ACETYLCHOLINE(ACh) CYCLE IN THE ELECTRIC ORGAN OF FISH※

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1957

※Contribution from the Shimonoseki College of Fisheries, No. 196

CONTENTS

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INTRODUCTION

The electric signs of nervous and muscular action were for a century, the only manifestations studied by electrophysiologist. But function of a living cell cannot be conceived in purely physical terms. For a through understanding of the chemical of bioelectricity, a knowledge of the biochemical reactions involved is essential. Biophysics and biochemistry are concequently, of equal importance and inseparable in any attempt to solve the problem.

Ideas as to the mechanism of generation of bioelectric potential developed in latter part of the nineteenth century, when it became known that semipermeable membranes may develop strong electric potentials. Many physiochemists laid the groundwork for the so-called "membrane theory", of nerve impulse, best formulated by Bernstein and Tchermak early in this century. This theory is still the basis of all modern concepts of the mechanism of conduction of nerve impulse. It assumed that the nerve axon is surrounded by a polarized membrane with a positive charge on the outside and a negative on the inside. During activity, the charge is reversed at the point where the impulse passes. This reversal has been demonstrated by direct measurements across the membrane in experiments with the giant axon of the squid (Curtis, 1942 and Hodgkin, 1945). A flow of current is generated from the resting to the active region, and thereby the adjacent region is stimulated. There the same process is repeated, and in this way successive points of the surface are stimulated, and the nerve impulse is propagated by these small currents along the fiber.

There exists a considerable concentration gradient of sodium and potassium ions on the both sides of the membrane: the concentration of sodium is high in the outside fluid compared with its concentration on the inside; the reverse is true for potassium ions. There is a constant flow of ions across the membrane even in the resting condition. The equilibrium is dynamic. During activity it occurs a rapid influx of sodium and equivalent amount of potassium leaks to the outside. According to data obtained in Nachmansohn's laboratory, the amount of sodium which enters per square centimeter of surface per

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impulse is 4.5×10^{-12} mole, which is in close agreement with figures obtained by a group of investigators in Cambridge, England (Hodgkin and Huxley, 1947, 1950; Keynes, 1949, 1950).

Recent observations of Hodgkin and his associates suggest that the entrance of sodium into the axon occurs during the ascending phase of the potential, whereas potassium leaks out during the descending phase (Hodgkin and Huxley, 1950). Their results support the assumption that for at rest the permeability of the membrane is high for potassium ions, much higher than for sodium ions. During activity, however, membrane becomes more permeable to sodium ions, about 500 times more than in resting state, according to the estimates of Cambridge group (Hodgkin and Katz, 1949).

The investigations described support the view that the ionic concentration gradients are the source of the electromotive force of action potentials in nerve and muscle fibers. However, this raises the fundamental question as to the mechanism by which the potential source of electrical energy, inactive at rest, becomes suddenly effective. As to the question, Nachmansohn and his associates made the remarkable hypothesis that there must be "trigger" mechanism by which the protein membrane separating the ion species becomes suddenly more permeable, so that the ionic concentration gradient may become an effective force. Something must happen at successive points of the protein which rapidly opens and closes the barrier to the flow of ions. The most likely assumption to explain the transitory change of the protein membrane is that of an extremely rapid chemical reaction. This was postulated about 20 years ago by Meyer (1937), on the basis of experiments with protein films. He arrived at the definit conclusion that permeability changes can be excluded as the primary cause of the action current. They must be produced by chemical reactions. He postulated as the primary event in conduction the release of a cation which produces the permeability change.

The change in the resistance of the membrane was experimentally established by Cole and Curtis on the basis of measurements of impedance change (1939). They calculated that during the passage of impulse the resistance of the membrane decreases from about 1000 ohms to 40 ohms per sq. cm. moreover, the temperature coefficients, Q_{10} of both the rising and falling phases of the action potential are high, 2 for the first phase and 3.2 for the second phase (Hodgkin and Huxley, 1949). This further supports the assumption that the action potential is associated with chemical reactions.

Meyer proposed the first hypothesis concerning the nature of the process postulated that a chemical reaction must precede the permeability change. Membranes are formed by protein chains. Appearance of amino groups may increase cationic movements. The difficulty of identifing these reactions is

readily recognized if two features of this process are kept in mind: the high speed and the small amount of energy involved. The permeability change of the membrane reaches its peak within 100 μ sec. A chemical reaction responsible for this change must have comparable speed. According to the heat measurements of Hill and his associates, the heat released during nerve activity is of the same order of magnitude of 10^{-9} g.cal. per square centimeter per impulse. Therefore, the metabolism of the specific compound responsible for the permeability change must be exceedingly small.

Since early in the century, two substances have been recognized to be specifically associated with nerve function : adrenaline and acetylcholine (ACh). The hypothesis of neurohumoral transmission, suggested in 1904 by Elliott, was assumed that sympathetic nerve endings liberated adrenaline which then acted as chemical mediator of nerve impulse to the effector cells. A similar role was later attributed to ACh in the transmission of nerve impulses from parasympathetic nerve endings to effector cell on the bases of important observation on Hunt, Magnus, Loewi, Dale, and others. The hypothesis was based, as in case of the automatic nerves, essentially on two : (1) the powerful effect of ACh when applied to the junctions and strict limitation of the foci; (2) the appearance of ACh exclusively in perfusion fluid of the synaptic junctions following nerve stimulation.

