic position of the specimens under investigation

Name of the specimen	Family	Order	Class	Feeding habits
Cyprinus carpio Linn. (American carp)	Cyprinidae	Cypriniformis	Actinopterygii (Teleostomi)	Omnivore
Ophicephalus punctatus Bloch.	Ophiocephalidae	Ophiocephaliformes	"	Carnivore

Name of the specimen	Family	Order	Class	Feeding habits
Bufo melanostictus Schneider (Indian toad)	Bufonidae	Anura	Amphibia	Carnivore
Mabuia carinata Schn. (Glass lizard)	Scincidae	Squamata	Reptilia	"

Preservation and dissection

For routine dissection to study the anatomy of the digestive system, either freshly killed animals or preserved specimens were used. For preservation, the abdomen was cut open and the specimens submerged in 6% formaldehyde solution for about a week or more. Such preservation facilitated the study of the different digestive glands and their ducts.

Fixation of tissues for histological studies

Tissues for histological preparation were collected from live fish and freshly killed specimens. Bouin's fluid was the fixative of choice, though Zenker's solution was occasionally used.

Sections were cut 5-7 microns thick and double stained with Delafield's Haematoxylin and eosin.

Sources of enzyme

a. Oesophagus

b. Stomach

Sources of enzyme

c. Intestinal bulb (C. carpio)

d. Duodenum

e. Anterior intestine
f. Posterior intestine
) (C. carpio and O. punctatus)

g. Intestine

h. Hepatopancreas (C. carpio and O. punctatus)

i. Liver

j. Pancreas

h. Bile

Determination of gut pH

Specimens were sacrificed one hour after feeding, and different zones of the gastrointestinal tract of freshly killed animals were opened. The gut contents of each zone were taken out by a spatula and the fluid there was squeezed out through a fine mesh silk cloth and collected in vials. About 0.5 ml fluid could be collected from each zone of the gastrointestinal tract. The pH of the fluid was read with the help of a pH meter.

Reagents used were of analytical grade. To minimise experimental error, an experiment with a particular enzyme collected from one animal was repeated at least for three times. Three such sets were prepared for each species. Therefore, the result expressed is an average of nine experiments.

Technique for preparation of enzymes

Measured quantity of tissue from different zones of the gastrointestinal tract and digestive glands of live fishes and anaesthetised toad and glass lizard were used at 20°C in an air conditioned room for enzymatic studies.

Prior to the collection of tissues for extraction of enzyme, microhomogenisers with measured quantity of neutral glass powder were kept submerged in freezing mixture in large glass beakers for a few hours.

Small measured pieces of oesophagus, stomach, duodenum, intestinal bulb, anterior intestine, posterior intestine, intestine, rectum, liver, pancreas and hepatopancreas were collected aseptically and as quickly as possible and put in chilled homogeniser. Equal amount of materials were collected from the same sources, water removed as far as practicable with the help of a filter paper, weighed in a torsion balance and the weight recorded.

The tissues were homogenised at subzero temperature for about 3 minutes in a mechanical homogeniser at a speed of about 1,000 rpm in which most of the cells were broken down and the major bulk of the enzymes there was released. The enzyme was extracted with measured quantity of buffer at appropriate pH, or double glass distilled water at neutral or desired pH for two hours at 4°C. The tissue

debris were centrifuged off at 5000 rpm and the slightly. translucent supernatant was either used immediately or the enzyme there was precipitated for a few minutes with chilled acetone at 60% concentration at subzero temperature. The precipitated crude enzyme was separated by centrifuging the mixture for 4-5 minutes at 5000 rpm in a refrigerated centrifuge. The supernatant rejected and the solid was dried in a vacuum desiccator and stored at subzero temperature for future use. In most of the experiments freshly extracted enzyme solution was used. In experiments with stored enzyme, vol./vol. enzyme solution was prepared at 4°C. The amount of buffer taken to dissolve the dried enzyme was equal to the volume of distilled water or buffer used in the extraction of enzyme from homogenised tissue. For bile the gall bladder was carefully removed and a puncture was made in it to collect the bile in a sterile vial.

SUBSTRATES

Amylopectin :

Amylopectin is a polysaccharide, which on hydrolysis for a short period is converted mainly to maltose. Being water soluble and permitting enzymatic reaction in a wide range of pH and at the same time estimation of both α and β amylases being possible the substrate is considered as an ideal one.

Bovine albumin :

A water soluble substrate permits enzymatic reaction in a wider range of pH. Bovine albumin both for its 99% purity and water solubility was considered as the ideal protein substrate and used.

