CHAPTER V

THE ACTION OF ACETYLCHOLINE AND SEROTONIN ON THE ISOLATED INTESTINE OF FISH

Pharmacological analysis of drug action on the gastrointestinal motility had led to the study of the action of those substances in a comparative basis when it became evident that a particular amine might cause either a contraction or a relaxation depending upon the specific anatomical location of smooth muscle and upon the species of animal from which it came. The action of acetylcholine and serotonin have been extensively studied in the mammals but few investigations have been carried out on fish. von Eular and Östlund (1957) studied the action of certain biologically occurring substances on the isolated intestine of fish and interesting differences on the responses of elasmobranch and teleost to such substances were reported. Gaddum and Szerb (1961) described a technique for biological assay of substance P and acetylcholine with gold fish intestine in a microbath and reported a remarkable sensitivity of fish intestine towards acetylcholine and related substances.

This provoked us to study the action of acetylcholine and serotonin on the isolated intestine of fresh water teleost fish commonly available in our locality. Curiously enough, the isolated pieces of intestine of Ophicephalus punctatus in a small perfusion bath contract vigorously in presence of small amount of acetylcholine and serotonin. Therefore an attempt has been made in this section to study the action of these substances on the isolated intestine of the particular species.

Materials and Methods :

Specimens of Ophicephalus punctatus weighing about 70 to 100 gms were kept in oxygenated fresh pond water in laboratory aquarium. Different parts of the intestine were isolated from the decapitated fish. Small strip of unstreched intestine of

1 cm in length was suspended in a 2 ml aerated fish Ringer solution at room temperature.

Perfusion Bath : Perfusion bath consisted of a glass tube (5 cm x 1 cm) open at both ends and the bottom of which was connected with a rubber tube to an over-head reservoir, through which the bath solution ran continuously at a rate of about 1 ml /minute. Drugs were applied by stopping the flow by clamping of the rubber tube when the volume of the solution contained in the bath remained constant at about 2 ml even after the addition of the drug. The solution of the bath was continuously aerated through sintered glass covering at the bottom. Drugs were added to the bath in 0.1 ml volume with the microsyringe and left in contact with the intestine for a constant time (2 min); then the flow of Ringer solution was restarted.

Isolated Fish Intestine : Isolated piece of intestine was suspended in the bath under unstreched condition in such a way so that one end of the intestine was attached to a fine nylon thread connected with the simple isotonic lever while a second thread attached to the other end of the intestine was tied to a very small hook at the bottom of the bath and fixed with plasticin.

Recording apparatus : The contractions of the intestine either spontaneous or induced by drugs were recorded through the isotonic lever made up of glass tube having 10 times magnification. Tension of the muscle was adjusted by putting plasticin on one arm of the lever and it was never more than 0.5 gm. If the tension was too low relaxation was poor and the tone continued to increase. Contractions were recorded on a slow moving kymograph at the speed of .25 mm/sec.

Composition of Fish Ringer : (Lockwood, 1961)

NaCl	- 126 mM	$NaHCO_3$	- 3 mM
KCl	- 4 mM	$Na_2HPO_4.12H_2O$	-18 mM.
$CaCl_2$	- 2 mM	$NaH_2PO_4.2H_2O$	- 2 mM
$MgSO_4$	- 2 mM		

The pH of the solution was maintained at 7.6

Drugs used : Acetylcholine Iodide -(Sigma Chemical Co.).
Atropine sulphate -(McFarlan Smith Ltd).
Serotonin-Creatinine sulphate -(Sigma Chemical Co).
Morphine sulphate -(Dey's Medical Stores Mfs. Pvt.Ltd).
Methysergide bimaleate - (Sandoz, A.G., Nurenberg)
Physostigmine salicylate-(C.H. Boehringer Sohn.In Gelheimam Rhein).

All drugs were prepared in molarity basis either with fish Ringer or distilled water and freshly prepared dilutions were used in each case.

Acetylcholine Iodide was dissolved in acetate buffer at pH 4.6 in concentration of 10^{-3}gm/ml, and from this stock solution different graded doses were prepared with fish Ringer solution. Serotonin creatinine sulphate was dissolved in N/10 HCl at 10^{-3}gm/ml concentration and different dilutions were made from this with Ringer solution.

