Activities of Acetylcholinesterase in the Electric Organ of the Skate Fish※

By

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According to the theories of Loewi and Dale, Acetylcholine (Ach) acts as the specific transmitter substance for nerve impulses to the effector organ or to a second neuron. A major difficulty in this chemical mediator theory lies in the problem of how Ach, released by the nerve impulese, can be rapidly removed within the brief refractory period which is of the order of milliseconds.

In 1941, Nachmanson and his co-workers suggested that the original theory must be altered to account for the rôle of esterase-Ach system, which closely parallels the electrical changes occurring everywhere at or near the surface. Particularly suitable for the demonstration of this relationship was the striking parallelism which has been established between the concentration of cholinesterase and the voltage and number of plate per cm in the electric organ of the electric ell, *Electrophorus electricus*.

The author hesitates in making any further criticism of such a brilliant contribution as Nachmansohn's hypothesis; but it is very interesting to correlate dynamically these phantasmic problem between the rôle of Ach cycle and mechamism of bioelectric activity in electroplaque.

The present paper presents data on a brief survey of the activities of Acetylcholinesterase (AchE) and a survey of the quantitative analysis of the substance contained in the electric organ of Japanese common skates fish, *Raja pulchra* and *R. tengu*, and offers new evidence to the activities of AchE in the caudal muscle related to the electric organ of the skate.

Methods

A. Manometric method in determining Acetylcholinesterase activity.

1. Use of Bacrof-Waruburg manometric apparatus.

The Acetylcholinesterase activity was measured by the Bacrof-Warubrug manometiric method, in the modification used here, described by Augustinsson (1944). The method is based on the manometric estimation of the volume of CO_2 evolved from a bicarbonate containing system by the acid formed in the ester hydrolysis. Conical flasks, each of 22~26 ml volume, with one side bulb were employed. The flask constants were determined by the calibration method using mercury. The manometers were filled with Brodie's solution, containing 23 g NaCl and 5 g sodium choliate in 500 ml water, a few drops of

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an alcoholic solution of thymol were added. The fluid was coloured with eosin but it tend to decompose in the manometer. The density of the solution is $1._{034}$, and 10000 mm. Brodie corresponds approximately to 760 mm Hg.

The flasks were carefully cleared; grease was removed with benzine. They were washed with water and placed in cleaning solution overnight. The cleaning solution was prepared by dissolving 50 g potassium dichromate in 35 ml hot water and adding conc H_2SO_4 to 1 liter. Finally, the flasks were rinsed several times with distilled water and dried in drying chamber.

The grease used in lubricating the joints were the admixture of anhydrous lanoline and waseline.

The flasks and manometers were shaken at about 90 complete osillations per minute. The shaking amplitude was about 7 cm. In most case, the temperature of the water was $37._5 \pm 0._{05}$°C unless otherwise stated.

2. Measurement of Activity.

The volume of the reaction mixture has always been $2._{00}$ ml. In the main compartment of the flask $1._{60}$ ml of the substrate solution was placed and in the side bulb $0._{40}$ ml of homogenate of the certain percent or a mixture of $0._{40}$ ml of the Krebs' Ringer-Bicarbonate (KRB) or a piece of the slice of tissue. Substrate and enzyme preparation were dissolved in KRB solution (Table 1.).

The hydrolysis was carried out in a gas mixture of 95% N_2 and 5% CO_2 by volume. $CaCO_3$ is formed if the solution is not in equilibrium with at least 5% CO_2, when the optimum conditions are changed and the evolution of CO_2 is disturbed. The solutions were saturated with the gas mixture and flasks filled after they had been attached to the manometers. Before enzyme solutions were mixed with the contents of the main portion of the flask the temperature eqilibrium was attained by shaking in the water thermostat for about 15 minutes.

The shaker was stopped. The first manometer was read, lifted from its mount, the contents were mixed at zero time, and the manometer was placed back on its mount and the shaker started again. At one minute intervals the contents of the other flasks were similarly mixed. Usually, each series included six to eight expermints. Each manometer was read at six to ten minutes intervals, one minute between each manometer-reading. Readings were made continuously for 40~60 minutes.