Whereas the hypothesis of neurohumoral transmission appeared acceptable to most physiologist in the case of the autonomic nerve endings, the situation changed when Dale tried to extend his hypothesis to the transmission of nerve impulses across synapses and neuromuscular junctions. In this case the idea encountered a vigorous opposition from many physiologists. A major objection stressed by Fulton is the similarity of the existable properties of cell bodies and axons as revealed by electrical signs. This makes it difficult to assume basically different mechanisms for conduction and transmission. A similar view was expressed by Erlanger in 1939 in Toronto at a symposium on the synapse. Analyzing some of the so-called peculiarities attributed to the synapse, such as latency, one-way transmission, temporal summation and facilitation, and others, he pointed out that all these phenomena can be demonstrated on axons.

The idea of neurohumoral transmission appeared unsatisfactory in many respects. On the other hand, it seemed necessary to explain the findings on which the hypothesis was built and to offer an interpretation which would reconcile these findings with the conclusions based on the study of the chemical manifestations. In this impasse a new approach became necessary.

Nachmansohn's brilliant investigations have made it possible to integrate ACh into the metabolic pathways of the nerve cell, to establish the sequence

of energy transformations associated with the conduction of nerve impulse, and to correlate chemical processes with the electric potentials. The original hypothesis of neurohumoral transmission has been replaced by the new Nachmansohn's conceptual scheme which integrates physical and chemical findings and reconciles the seemingly contradictory observations (Nachmansohn, 1952, and Nachmansohn & Wilson, 1951). The hypothesis of "neurohumoral" transmission assumed that ACh was released from the nerve endings and acted upon the effector cell as a mediator. The roll of ACh was liberated exclusively to the synapse. According to new conceptual scheme the action of ACh is not an inter but an intra cellular process occurring within the conducting membrane. Its action is responsible for the alternations of the membrane leading to the increased ion permeability postulated by all modern theories of the impulse conduction. It forms an integral part of the elementary process by which bioelectric potentials are generated in the axon in the nerve terminal, and in the postsynaptic membrane. But the propagating agents along the axon and across the synapse are the electric currents.

It is obvious that the study of the chemical reactions connected with an event of this kind must offer even more serious difficulties. No adequate methods are available for directly determining chemical compounds appearing in such minute amounts and for such short period of time. There is, however, another possible approach. Nearly all chemical reactions in the living cell are effectivated by enzymes. The study of enzymes *in vitro* has elucidated many chemical reactions, known to occur in living cells, which could not be followed by direct chemical determination of the compounds metabolized. Especially for an event occuring with such a high speed as the propagation of the action potential, analysis of the enzyme systems involved appeared to be most promising approach.

Enzyme studies alone are, however, not sufficient for the elucidation of a biological mechanism, since there are so many simultaneous enzymatic reactions in the complex system of the living cell. The rise of dynamic biochemistry during last about 30 years, made possible a new approach to cellular function. Many complex intermediate reactions were analyzed and sequence of single steps was established. The most conspicuous example of such an approach is the development of muscle and nervous physiology. Through the pioneer work of Meyerhof, Hill, Lipmann, and Nachmansohn etc., many physical and chemical changes have been correlated, and our concept of the biochemical mechanism of electricity has, according to an expression of Nachmansohn, gone through a real "revolution".

It appears necessary to investigate the enzyme associated with the breakdown and synthesis of ACh, to establish the sequence of energy

transformation during the activity, to integrate ACh into the metabolic cycle of the electric tissue, and to correlate the chemical reactions with the electric manifestations.

MATERIAL AND METHODS

I. DETERMINATION OF ACETYLCHOLINESTERASE ACTIVITY

In the quantitative determination of acetylcholinesterase activity, the rate of hydrolysis of added ACh is measured. Formerly biological methods were chiefly used, but today chemical ones are more in favour. In the biological methods high enzyme and low substrate-concentrations are employed, whereas in the chemical ones the substrate is in excess and the enzyme in dilute solution. Because of these differences, comparable results are not obtained with the two methods (Mahal, 1938).

A. Biological methods

The evanescence of ACh is followed pharmacologically by measuring its action on isolated intestine (Abderhalden & Crivetz, 1926, Ajlmark & Kornerup, 1939) heart (Loewi & Navaratil, 1926, Plattner & Hintner, 1930. Helianer, 1936), frog muscle (Scheiner, 1936, Crivetz, 1945), leech muscle (Minz, 1932), frog rectus (Martini & Torda, 1937), or by measuring the blood pressure (Danielopolu & Popesco, 1946).

B. Chemical methods

1. Titration methods

The liberated acetic acid is titrated with an alkaline solution of known concentration.

i) Indicator methods. Various indicators have been recommended, e. g., phenolphthalein, phenol red, cresol red, or thymol blue. The method has been described by Renshaw & Bacon (1926), Stedman, Stedman, & Easson (1932) White (1933), Vahlquits (1935), Hall, & Lucas (1937), Roepke (1937), Pighini (1936), Ebaerhard, & Silverman (1939), Bovet & Santenoiss (1941), and others. In connection with enzymatic-histochemical investigations, a micro-titration method for assay of ChE has been described by Glick (1937, '38). Glick's method has been modified by Sawyer (1943). In using dyes as indicators, it is often very difficult to observe a clearly defined colour change. In addition, many other troublesome precautions must be observed, not the least being the possible influence of the indicator on the enzyme (cf. Bernheim

& Bernheim, 1939).

ii) Electrometric methods. A more convenient method than using indicators is to titrate electrometrically. It is advisable to use a quinhydrone electrode (Socoz & Cattaneo, 1937, Barbour & Dickerson, 1939), glass electrode (Glick, 1937, Alies & Haves, 1940, Sanz, 1944, Schummelfeder, 1947), or antimony electrode (Delaunois & Casier, 1945). Electrometric titration, details not given, was also employed by Gliman, Carlson, & Goodman (1939).