The effect of acetylcholine was also tested with the isolated perfused fish gut after cooling the tissue for 24-48 hours at 2°C in fish Ringer solution.

Results :

Isolated fish gut in normal Ringer solution within the bath was found to contract spontaneously rhythmically for sufficient length of time though the basal tone in each case tended to vary from time to time. Although different parts of the same intestine contracted vigorously, the portion of the intestine near the stomach showed appreciably better contraction. The height of the contraction could be inhibited by increasing the tension of the lever from 0.5 to 1 gm or more, in that case the basal tone after relaxation had also decreased. The spontaneous contraction of the isolated intestine could be inhibited by changing the composition of the fluid while tone remained at the same state or the initial response of the intestine towards repeated acetylcholine was abolished. In order to avoid this, attempt was made to study the response of repeated doses of acetylcholine on the intestinal preparation while immersed in

Ringer solution containing lowered concentration of calcium. Hypocalcium Ringer solution was found suitable to reduce or inhibit the spontaneous rhythmic contraction of the intestine while the response towards lowered doses of acetylcholine was not greately affected, rather it was slightly potentiated. In hypodynamic perfusing solution, i.e. when the calcium concentration in the perfusing solution was reduced to half (1 mM) keeping the composition of the other constituents same, the isolated intestine preparation showed no spontaneous contraction (Fig 23) Addition of low doses of acetylcholine produced immediate contraction followed by full relaxation and full recovery took place within 2-3 minutes without significant alteration of the basal tone. Therefore, in subsequent studies with acetylcholine or serotonin, hypodynamic fish Ringer solution was used as a perfusing medium.

Since with each administration of drug, the entire bath fluid had to be replaced the Ringer fluid within the reservoir was also modified by reducing the calcium content. The pH of bath fluid remained at 7.6. The small increase in pH (8.2) caused contraction of the muscle and slight decrease in pH (6.2) relaxed it. Slight change in temperature in between 20-25°C did not have much difference in response while the effect of anoxia was profound. However, the effect of changes in temperature. pH and anoxia have also been studied in detail and reported in other section.

Action of acetylcholine :

Small doses of acetylcholine starting from 1×10^{-10} gm/ml to 1×10^{-6} gm/ml (in 0. 1 ml volume per 2 ml bathing fluid) caused immediate contraction in all intestinal preparations of the present fish studied in hypodynamic Ringer solution.

The contraction started within 10 to 20 seconds after the application of the drug, and disappeared within 1 to 2 minutes. Following washing out of the bath the tone of the muscle returned to the initial stage. The highest sensitivity towards the acetylcholine response of the gut was obtained in concentration as low

as 10^{-8}gm/ml in various parts of the intestine; the sensitivity of the upper part of the intestine (below the gastric pouch) was found to be greater in comparison with the lower parts.

The minimal concentration of acetylcholine that had been found to elicit appreciable contraction in quiescent preparation was approximately within the range of 10^{-14}- 10^{-12}gm/ml. This was further potentiated if the preparation was pretreated with physostigmine. Similarly, optimal concentration of acetylcholine that produced the contraction of isolated foregut immersed in hypodynamic Ringer solution was found to be 10^{-8}- 10^{-7}gm/ml. (Fig.24). Repeated applications of either the minimal or the optimal doses of acetylcholine produced a gradual decrement of response after 4th or 5th time of application within short intervals (within 5 minutes) when the muscle was bathed in normal Ringer solution. But if the interval between the two applications of acetylcholine was prolonged beyond ten minutes such decrement did not occur, though the individual response to two identical doses of acetylcholine varied with each other greatly. The smaller concentration of acetylcholine showing an appreciable effect in normal Ringer solution was greater in hypodynamic Ringer solution or when the muscle was pretreated with physostigmine. The effect of acetylcholine in increased concentration over a tenfold range showed increased contraction. The application of acetylcholine in 1 x 10^{-10}gm/ml concentration at 5 minute intervals in hypodynamic Ringer solution did not decrease the response remarkably. Thus the effect of acetylcholine within the range 10^{-12}- 10^{-7}gm/ml was fairly constant over a long period of time and a satisfactory assay could be carried out within that range provided the bathing solution was made deficient of calcium. With higher concentration of acetylcholine (10^{-7}- 10^{-4}gm/ml) responses towards frequent or repeated application were reproducible, but not identical. When the acetylcholine in range of 10^{-8}gm/ml was left in the bath in normal Ringer solution for longer time, the fish gut remained in maximally