The results were recorded in tabular form. And, the amount of CO_2 expressed in μl was plotted against time. The initial slope of the curve (in most cases a straight line), minus the slope of the curve for non-enzymatic hydrolysis was then taken as an expression of the enzyme activity. The extrapolated 60 min. value, minus the amount of CO_2 evolved during the same time period by non-enzymatic hydrolysis, has been used as unit in expressing the esterase activity (b_{60}). Expressed in μ mol substrate hydrolized during the same time period, the activity is $b_{60}/22._4$ μ mol. 1 μl CO_2 corresponds to $8._1$ μg Ach chlorid, or $1._0$ mg Ach chlorid$=123._5$ μl CO_2.

Materials

A. Buffer solutions

Table 1. Composition of Krebs' Ringer-Bicarbonate (KRB)

Solution	%(w/v)	ml	molarity
NaCl	0.90	100	1.5×10^{-1}
KCl	1.15	4	
$CaCl_2$	1.22	3	
KH_2PO_4	2.11	1	
$MgSO_4 7H_2O$	3.82	1	
$NaHCO_3$ *	1.30	21	0.4×10^{-1}
Total		130	

* $NaHCO_3$ solution saturated with N_2-CO_2 gas mixture, was pH 7.4

In most cases, the experiments have been carried out in a Krebs' Ringer-Bicarbonate (KRB), the composition of which is given in Table 1.

The solution, made by substance of highest purity and distilled water, was used for dissolving the substrate and diluting the enzyme preparations. In some cases it was also employed in extracting the enzyme from the disintegrated tissue. Fresh KRB was always prepared before used, since the solution deteriorated if kept.

B. Substrate

Table 2. Substrate

Substrate	Abbreviation	Formula	Mol Wt	Stability	
				in air	in water
Acetylcholine * Chloride	Ach	CH_3 CH_3>N-CH_2-CH_2-O- CH_3 | $COOH_3$ Cl	181.66	very hygroscopic	very unstable

* Merck &Co.

Acetylcholine was usually used in the form of the chloride. The substrate used are listed in Table

For each experiment fresh solution of the substrate diluted with KRB was made and spontaneous hydrolysis detrermined. A 0.1 M Ach was imployed unless otherwise stated.

C. Enzyme preparations

Enzyme preparations chiefuly used were slice-pieces of the electric tissue and their homogenate in the same way as described previously (Kuwabara, 1955).

Results and Discussion

Electric organ :

The activity of AchE in the electric organ was symbolized by QAch* , which

* Nachmansohn's Q and A : A very useful unit of AchE activity is Nachamansohn's Q (Nachmansohn and Lederer, 1939 b). This is mg Ach hydrolyized in 60 min. by 100 mg tissue at 20° C. In later investigations Rotenberg and Nachmansohn (1947) have used the unit A or mg Ach hydrolyized in 60 min. by 1 mg protein. In this paper AchE activity is symbolized by QAch which presents Nachmansohn's Q at 37.5°C.

Table 3. The activities of Acetylcholinesterase in slice and its homogenate of the electric organ of *Raja pulchra Liu*
Conditions : Substrate, 0.01M Ach (last conc.*); gas, 95% N_2 and 5% CO_2
OAch : hydrolized Ach mg per 60min. by dry tissue at 37.5°C

Materials	No.	Wet weight (mg)	Dry Weight (mg)	CO_2 out put per hr μl	QAch (wet)	QAch (ave.)	QAch (dry)
Slice	1	93 46	4.3 2.3	924 442	72 70	71	1,480
	2	46 39	4.2 4.5	1098 727	173 156	165	1,610
	3	53 51	4.2 4.6	912 900	125 128	127	1,180
	4	48 46	4.7 4.0	1050 930	159 147	153	1,480
	Average						1,440
Homogenate	5	50		666	97	99	
	6	50		684	100		
	7	90 90		642 1248		52 101	

* last concentration of the substrate is o.o1 M Ach (KRB).

represents mg Ach hydrolized in 60 min. by *g* dry tissue. As shown in Table 3, the average value of QAch is 1440, that is to say, the organ can split in 60 minutes an amount of Ach equivalent to 1~1.5 times their own weight.