Estimations of enzymatic activity by biological assay for residual ACh ion in reaction mixtures have inherently low precision and are much more time-consuming and expensive than those depending on chemical methods. The eight chemical methods of enzyme assay which have been used depend on the fact that one of the products of the reaction is acetic acid. They differ in details of temperature, pH, and substrate kind and concentration, but have in common the fact that relatively high substrate concentration is necessary to provide sufficient acid on hydrolysis for chemical detection. Continuous titration, with a glass electrode-vacuum tube potentiometer sensitive to 0.01 pH unit, was selected as the chemical technique or the greatest precision, simplicity, and verstility, with least danger of interference from added reagents or manipulation. The titration volume was 25 ml. and standard substrate concentration was 0.160 M. The titration vessel was a jacketed gas tube paddle. Electrodes were immersed in the solution, their design and small size insuring their rapidly coming to temperature equilibrium and, the calomel half-cell liquid junction was then rinsed afresh to maintain the saturation of KCl solution.

After the warmed reagents were mixed, the pH was quickly adjusted and maintained within 0.10 unit by small additions of 0.02N NaOH from a 5 ml burette. Reading to the nearest second of time required for consumption of measured amounts of alkali furnished data from which total hydrolysis rates could be calculated. Readings were continued until the desired accuracy was obtained. Usually 10 to 20 minutes gave values duplicable within ±2 per cent. "Rates" were calculated as volumes of 0.0200N NaOH used in 20 minutes, in conformity with the Stedman procedure. The value, blank rate under standard conditions with ACh ion as substrate, was 0.085 ml. The iodides of ACh and its derivatives were used for preparing the substrate solutions, as they are stable and nonhygroscopic. In contradiction to the results of Roepke (1937), it was found by trial that iodide ion did not interfere. The 0.0200M stock of ACh iodide ordinarily used was adjusted to pH 4.0. The solution changes only just detectably at room temperature during a month, presumably by addition, since calculation by extrapolation from reaction rate constants measured at higher pH values indicated the hydrolysis due to OH-ion to be

less than 1 per cent per year at this pH and a temperature of 37°C.

2. Manometric method

i) Warburg method. The Warburg manometric method is the most convenient for following the hydrolysis of ACh, and it is also the most suitable for use in comparative and serial experiments. Ammon (1933) was the first to use this method in assaying ChE activity. The basis of the method is the manometric estimation of the volume of CO_2 evolved from a bicarbonate-containing system buffered against CO_2 by the acetic acid formed in the hydrolysis of ACh. This method is now used almost exculsively. The AChE activity was mainly measured by this method, in the modification used here, described by Augustinsson(1944). Conical flasks, each about 22—26 ml. in 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 choleate in 500 ml water; a few drops of an alcoholic solution of thymol were added. The fluid was coloured with methyl red. The density of the solution is 1.034, and 10 000mm. Brodie corresponds approximate to 760 mmHg.

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 was the admixture of anhydrous lanoline and waseline. The flasks and manometers were shaken at about 90 complete oscillations per minutes. The shaking amplitude was about 7cm. In most case, the temperature of the water thermostat was 37.5°± 0.05°C unless otherwise stated.

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 the enzyme sclution or mixture of 0.20 ml of the enzyme solution and 0.20 ml. of the inhibitor solution. Substrate and enzyme preparations were dissolved in a KRB's solution. Substrate and enzyme preparations were kept in lower temperature before use. The hydrolysis carried out in the solution is not in equilibrium with at least 5 per cent 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 the flasks filled after they had been attached to the manometers. Before the enzyme solutions were mixed with the contents of the main portion of the flask the temperature equilibrium 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 experiments. Each manometer was read at six to ten minutes intervals, one minute between each manometer-reading. Readings were made continuously for 30—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 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. One μl CO_2 corresponds to 8.1 μg ACh chloride, or 1.0 mg ACh chloride may estimate 123.5 μl CO_2.

ii) Barcroft differential method has been employed by Stedman (1939).

iii) Van Slyke method has been used by Rinkel & Pijoan (1938); it was employed in a modified form by Friend & Krayer (1941).

iv) Microchemical gasometric method. The principle of the Cartesian diver has been successfully applied to the study of the ChE activity by Linderstrm-Lang & Glick (1938).

3. Other methods

A step-photometric method, based on the ferric-chloride reaction of acetic acid, has been described by Abdon & Uvnas (1938). This method is one of those used by Ali (1940) and by Pirolli (1941). Another photometic method using *m*-nitrophenol as indicator has been evoked by Croxatto, Croxatto, & Huidobro (1939). An opalescence method was introduced by Gal (1940).

II. DETERMINATION OF ACh AND RELATED ESTERS

In spits of its physiological importance ACh has been determinable chemically only after isolation by time-consuming procedures. Of necessity workers in this field have reported to pharmacological bioassays whose specificity seems open to doubt. The finding that choline is readily acetylated by the action of coenzyme-linked enzyme in which ATP serves as an energy source (Nachmansohn, 1949) lends added interest to the development of adequate analytical procedures for ACh (Hestrin, 1949).

A. Reagents

(1) Hydroxylamine. Hydroxylamine hydrochloride, 2M, the solution should be stored in the cold.

(2) Acid. Concentrated hydrochloric acid, sp. gr. 1.18, diluted with 2 parts by volume of water.

(3) Alkali. Sodium hydroxide, 3.5N.

(4) Iron. Ferric chloride, 0.37 M, in hydrochloric acid 0.1N. As reagent iron, ferric chloride crystals, ($FeCl_2 \cdot 6H_2O$), of Merck & Co. was used.

(5) Standard solution. ACh chloride, 0.004 M, in sodium acetate solution, 0.001N, of pH 4.5. This standard may be kept in the refrigerator for a week without measurable loss.