contracted stage which was then followed by gradual relaxation or lowering of the tone even when the drug was not washed out by flushing with fresh solution. After recovery of the basal tone flushing of the bath with fresh hypodynamic Ringer solution caused another contraction indicating that acetylcholine remained attached with the tissue and perhaps have been activated under low calcium, although the second contraction was smaller than the first. But further contractions were not obtained by continuous successive washing with that solution.

Contraction of fish gut caused by acetylcholine between 10^{-10}gm/ml&10^{-8}gm/ml was abolished by atropine at the range of 10^{-8}gm/ml (Fig. 25). In atropine (10^{-8}gm/ml) pretreated intestine, acetylcholine in range 10^{-6}gm/ml produced contraction. Treatment with lower concentration of physostigmine at the range of 10^{-8}gm/ml was able to potentiate the action of acetylcholine. The optimum concentration of physostigmine demonstrating potentiation of acetylcholine response was approximately 10^{-8}gm/ml when incubated for 5 minutes. (Fig.26). But pretreatment with higher doses of physostigmine (10^{-6}gm/ml) produced some irregular motility and lesser potentiation towards acetylcholine response. Repeated treatment or incubation for a prolonged period with physostigmine failed to produce significant potentiation of acetylcholine action as found in cases of treatment with single dose for 3 to 5 minutes only.

There was no remarkable change observed in response of the acetylcholine action in specimens cooled for 24 hours, but prolonged cooling beyond 36-48 hours reduced the recovery of spontaneous rhythmic contraction or the acetylcholine response. However, after 3 days of cooling the fish intestine had lost its ability to contract spontaneously when the response to acetylcholine in larger doses was obtained after a long period of suspension in bath at room temperature. (Fig 27).

Action of serotonin

Addition of serotonin (10^{-12}gm/ml to 10^{-10}gm/ml) caused alteration of spontaneous activity of fish gut. In most cases

there was slight rise in tone followed by increased spontaneous contraction. Washing out the bath produced return of the tone and activity to the normal state. The distinctive feature of serotonin action was stimulation characterized by sharp increase in tone and marked contraction. This action of serotonin was noted at the concentration of 10^{-10}gm/ml to 10^{-9}gm/ml. Considerable stimulation occured immediately and along with spontaneous contraction maintained at higher tone until the washing of the tissue. Higher concentration of serotomin at the 10^{-6}gm/ml caused significant contraction with increased tone but spontaneous contractions were inhibited. Recovery following washing occured within five minutes at lower doses (10^{-10}gm/ml), while at higher doses (10^{-6}gm/ml) it took more than 30 minutes for complete recovery.

As long as the muscle was sensitive to the acetylcholine it also responded to small doses of serotonin. With gradually increasing the dose of serotonin it was possible to obtain stronger contraction even in the depressed and cooled preparation. The cooling of intestine for 24 hours depressed the sensitivity of the muscle to serotonin at lower concentration. However, prolonged cooling made the muscle less sensitive and finally insensitive to serotonin and acetylcholine both. The effects of serotonin at different concentrations in different preparations were mostly uniform.

Although the sensitivity to serotonin was lower compared to that of acetylcholine, with the increasing dose of serotonin the responses were markedly high.

Methysergide, a well known reliable antagonist, was found to depress or block the serotonin response. Low doses of methysergide (10^{-10}gm/ml) reduced 40%of the serotonin response at concentrations of 10^{-14}gm/ml to 10^{-12}gm/ml, but higher doses of methysergide (10^{-8}gm/ml) was required to abolish the effect of serotonin at concentration of 10^{-10}gm/ml. Pretreatment with methysergide for prolonged time had same blocking effect towards serotonin as caused by treatment for 5 minutes. Morphine at low

dose (10^{-10}gm/ml) was able to block partially the serotonin response by about 30% at the dose of 10^{-10}gm/ml. Whereas 10^{-6}gm/ml of morphine reduced 45% of the response with similar dose of serotonin (Fig.28,29). Further higher doses of morphine failed to produce any blocking of serotonin response while the drug itself caused marked contraction of the intestine.