Considering from the quantity of a pure enzymatic protein it may possible to notify how higher is the activity of AchE in the electric organ, for it might be able to hydrolyize a great amount of Ach within the brief refractory period.

According to newly received letter of T. Sekine, the activity of AchE in boar spermatozoa is rather high : ZAch***, 0.586 or QAch, 70~80 which is of the same order of magnitude as that estimated previously for some mammalian brains. Therefore, activities of AchE in the electric organ of the skate is more than 20 times as higher as those of boar spermatozoa and of some mammalian brains.

In order to compaire with the activities between the slice of the tissue and its finely ground homogenate, small samples was ground in a glass homogenizer

Table 4. Extraction of Acetylcholinesterase in the electric tissue of *R. pulchra*. Activity : CO_2 out put (μl)/10 min./100mg wet tissue. Supernatant : 3000 RPM/min./10min. Homogenate : 10% homogenate.

Material No.	Homogenate Activity (ave.)	Supernatant Activity (ave.)	Extraction (%)
1	225	178	79.2
2	119	99.3	83.4
3	231	116	71.8
Average			78.1

*** ZAch : hydrolized Ach mg per 60 min. per 10^6 boar spermatozoa (Sekine, 1954).

of Potter-Elvehjen type. For homogenated samples, however, are not appeared increased activities of the enzyme (Table 3), whereas some of other reports clearified increasing tendency in certain nervous tissue.

When 5% homogenate of the ground tissue put into the centrifuged tube it was then centrifuged for 10 min. at lower tempreature. As showing in Table 4, the activity of its supernatant was lower than that of the homogenate. The rate of extraction of the enzyme in the organ is in close agreement with figures obtained from the spermatosoa (Sekine, 1954).

Table 4. Activities of Acetylcholinesterase in slice of the electric organ of ***Raja tengu***; cut off successively from the top to distal end. Length of animal 85.0 cm. Length of the organ 21cm. D=distance from the top of the organ to the slice cut off. Ww=Wet weight of slice. Wd=Dry weight of slice. Q Ach=Hydrolized Ach mg per hr. per dry weight. (Each value were determind in averae of five cases.)

D (cm)	Ww (mg)	Wd (mg)	CO_2 out put per hr. μl	QAch (D) (dry)
1	30	4.0	708	1,439
5	35	3.5	583	894
10	50	4.0	268	544
15	50	4.1	255	413
20	53	4.0	140	277
Average				713

Pieces of the slice were taken with the slicer successively at 5 positions from top to distal end of the organ, and activity of AchE was determined. The results obtained are given in Table 4. The highest activities of AchE is found in the region near the head end of the organ ; it decreases continuously towards the caudal end. The results obtained in this experiments are in close agreement with figures obtained by a group of investigators in Columbia, New York.

As described previously, the electric elements of the skate fish was microscopically observed consisting of five functional components, of these, electric plate, striated and alveolar layers were regarded as an electric disc. The elctric plates is made of layer of plotoplasm, containning numerous nervous which divide dichotomousely as they pass backwards through a suporting connective tissue framework. Therefore, the greatest activities of AchE should be presumably at the electric plate which are at the innervated side of the disc. In order to get more

Tadle 6. Activities of Acetylcholinesterase in successive slice of piece of the electric organ on distance of 5 cm from the top of the organ. About 100 μ in thickness of slice were prepared with slicer.
(Each value were determind in average of five cases.)

Slice No.	Wet Weinght (mg.)	Dıy Weight (mg.)	CO_2 out put per hr. μl.	QAch (dry)
1	84	4.1	589	1,163
2	60	4.0	489	933
3	60	4.0	581	1,220
4	70	4.2	542	1,057
5	65	4.0	600	1,214
Average				1,347

information about the variations which may occur in a given piece due to uneven distribution of innervated surface, activities of AchE was determined in succssecive slice-picce of the tissue cut into 0.1 mm thick with slicer at lowr temperature. Pieces were taken at distance of about 5 cm. from the head of the organ. The values obtained (in Table 6) were great variated from one slice to another in a regular rhythm. It makes possible assumption, as Nachmansohn also pointed out, that this rhythmic changes is not incidental but should be corresponded to rhythmical change in the electric plate.