B. Procedure

Alkaline hydroxylamine reagent is prepared freshly before use by mixing equal volumes of Reagents (1) and (3). The mixture keeps for about 3 hours at room temperature. Two ml of alkaline hydroxylamine reagent are added in a test-tube to 1.0 ml of the solution to be analyzed. After at least 1 minute or longer if desired, the pH is brought to 1.2±0.2 with 1.0 ml of acid, and 1.0, of the iron solution is added. The density of the purple-brown color is prompt determined at 540 mμ. The formation of the bubbles of colorimeter cell is avoided if the mixture has been swirled adequately after the addition of each component. Solutions too dark to read are brought to suitable density by diluting with ferric chloride, 0.074M, in 0.1N hydrochloric acid. If the color reading is low, the analysis may be carried out on a larger ester aliquot with reagents of a suitably higher concentration. Correction for non-specific color is made by repeating the procedure as described, except that the order of addition of hydroxylamine, alkali, and acid is reversed. With this order of addition of the various components, esters do not form any hydroxamic acid. Protein in the sample analyzed is generally precipitated when the acid and iron additions are made and may be removed either by filtration or brief centrifugation. Formation of flocculent precipitate may be hastened by the addition of trichloroacetic acid. The extinction, after corrected for non-specific absorption, is converted to concentration with the help of a proportionality factor which is found by applying the same procedure to a standard solution. When buffer is present in the unknown, the strength of an added component should be adjusted to compensate for the effect of the buffer on the pH. If the ester sample contains iron-binding buffer salts, an increase in the iron concentration may be used to insure the maximum development of color. If necessary, a correction is applied for the effect of buffer salts on the optical density. In a case in which the color density of the blank is undesirably high, the efficiency of lower iron concentration or lower final pH than indicated as a means of reducing the relative color

density of the blank should be determined. The upper limit of measurement exceeds 5μM and the lower limit is about 0.04μM of ester per ml. of final solution.

III. ASSAY METHOD OF CoA ACTIVITY

CoA activity was measured by three assay methods; (1) the assay based on the acetylation of sulfanilamine by crude extracts of pigeon liver so-called the "crude" assay system, as described by Kaplan & Lipmann; (2) a "purified" assay system which is the same as the previous one except that enzyme system is fractionated with ammonium sulfate between 0.4 and 0.7 saturation (Kaplan & Lipmann, 1947); (3) the transacetylase assay which is based on the CoA-dependent arsenolytic decomposition of acetyl phosphate by the enzyme phosphotransacetylase from *Clostridium kluyveri* extracts (Stadtman, Novelli, & Lipmann, 1951). Inorganic phosphate was determined by the method of Fiske & Subbarow (1925), acetyl phosphate by method of Lipmann & Tuttle (1945), and CoA-bound pantothenate by the method of Novelli, Kaplan, & Lipmann (1949). Total adenosine, and free adenosine-5'-phosphoric acid were determined by the spectrophotometric method of Kalckar (1947) by specific enzymatic determination procedures. Protein was measured by TCA turbidimetric method of Bucher (1947).

IV. PREPARATION OF ACETYL CoA

Acyl coenzymes were prepared by adding the corresponding acid anhydrides to the coenzyme in slightly alkaline medium as described by Simon and Shemin. The amount of acyl CoA formed is determined by two methods : (A) the hydroxamic acid formation at neutral pH, at which presumably only the acyl group on the SH is tested, and (B), the nitroprussid test for free SH groups described below. The hydroxamic acid test was calibrated against ACh solution. According to both methods 95% to 100% of the CoA was acylated.

V. ASSAY OF THE REACTIONS MEDIATED BY CHOLINE ACETYLASE BY THE NITROPRUSSIDE TEST FOR FREE SH GROUPS

If the reaction mixture contains acetyl CoA and choline as substrates and choline acetylase as enzyme, the enzymatic activity may be tested either by bioassay of ACh formed, using the frog's rectus muscle, or determining the remaining acetyl CoA by the use of hydroxylamine in neutral solution or by determining the appearance of free SH groups of CoA. Of the two chemical

tests the latter is more sensitive and was used by adapting the method described by Grunert & Phillips to the Nachmansohn's special conditions.

The reaction mixture contained only acetyl CoA, choline and enzyme in buffer (0.1M K_2HPO_4 of pH 7). The total volume was 1.0 ml. An aliquot of this solution (0.2 ml) was added to 0.1 ml of 0.33 N HCl and 1.2 ml of saturated sodium chloride solution. Solid NaCl (0.2g) was added to keep the system saturated. Following the inaction of the enzyme, 0.1 ml of 0.03 N NaOH was added. The protein precipitate was removed by centrifugation; at this stage the mixture remains stable. An aliquot (0.8 ml) was used for spectrophotcmetric detremination. To the aliquot were added 0.1 ml of 0.067 M sodium nitroprusside and 0.1 ml of a solution containing 1.5 sodium carbonate and 0.067 M NaCl. The optical density was determined in the spectrophotometer at 520 mμ exactly 30 seconds after the addition of the base. To correct for slight current changes in the spectrophotometer control tubes containing saturated NaCl, the nirtoprusside and cyanide-carbonate reagents were tested between every 3 or 4 assays. If the assay mixture was not saturated with NaCl, a significant decrease in color was observed, whereas the presence of metaphosphoric acid was not found to be necessary for the experimental conditions used. There is a slight decrease in optical density if the phosphate concentration in the acid system exceeds 0.01 M, probably due to the effect of the buffer on the final pH, since the optimal pH of the colorimetric test is 10. The nitroprusside test was calibrated against standard glutathione solution under identical conditions and with the same constituents as in the tests themselves.

If the color developed per SH group is assumed to be the same in both cases, 1 mg of the Pabst CoA preparation would contain about 750 μg CoA, or close to 1 μM. In the calculations 1 mg of the CoA preparation was assumed to contain 1 μM. of CoA.