Atropine in any concentration did not show any blocking effect towards serotonin response. Atropine in concentration just low enough to abolish completely the stimulating effect of acetylcholine (10^{-8}gm/ml) failed to inhibit or block the serotonin response.

Similarly, methysergide pretreatment though caused specific blocking towards serotonin response did not show any depressant action towards actylcholine response.

Assay of acetylcholine and serotonin on fish gut:

The preliminary experiment with the fish intestine of Ophicephalus punctatus as described above, had indicated that isolated piece of fore-gut of the particular fish contracted vigorously in presence of minute quantity of acetylcholine (10^{-10}gm/ml) or serotonin (10^{-8}gm/ml) and that this response was repeatedly demonstrable with the same material for the assay of acetylcholine or serotonin, so that a sensitive and specific method for the bio-assay of acetylcholine and serotonin could be established. The suitability of such method was further compared with the other common bio-assay methods like frog rectus preparation, guineapig ileum and molluscan heart perfusion. (MacIntosh and Perry,1950).

Methods :

Methods used were essentially those described above. After decapitating the living Ophicephalus punctatus weighing on average 60-80 gms, small strips of intestine, below the pyloric end of the stomach and pyloric caeca, were isolated and quickly

flushed with Ringer solution to remove the debris within the lumen. Two strips of fish gut of approximately equal length(1 cm) were cut. Each strip was suspended in a 2 ml muscle chamber filled with fish Ringer solution. The chamber was aerated continuously with 95% oxygen and 5% carbon dioxide. The pH, temperature and composition of the buffered bicarbonate hypodynamic Ringer solution remained same as described before. The perfusion assembly and the method of recording of the contraction were the same. The intestinal strips were kept under a constant tension of 500 mg with plasticin. A 30 minutes stabilisation period was allowed before initiation of the experiment. The known and unknown amounts of acetylcholine, serotonin solution were added to the bath in 0.05 ml volume delivered through a microsyringe and the drug was left in contact with the intestine for a constant time of 1 minute after which bath fluid was changed. Controlled contractions of approximately equal magnitude were obtained by alternately administration of known and unknown concentrations of acetylcholine, an interval of 10 minutes being allowed between the addition of two doses.

For bio-assay proper and establishing the range of sensitivity, the minimal concentration of acetylcholine or serotonin that would cause an appreciable contraction of the intestine was used.

Drugs used :

1. Acetylcholine Iodide-(Sigma Chemical Co).
2. Serotonin Creatinine sulphate-(Sigma Chemical Co).
3. Physostigmine salicylate-(C.H.Boehringer Sohn ingelheim am Rhein)
4. Morphine sulphate - Dey's Medical Stores(Mfs.)Pvt.Ltd.
5. Atropine sulphate-(McFarlan Smith Ltd).
6. Methysergide bimaleate - (Sandoz, A.G., Nurenberg).

The standard concentration of acetylcholine solution was prepared in acetate buffer at range of 10^{-3} gm/ml and was kept at 4°C from which the different test standards of known doses were prepared with buffered Ringer solution before

addition to the bath. The stepwise dilutions from the standard concentration were prepared until it reached 10^{-14} gm/ml. Serotonin creatinine sulphate was dissolved in N/10 HCl and diluted to 1 /ug/ml and stored at 5°C. Different dilutions of serotonin were prepared with normal Ringer solution at pH 7.6 just prior to addition to the bath. The test standards at different dose ranges were used as follows :

Sl.No.	For Acetylcholine (in gm/ml)	For Serotonin (in gm/ml)
1.	$1.0 \times 10^{-(n+1)}$	$1.0 \times 10^{-(n+1)}$
2.	$1.4 \times 10^{-(n+1)}$	$1.2 \times 10^{-(n+1)}$
3.	$2.0 \times 10^{-(n+1)}$	$1.6 \times 10^{-(n+1)}$
4.	$2.9 \times 10^{-(n+1)}$	$2.4 \times 10^{-(n+1)}$
5.	$4.2 \times 10^{-(n+1)}$	$3.5 \times 10^{-(n+1)}$
6.	$6.0 \times 10^{-(n+1)}$	$5.0 \times 10^{-(n+1)}$
7.	$8.5 \times 10^{-(n+1)}$	$7.2 \times 10^{-(n+1)}$
8.	$1.0 \times 10^{-(n)}$	$1.0 \times 10^{-(n)}$