Phenyl benzoate :

It is an ester. It is prepared with the action of Benzoyl chloride and phenol. On esteolytic action phenol is produced as one of the end products. Phenol can be correctly determined due to its high molar efficiency,

i.e. $\frac{\text{molar concentration of phenol}}{\text{molar concentration of unknown}}$ giving same colorimetric readings.

ANALYTICAL PROCEDURES

Amylase (Noelting and Bernfeld, 1948).

Reagents used

1. 1% Potato amylopectin solution.
2. 3,5-dinitrosalycilic acid.
3. 2N NaOH solution.
4. Rochelle salt.
5. 0.02 M phosphate buffer.
6. Enzyme solution.

Procedure

The assay method is based on the increase in reducing power and is applicable for both α and β amylases. One ml enzyme solution extracted in phosphate buffer of appropriate pH and 1 ml of the substrate solution (amylopectin) were mixed.

One drop of toluene was added to prevent microbial contamination and the mixture was incubated for 10 minutes at 37°C. Toluene slightly inhibits enzymic activity of amylase which was ignored. The enzyme reaction was interrupted by adding 2 ml of dinitrosalicylic acid reagent. The tube containing this mixture was heated for 5 minutes in boiling water bath and then cooled in running tap water. After addition of 20 ml distilled water the optical density of the solution containing the brown reduction product was determined photometrically, by means of green filter (520 m μ). A blank was prepared in the same manner without enzyme. A calibration curve established with maltose (0.2 to 2 mg in 2 ml of water) was used to convert the colorimeter readings into mg of maltose.

Proteinase (Hunt, 1948)

Reagents used

1. Trichloroacetic acid, 0.35 N solution. This is approximately 6% W/V standardized to 0.35 N.
2. Sodium hydroxide 0.25 N.

3. Hydrochloric acid pH 2.1
4. Folin and Ciocalteu reagent.
5. Standard phenol solution 5 mg/100 ml solution.
6. Bovine albumin solution. 5.6 grams of bovine albumin was dissolved in 100 ml appropriate buffer. The solution was stirred and filtered through cotton wool.
7. Enzyme solution.

Procedure

Estimation of proteinase activity was based on the measurement of total protein hydrolysed. Four tubes were taken. 5 ml of bovine albumin solution was put in each tube (1 + 2). 10 ml of trichloroacetic acid solution was put in each tube (3 + 4). 1 ml of enzyme solution was added to each tube (1 + 4). One drop of toluene was added to each to prevent microbial contamination and the tubes were incubated for 15 minutes at 37°C in a water bath.

Content of tube (1) was mixed with that of tube (3) and the content of tube (2) with that of tube (4) by turning upside down several times.

The tubes were left in the water bath for about 4 minutes. Tubes (1 + 3) were test and tubes (2 + 4) were control. The materials were filtered.

15 to 30 minutes after the beginning of filtration, 2 ml of the filtrate was pipetted into 50 ml stoppered cylinder containing 20 ml of 0.25 N sodium hydroxide, and mixed by gentle rotation.

1 ml of Folin and Ciocalteu reagent was added and mixed by gentle rotation again. In 30 minutes the colour was read using a red filter or transmission at 680 m μ .

The difference between the test and the control gives a measure of the proteolytic activity. As standard, 2 ml of a freshly prepared phenol solution, containing 5 mg/ 100 ml was mixed with 20 ml 0.25 N, NaOH, and immediately 1 ml of Folin and Ciocaltau reagent was added. Reading in a colorimetre was taken after 5 - 10 minutes. A blank omitting the phenol was put up. The enzyme extract contains 50 units of protelytic activity per ml when the colour produced in the above estimation equals that of the standard.

Esterase (Gomori, 1949)

Reagents used

1. 2% Phenyl benzoate (Fluka) solution.
2. Sodium phosphate buffer (0.1 M pH 6.3).
3. Saturated borax solution in 15% alcohol.
4. 0.25% Diazo reagent - Fast Red salt (Fluka) solution.
5. Enzyme solution.

Procedure

1 ml of enzyme solution was added to 5 ml of buffered substrate, previously warmed to 37°C. The mixture, together with the blank consisting of 5 ml of the buffered substrate without enzyme solution, was incubated for one hour at 37°C. At the end of incubation the two tubes were placed in ice cold water and 1 ml of the enzyme solution was added to the blank. Then 4 ml of the borax solution was added, followed by 0.5 ml of Diazo reagent, with mixing after each addition and the orange-red colour measured after 30 minutes, using 490 mμ, blue green filter. A calibration graph was made by treating 5 ml samples of dilute phenol solution with 1 ml of water, 4 ml of borax solution and 0.5 ml of Diazo reagent. Results are expressed as micromoles of esteric linkages split in one hour by 1 ml of enzyme solution.