Acyl CoA breaks down at pH 10, liberating SH groups. There is, therefore, increase in colour between the reading of the time of addition of the alkali and 30 seconds later. This increase is proportional to the amount of acyl CoA present at the time of addition of alkali.

Since the quantitative measurement of ACh was based upon the appearance of mercaptan groups during the course of the reaction, it is necessary to establish the validity of the test. If, as is believed, the colour is produced by a complex involving the mercaptan, then the molar extinction coefficient should depend upon the precise nature of the mercaptan and colorimetric assay would require calibration with CoA. This latter procedure requires a sample of known composition and this was not readily available to us. However, it appeared likely that the molar extinction coefficient for glutathione might not differ greatly from that of CoA and could be used for the assys. Consistent by

measuring the acetylation in an independent way, namely by assaying for ACh directly: This latter comparison is necessary for establishing the validity of the method in general even aside from the question of extinction coefficients.

VI. BUFFER SOLUTION

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

Table 1. Composition of the bicarbonate krebs' solution (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	
$MgSO_47HO_2$	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, at pH 7.4

The solution, made by substances of the highest purity and distilled water, was used for dissolving the substrate. In some cases it was also employed in extracting the enzyme from the disintegrated tissue. Fresh KRB's solution shoul be always prepared before use, since the solution deteriorates if kept.

VII. SUBSTRATES

The substrates used are listed in Table 2, which show the abbreviations used in this paper and morecular weights of the various substances.

Table 2. Substrates

Substrate	Abbreviation	Formula	Mol. wt.
Acetylcholine chloride	ACh	$[(CH_3)_3N\text{-}CH_2\text{-}CH_2\text{-}O\text{-}COCH_3]Cl$	181.66
Acetylcholine bromide		$[(CH_3)_3N\text{-}CH_2\text{-}CH_2\text{-}O\text{-}COCH_3]Br$	226.12
Acetylcholine iodide	ICh	$[(CH_3)_3N\text{-}CH_2\text{-}CH_2\text{-}O\text{-}COCH_3]I$	273.13
dl-Acetyl- -methylcholine chloride (Mecholy)	MeCh	$[(CH_3)_3N\text{-}CH_2\text{-}CH(CH_3)\text{-}O\text{-}COCH_3]Cl$	195.69
Carbaminoylcholine chloride (Doryl, Lentin)	CbCh	$[(CH_3)_3N\text{-}CH_2\text{-}CH\text{-}O\text{-}CONH_2]Cl$	182.65
Benzoylcholine chloride	BzCh	$[(CH_3)_3N\text{-}CH_2\text{-}CH_2\text{-}O\text{-}COC_6H_3]Cl$	243.73
N-Acetyl-*p*-amino benzoyl-choline chloride	AAmB Ch	$[(CH_3)_3N\text{-}CH_2\text{-}CH_2\text{-}O\text{-}COC_2HNH\text{-}COCH_3]Cl$	300.78
Salicylcholine chloride	SaCh	$[(CH_3)_3N\text{-}CH_2\text{-}CH_2\text{-}O\text{-}COC_6H_4OH]Cl\cdot H_2O$	277.74
Acetylsalicylcholine chloride	AsaCh	$[(CH_3)_3N\text{-}CH_2\text{-}CH_2\text{-}O\text{-}COC_6H_4O\text{-}COCH_3]Cl$	301.77
Acetylaneurine chloride hydrochloride	AAn	$[C_{14}H_{19}O_2N_4S]Cl\cdot HCl$	379.31
Acetylsalicylic acid	ASa	$HOOC\text{-}C_6H_4\text{-}O\text{-}COCH_3$	180.15
Tributyrin	TB	$C_3H_5O_3(CO\text{-}CH_2\text{-}CH_2\text{-}CH_3)_3$	302.36
Ethyl acetate	EA	$C_2H_5O\text{-}COCH_3$	88.10

EXPERIMENTAL RESULTS AND DISCUSSION

I. ELECTRIC FISH

A. A Particularly favorable tool

Nature has created a model in which muscle fibers have lost their contractile elements and have kept as sole functional activity the conductive elements: the electric disc or electroplax of certain electric organ of fish. These tissues offer for many reasons a particularly favorable tool for the study of various physical and chemical factors underlying conduction. Although the electroplax represents more precisely a special kind of conducting muscle fibers, their study has much wider implications. If one believes in biochemical unity of life, these most powerful bioelectric generators, which nature has created, are a favorable material for studying physiochemical mechanisms of generation of bioelectric potentials in general.

Yet, it was in 1772 that Welsh demonstrated before the Royal Society of London that the powerful shock of certain fish, known since ancient times, is an electric discharge. It was the first demonstration of animal electricity, and attracted the attention of many physicists, like those of Cavendish, Farday, Davy, and others. When, however, the question was raised on the Galvani's experiments whether electricity plays an important role in nerve and muscle activity, the picture changed, and biologists too became interested in this fish. Galvani himself in the last year of his life worked on electric fish, and during the last century physiologist, especially DuBios-Reymond, investigated different aspects of the electric discharge in these fish.

The most important feature of electric organs is generally accepted as the nature of their activities is identical with action current of nerve and muscle. The cellular units of the electric tissue are the electric plates. Each plate develops during the discharge a potential difference of about 0.1 volt, which is of the same order of magnitude as that found in ordinary nerves. It is only the arrangement of these plates in series by which these organs are distinguished from ordinary nerves. The great differences of the total discharge in various species do not depend on the units, which show only relatively small variations, but on the shape and dimensions of these species.

The group of electric fishes comprises a number of very different varieties, both in fresh water and in marine: All of them possess special organ capable of producing transient electric discharges, which, in some species are quite weak, but in others, are powerful enough to give a severe shock. These organs vary

widely among the different species in their shape and size and in their position and orientation in body of the fish.