The value of n varied from 9 to 12 in case of acetylcholine and 7 to 10 in case of serotonin. Before the begining of the bioassay proper the effect of different standard concentrations of acetylcholine or serotonin were tested simultaneously with both the preparations in order to establish the range of effect and the standard response curve. This was repeated with 10 animals. In both the series of experiments, one piece of intestine was used for the entire series of test standard while another one was employed for the unknown. This was performed as the sensitivity of the individual preparation varied after repeated trial. Lastly, the alternate application of test standard and unknown was used with the same intestine for corroborting the results with the other. The unknown standard solution of acetylcholine was prepared by another worker and the concentration range was kept unknown to the experimenter until the termination of the experiment.

Calculation :

From a test standard curve at the above dose ranges the concentration range of the unknown was calculated. Starting from the smallest dose the effect of acetylcholine of the test or unknown standard was tested over a tenfold range and when the effect of both the unknown and the test standards became fairly constant as observed from the contractile height, the particular dose range was retested 3 times. The accuracy of the above test was determined by double blind test. The data obtained were calculated for analysis of variance and significance of difference from the parallel curves. The potency ratio and the index of precision were estimated from the ratio of standard deviation of a single response. Calculation of the analysis of variance and the regression curve were obtained from different groups of results. The procedures for the above calculations followed the model of Maddrell (1968).

To determine the specificity of the assay, the effect of test standard of each drug was tested with physostigmine or atropine pretreatment in case of acetylcholine, and methysergide pretreatment in case of serotonin.

Results :

Acetylcholine:

The smallest effective dose of acetylcholine was found to be 25 to 50 picograms which caused a contraction within 10 to 20 seconds after each application and disappeared in 1 to 2 minutes. The smallest concentration to have an effect was generally 2.4×10^{-11}gm/ml but some preparations were more sensitive than this when 1×10^{-12}gm/ml gave appreciable contraction. In physostigmine pretreated intestine the effect of acetylcholine at this dose was more pronounced but the doses lower than this did not elicit any appreciable response.

The effect of acetylcholine in the dose range 2.4×10^{-12} to 4.2×10^{-12}gm/ml was fairly constant over a long period and satisfactory assay could be carried out. The concentrations of

standard were 2.4 x 10^{-12}, 4.2 x 10^{-12} and 6 x 10^{-12} gm/ml given every five minutes with each dose being repeated two times.

This action of acetylcholine was abolished by atropine (10^{-8}gm/ml) so that the dose of acetylcholine had to be increased about 1000 times to get the similar effects. When acetylcholine was left in the bath for a longer time (2 to 3 minutes) the contraction height lasted only for one minute and then gradually disappeared although the drug was not washed out. The highest effective dose was found to be 1 x 10^{-7}gm/ml. After treating with the highest dose and following washing, the effect of the minimal concentration of acetylcholine (2.4 x 10^{-12} gm/ml) could not be elicited, but after a rest of 30 minutes and repeated washing the effect of minimal dose of acetylcholine was revived.

The log dose of acetylcholine response curve is usually steep but reasonably constant (Fig.30). The analysis of variance shows that there was no significant difference of parallelism but the variance due to repetition is highly significant since the responses became smaller during the test . In one 2-plus-2 point assay 8 doses were given with dose ratio of 1.5. The potency ratio was found to be 45% and the index of precision was estimated with 0.0232.

For comparative analysis the effect of low doses of acetylcholine was studied with isolated toad's rectus abdominis preparation and guineapig ileum preparation at 37°C. The minimum effective dose of acetylcholine on physostigmine pretreated rectus abdominis preparation was found to be 1.6 x 10^{-9}gm/ml and in case of guineapig ileum it was 3.5 x 10^{-8}gm/ml.