AchE activity in the caudal muscle connected with the electric organ :

Table 6. Activities of Acetylcholinesterase in the caudal muscle connected with electric organ of *Raja tengu*. Muscle was taken from the portion at just front of the organ.
l = lateral muscle. d = dorsal muscle. v = ventral muscle.
(Each value were determind at average of five cases.)

Muscle	W (w) (mg)	W (w) (mg)	CO_2 out put per hr μl	QAch (D) (dry)
l	65	12.1	685.7	485
d	85	15.5	28.6	14
v	55	14.5	32.4	18

It is not too much to say that the electric organ of the skate fish occupies the most parts of the tail which are short or large, so its tail itself is electric organ.

Since the time that Ewalt studied the ontogenetical development of the electric organ of *Raja batis*, there has been no doubt as to the genetic relation between the two tissues, the organ and the dorso-lateral muscle of the tail. Especially the skate fish which are known as the fish with weak electric power in which the disc retains the striated layer of their muscle characteristics even when the formation is perfect.

As described previously, the lateral muscle should be transformed into the electric organ of skate fish. It was thought possible that AchE of the lateral muscle at the just front of the elecric organ might be higher than those of ventral- and dorsal-muscles located at same portion. Therefore, AchE activies were determind in a few samples of the muscle at just front of the organ. The data obtained are given in Tadle 6. The relatively higher activity of AchE were found in "lateral" : QAch, 485. Conversely, in the dorsal and ventral muscles located at same portion have a lower activities than the fomer : QAch value was 14 and 18 respectively.

The quantitive analysis of the substances contained in the electric organ :

In order to be acquainted with the quantities in the substances contained in the electric organ, only a few samples had been examined, it is true, the quantitative analysis was determined. Results obtained are showing in Table 8. The existence of such a high concentration of the enzyme appears particularly significant in view of the high water (92%) and low protein (1~2%) of the organ.

Table 7. Data of the quantitative analysis of the substances contained in the electric organ of *R. pulchra Liu.*

Substance	Material	Quantities in tissue g (wet)			Method.
Sample No.		I	II	III	
water	tissue	92.8	92.1%		weight analysis
total N	homogenate			2.27mg	micro-Kjeldahl
none protein N	supernatnat			0.19mg	〃
protein				1.3%	caluculated
sugar	supern.			0.94mg	Hagedorn-Jansen
creatinine	homog.			120γ	Jaffe
alkaline phosphatase	〃			35.8 B.U.	Bodansky
NH_3	supern.			182γ	microdiffusion
P	ash	317	289γ		Gomori
total Ca	〃	752	790γ		Yanagisawa

Such a results obtained in this analysis were very similar with that of the electric organ of the electric eel, *Electrophorus electricus* reported by Columbia group. During the transportation of the fish by land and sea, the sugar should be decomposed, whereas it was fairly analized. Although, it makes possible assumption that almost of them may be reductive substances other than the sugar.

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Summary

1) The activities of Ach-E were estimated on the electric organ of the skate fish. The average value of QAch is 1400, which is more than 20 times as that of boar spermatozoa and some mamlian brain.

2) When AchE activities were determined at five portions from the head to distal end of the organ, the high activity of AchE is found in the near the head of the organ, it decreases continuously towards the caudal end.

3) In order to get more information about the variation which may occure in certain pieces of same portion, AchE activity were estimated in successive slice of a piece. As Nachmansohn was pointed out on electric ell too, the rhythmical change of AchE obtained may be corresponded to a change of tne fine structure of tne electric discs.

4) Considering the phylogenetical development of the electric organ, AchE activities of caudal muscles connected with the organ were estimated. The highest activies were found in the "lateral" muscle which may be transmitted to the organ: QAch was 485, which was more than about 60 times as that of dorsal and ventral muscles located in the same portions.

5) As shown in the data on the quantitative analysis of the substances contained

in the electric organ of skate fish, high water (92%) and lower protein (1~2%) content of the organs, whereas specific cholinesterase (AchE) is so highly concentrated in its organ.

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