The arrangement of the electric disc has its highest geometrical regularity in the electric rays, *Torpedo* and *Narke*. In the electric organ of these genera, they are piled in columns, and average one of which contains about 300 electroplaxes in *Narke japonica*, consequently, the discharge is, on the average, 30 volts.

Each column extends from the ventral to dorsal surface of the body. A number of them, side by side, form each of the two electric organs, which lie in the disk-like body of the fish to right and left of body cavity, just outside the line of gill slits. In each organ, there are four hundred columns in *N. japonica*. During the discharge, the current transverses each organ in direction from its ventral to its dorsal face. Thus, the columns of electroplaxes discharge in parallel, while each column, the electroplaxes act in series. Other species in our country, though its electric activity is yet indistinct, *Rajidae* has been known as electric fish which holds an electric organ in its tail. Full detail observations of the electric organ of this fish will be published elswhere.

In species with the most powerful electric organ known, electric eel found in the Amazon river, several thausand plates are arranged in series from head to caudal end of the organ. Thus, the voltage of a discharge is, on the average, 400 to 600 volts. In *Gymnotorpedo oceidentalis*, another species first described by Storer (1849), 150 to 200 volts was measured by Amberson and Edwards.

It was shown by Babuchin, in 1870, that the electric organ has evolved phylogenetically from striated muscles. The only exception is possibly the organ of *Malapterurus*, the origin of which is doubtful. In strong electric organs the contactile elements have completely disappeared, but they exist as rudiments in the plates of weak electric organ.

The electric plates are therefore more exactly homologous, at least functionally, to the motor end plates. As has been described by several authors in the last century, the electrolemma surrounding the nerve ending in the electric organ shows, on side toward the cell interior, a very remarkable structure, formed by a layer of rods, the "palisades" of Remak. It has been shown that the postsynaptic membrane at the motor end plate has interesting similarities with the postsynaptic membrane surrounding the nerve endings in the electric tissue (Couteaux, 1947; Gresciteii, Koelle, & Gilman. 1946) The postsynaptic membrane surrounding the nervous terminal in the electric plate was able to demonstrate by using Janus green or methyl violet the existence of a very peculiar structure of sarcolemma surrounding

the nerve endings but separated from them by a layer of neurogia.

On the other hand, the observations of Gopfert and Schaefer (1937) and the work of Eccles and his associates (1946) have revealed the existence of a special end plate potential generated in postsynaptic membrane. Since the electric plates are homologous to the motor end plate the discharge in electric tissue must be considered more precisely as homologous to the end plate potential. This view finds strong support in the remakable similarities of the morphological correlation of these two potentials.

All the typical features of the discharge are the same as known from the action potential in neurophysiology; latency, duration of the discharge, and the refractory period are of the same order of magnitude as in the nerve action. A full description of earlier work can be found in many handbooks and textbooks (Rothenberg, 1928).

Krogh (1929) stated that nature has created quite a number of animals for special physiological problems. The electric tissue appears indeed to be most suitable material for correlating chemical reactions with electrical manifestations. The energy required for the propagation nerve impulse is extremely small. The initial heat per impulse is of the order of magnitude of 10^{-7} to 10^{-8} g-calorie.

It is therefore, in ordinary nerves, the electrical processes are on such a small scale and associated with such small amount of energy that the available methods are inadequated for studying the underlaying biochemical reaction. The electric tissue is magnificent tool for correlating metabolic reactions with the generation of bioelectricity.

In 1937, the electric tissue of *Electrophorus electricus* was introduced by Nachmansohn as material for the study or the role of ACh in the mechanism on nerve activity, especially in view of the homology with the neuromuscular junction. DuBois-Reymond who devoted several decades to investigation on electric fish, made in 1877 the following interesting statement: The unusual interest always aroused by electric fish has been still increased by the recognition that the property of these animals, long a matter of curiosity and amazement, is not something unique, but appears to be special application of a common feature, frequently encountered in animal kingdom. Since we have learned that all nerves and muscles of all animals are capable of electric effects, electric fish indeed lost somewhat of their wonder : but this may greatly contribute to the solution of great problems of the general physics of nerve and muscle.

B. The Quantitative analysis of the substances contained in the 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 pursued. Results obtained are in Table. 3. The existence of such concentration of the enzyme appeared particularly given

Table 3. Data of quantitative analysis of the substances contained in the electric organ of common ray.

Substance	Material	Quantities			Method
Sample No.		I	II	III	
water	tissue	92.8	92.1%		weight analysis
total N	homogenate			2.27mg	micro-Kjeldahl
non-protein N	supernatant			0.19mg	〃
protein				1.3%	calculated
sugar	supern.			0.94mg	Hagedorn-Jansen
creatinine alkaline	homog.			120	Jaffe
phosphatase	〃			35.8B.U.	Bodansky
NH_3	supern.			182γ	microdiffusion
P	ash	317	289γ		Gomori
total Ca	〃	752	790γ		Yanagisawa

significant in view of the high water (92%) and low protein (1—2%) of the organ. Such a results obtained in this analysis were very similar to that of the electric organ of the electric eel, *E. electricus* reported by Columbia group. During the transportation of the fish by land and sea, the sugar should be decomposed, whereas it was fairly analyzed. Therefore, it makes possible assumption that almost of them may be reductive substances other than the sugar.