Acetylcholine dose response curve on the normal and atropine pretreated fish gut is illustrated in figure (31a) from which it appears that nearly 60% to 80% depression of the contraction could be attained by atropine pretreatment.

The acetylcholine dose ratio in phvsostigmine pretreated intestine was significantly greater at the higher ranges (Fig.31b).

Although different preparations had widely different sensitivity to repeated doses of acetylcholine when used at a particular dose range, its dose ratio was more or less constant and indepedent when graded doses were used.

Serotonin :

The minimal concentration of serotonin causing appreciable contraction of the fish gut was between 1 x 10^{-10} gm/ml and 5 x 10^{-9} gm/ml. Pronounced response was maximally obtained at 1 x 10^{-6} gm/ml of serotonin. Curiously enough the effect of serotonin was more pronounced at lower dose ranges in the acetylcholine pretreated intestine i.e, when it was sensitized with effective dose of acetylcholine.

The response to lower doses was reduced within 1 minute and to higher doses within 30 minutes. Repeated administrations of serotonin at particular dose followed by washing each time showed the same response upto 5 or 6 times. Further, the effect of low dose of serotonin did not change much in those tissues which were pretreated with high doses. There was no change in the sensitivity towards serotonin response following atropine (10^{-6} gm/ml) pretreatment, but considerable blocking effect could be seen with methysergide or morphine treatment.

Repeated application of serotonin did not show tachyphylactic response, but after repeated administrations of the drug recovery to normal tone was delayed. This was clear from the delay in return to base line, often it took more than 30 minutes inspite of repeated washing. With higher concentration, following revovery of the basal tone often was greatly reduced.

As the spontaneous contraction was enhanced following serotonin treatment for assay purpose, the bathing solution used was made hypodynamic by reducing the calcium ion content

in the Ringer solution to half (1 mM). The effect of serotonin on the fish gut, when perfused with the above Ca^{++} deficient Ringer solution, was more pronounced and was repeatedly demonstrable for a longer period of time when compared to those of acetylcholine and satisfactory assay of serotonin could be carried out easily at the dose range of 1×10^{-9} to 1×10^{-7}gm/ml. The standard doses of serotonin starting from 1.4×10^{-10}gm/ml to 6×10^{-7}gm/ml were used at 15 minutes interval, each dose having been repeated 3 times.

The serotonin dose response ratios before and after treatment with methysergide and morphine are given in the figure 32. In comparing the sensitivity of other standard methods, like guineapig ileum and molluscan heart preparations with the fish gut perfusion method, it was found that minimal effective concentration of serotonin in case of guineapig ileum was at the range of 1.4×10^{-6}gm/ml and molluscan heart at the range of 1.4×10^{-8}gm/ml whereas in case of fish gut it was 1×10^{-10}gm/ml.

Comparative sensitivity :

For a comparative analysis of the range of sensitivity in the bio-assay of the acetylcholine and serotonin with fish gut, the data obtained from the different experimental preparations like toad rectus abdominis, molluscan heart, guineapig ileum, rat uterus and fish gut (of Ophicephalus punctatus) are pooled together and illustrated in table-IV which shows maximum sensitivity of acetylcholine on Ophicephalus punctatus intestine. The fish gut of present species was also sensitive towards serotonin. Thus the intestine of Ophicephalus punctatus perfused with Ca^{++} deficient Ringer solution in a simple microbath at room temperature, may convieniently be used for the biological assay of minute quantity of acetylcholine and serotonin - present in biological tissues. The sensitivity and specificity of the assay appeared to be quite high although the effect of the interfering substances which

Table - IV

The relative sensitivity of different tissues towards acetylcholine and serotonin as utilised for bioassay purpose depicting the effective minimal concentration (in ng/ml) of acetylcholine and serotonin for eliciting appreciable contraction on different tissues.

	Rectus abdominis	Guineapig ileum	Rat Uterus	Molluscan heart	Ophicephalus punctatus
Acetylcholine	50	100	250	5	.01 - 1
Serotonin		400	10	10	1 - 2

were present in the tissue extract or fluid should be elucidated. However, if suitable antagonists to those substances were added, it might be insensitive to other substances and the assay of either acetylcholine or serotonin could be carried out.