II, ACETYLCHOLINESTERASE (AChE) IN THE ELECTRIC ORGAN OF THE FISH

A. Historical note of cholinesterase(ChE)

Interest in the group of enzymes now known as the cholinesterases may be said to have started in 1914 when Sir Henry Dale first suggested that the transient action of acetylcholine *in vivo* was probably due to its destrution by an esterase in the blood. Twelve years later, in 1926, Loewi & Navratil succeeded in demonstrating the presence of such an enzyme in tissue extracts, and in 1932 the term "choline-esterase" was introduced by Stedman, Stedman, & Easson to describe enzymes that hydrolyze acetylcholine. It soon became apparent, however, that the cholinesterases from different sources, and even from different tissues in the same animal, show striking differences in properties. Despite much work, the specificity of these and other esterases

remains an outstanding problem in this field, with which is linked the broader question of their physiological functions. Alles & Hawes (1940) demonstrated that the cholinesterase of human erythrocytes is much more active in hydrolyzing acetylcholine at low substrate concentrations than the enzyme in human plasma. They also described differences in substrate

Table 4. Various Vertebrate Tissues Investigated for their Cholinesterase Activity

Tissues (Animals)	ChE Activity	Type	Bibliography *
Heart	++	I	45, 15, 64, 63, 24
		II	70, 17
Lung	+		65, 24, 42, 41
Salivary glands	++	I	52, 64, 24,
	+++	II	
Saliva	−		65, 9,
Stomach (pig, frog)	++		20, 21, 26,
Gastric juice (man, rabbit)	−		9
Pancreas	++	I	65, 2, 18, 44, 47,
			64, 7,
	++	II	23,
Pancreatic juice	++	II	18,
Liver	+	I	65, 2, 29, 50, 14, 64, 28,
		II	18, 34,
Gall bladder, bile	+++		65, 38, 24,
Intestine	−		43, 65, 30, 24, 26, 12, 35,
Spleen	++	I	10, 47, 26,
		II	
Kidney	+	I	65, 51, 64. 24, 19, 40, 42,
	+	II	
Adrenal glands	++	I	65, 64, 24,
	+	II	
Bladder, ureters (calf)	++		16,
Urine(man)	−		65, 2,
Testis (man, pig)	−		24, 26,
Sperm, prostate, seminal vesicles (man)	+		77,
Spermatic fluid from shark (*Scyllium*)	+++		
Ovary, oviduct (man, pig)	+	I	36,
		II	24, 39, 40,
Uterus	+	I	
		II	65, 50, 24,
Placenta (man)	+		
Milk (cow)	−		62, 72,
Thyroid gland	+	I	65,
	++	II	65, 24, 23, 40,
Carotid body (cat)	+	I	53,
		II	
Thymus	+++	I	65, 24, 26, 40, 41,
Eye: various parts of			65, 24, 27, 56, 31, 4, 37,
Ear: perilymph (pigeon)	+		48,
Skin (man, cat)	+	I	71, 78,
Hibernating gland (cat)	+	I	68,
	++	II	68,
Red bone-marrow (cat)	+	I	
	+	II	

specificity between the cholinesterases in these two tissues, acetyl-α-methylcholine being hydrolysed by both enzymes, but acetyl-β—methylcholine only by the erythrocyte enzyme. Further differences in the specificity and kinetics of cholinesterases from different sources were described by Glick (1941), Richter, & Croft (1942) and Zeller & Bissegger (1943). Mendel and his colleagues in Toronto next proposed a classification of the cholinesterases into two main types, which they termed "true" and "pseudo" cholinesterase (Mendel & Rudney, 1943a). The "true" cholinesterase, present in erythrocytes and in the nervous system, was thought (i) to hydrolyze only choline esters, (ii) to be partially inhibited by high concentrations of acetylcholine, and (iii) to hydrolyze acetyl-β-methylcholine but not benzoylcholine; "pseudocholinesterase", present in the serum and the pancreas was thought (i) to hydrolyze both choline and non-choline esters, (ii) to show maximal activity at high acetylcholine concentrations, and (iii) to hydrolyze benzoylcholine but not acetyl-β-methylcholine.

Mendel's proposal of these two main types of cholinesterase received further support when the effect of inhibitors on cholinesterase activity was studied. Use had been made for many years of the inhibition of cholinesterase activity by eserine (Englehart & Loewi, 1930) and by prostigmine (Easson & Stedman, 1937), and, in general, these are highly effective inhibitors for most of the mammalian cholinesterases, irrespective of their type according to Mendel's classification. A large and rapidly growing series of compounds, however, is now known which selectively inhibit either true cholinesterase or the pseudo-cholinesterase. Among the earlier compounds to be studied, caffeine (Zeller & Bissegger, 1943), bis-β-chloroethyl-*N*-methylamine, or "nitrogen mustard" (Thompson, 1947 ; Adams & Thompson, 1948), and the synthetic prostigmine analogue Nu. 1250 (*N*-*p*-chlorophenyl-*N*-methylcarbamate of *m*-hydroxyphenyl-trimethylammonium bromide) are all selective inhibitors of the true cholinesterase. Percaine (Zeller & Bissegger, 1943), tri-*o*-cresyl phosphate (Mendel & Rudney, 1944 ; Earl & Thompson, 1952), di-isopropyl fluorophosphonate, or DFP (Mazur & Bodansky, 1946; Hawkins & Mendel, 1947) and another prostigmine analogue Nu. 683 (dimethylcarbamate of 2-hydroxy-5-phenylbenzyltrimethylammonium bromide) all selectively inhibit the pseudo-cholinesterase. Although tri-*o*-cresyl phosphate was first shown to inhibit horse serum cholinesterase by Bloch in 1941, the introduction of DFP, whose pharmacological action was described by Adrian, Feldberg, & Kilby (1947), together with the development of the alkylpolyphosphates and aryldialkylphosphates as insecticides, has led to the intensive study of the group of organo-phosphorus anticholinesterases.

Many of these compounds are highly effective in extremely low

concentration; for example, tetraethyl pyrophosphate (TEPP) is an effective inhibitor in concentrations as low as 10^{-10}M. They differ from eserine (Matthes, 1930) or prostigmine, in that they appear to inhibit cholinesterase irreversibly (Mackworth & Webb, 1948) by reacting stoicheiometrically with the active centres of the enzyme. It is thought that a phosphonium ion

Table 5. Characteristics of the Two Acetylcholine-Hydrolyzing Enzymes

Characteristics	ChE I	ChE II	Bibliography *
Nomenclature	Specific ChE "True ChE" "e-Type" Cholinesterase	Non-specific ChE "Pseudo-ChE" "s-Type" Unspecified esterase	25 46 79 58
General distribution	Erythrocytes, nerve tissues, thymus, etc.	Blood serum, pancreas, glands, etc.	3, 3, 4, 5
Optimum pH	7.5—8.0	8.5	1, 3, 4,
Isoelectric point	4.65—4.70 (erythrocytes)	4.36 (serum)	3, 5,
Permanence at different pH	less	more	3, 6,
Inhibition by excess of substrate	+	—	1, 53, 38,
Activatior by NaCl	+	—	57, 3,
Hydrolysis of :			1, cf, 22, 55,
tributyrin	—	+	76, 53,
acetyl- methy-Ch	+	—	49, 75,
benzoyl-Ch	—	+	54, 1,
phenylacety-Ch	—	+	66,
acetoacetyl-Ch	—	+	11,
Inhibition by:			
quaternary N-bases	+ +	+	67, cf, 13,
tertiary N-bases	+	+ +	67, 13,
dimethylcarbamate of 2-hydroxy 5 phenyl-benzyl-trimethyl-ammonium bromide	—	+	32,
caffeine	+ +	—	53, 59, 13,
percaine, irgamid	+	+ +	53, 13,
priscol	+ +	+	69,
privine	+	+ +	69,
quinine	+	+ +	60,
intocostrin (curare)	+	+ +	
paludrine	+	+ +	8,
di-*iso*propyl fluorophosphate (CFP)	+	+ +	73, 74, 61,

$[(RO)_2PO]^+$ is split off, and that this then combines irreversibly with part of the active centre.

The cholinesterase itself catalyzes the formation of the ion which ultimately destroys its activity; as Whittaker (1951) has put it, the enzyme "commits suicide". The kinetics of the inhibition has been studied in detail by Aldridge (1950), who believes that two reactions take place:

$$E + I \rightleftharpoons EI \rightarrow EI'$$

first, the formation of a dissociable enzyme-inhibitor complex, EI, which then undergoes a monomolecular transformation into an undissociable complex, EI'. Although Mendel's division of these esterases into the two types, true and pseudocholinesterase, has been of great value in that, in many cases, it allowed a greater precision in the definition of the enzyme concerned in acetylcholine hydrolysis in a given tissue, more recent work has shown that there are certainly more than two types of cholinesterase, as judged by the criteria usually employed in defining the true and pseudo types. For example, it soon became clear that many so-called true cholinesterases (or "specific" according to the nomenclature reported by Nachmansohn & Rothenberg, 1945) hydrolyze triacetin more rapidly than acetylcholine, and that this triacetin hydrolysis is highly sensitive to anticholinesterases and is present in preparations freed from aliesterase (Mazur & Bodansky, 1946; Holton, 1948: Blaschko & Holton, 1949). Cholinesterases that do not fulfil the original criteria regarding activity towards "selective" substrates have also been described. Thus, swine serum contains a cholinesterase which hydrolyzes neither acetyl-β-methylcholine nor benzoylcholine (Levine & Suran, 1950), while chicken serum cholinesterase hydrolyzes both of these so-called selective substrates at quite rapid rates (Earl & Thompson, 1952).

In view of these findings, Adams and Whittaker in 1948 commenced a systematic survey of the substrate specificity of partially purified true and pseudo-cholinesterase. They confirmed the earlier claims that true or "specific" cholinesterases are capable of hydrolyzing many non-choline esters. Their study of the effect of variations in the number of carbon atoms in the acetyl group of the esters yielded some interesting results ; they found that, both for choline esters and for aliphatic esters, the true cholinesterase hydrolyzes acetates more rapidly than propionates, and butyrates only very slowly indeed (Adams & Whittaker, 1949a); with pseudocholinesterase, on the other hand, butyrates are hydrolyzed faster than propionates, and acetates more slowly still (Adams & Whittaker, 1949b). Changes in the configuration of the alkyl group of the ester molecule were also of interest, in that 3 : 3—dimethylbutyl acetate, the carbon analogue of acetylcholine was hydrolyzed very much more rapidly than the other esters studied, both by true and pseudo-cholinesterases. Thus, both types of enzyme are "unspecific" (Nachmansohn & Rothenberg, 1945) in that they both hydrolyze non-choline esters, although with both enzymes there is a marked preference for those aliphatic substrates which approach most closely the configurations of choline esters. The idea that pseudo-cholinesterases are less specific than true cholinesterases therefore receives no support from these studies. On the basis of their findings Adams and Whittaker proposed a classification of these enzymes according to the acyl

group in the substrate molecule optimal for their activity, true cholinesterase being "aceto-cholinesterase" and the pseudo type "butyle-cholinesterase". Unfortunately, when a wider range of animal species is studied, it becomes clear that the cholinesterases cannot be divided into only two types on a basis of substrate specificity, since Ord & Thompson (1951) have shown that the optimal acyl group for the cholinesterase of rat heart is propionyl, though it would seem from inhibitor studies that this enzyme is a pseudo-cholinesterase.

Despite the criticisms that have been levelled at Mendel's original classification, a case can still be made out for retaining the terms true and pseudo-cholinesterase, particularly if the criteria for classification are relative sensitivities to inhibition by selective inhibitors as well as substrate specificity.

In recent years considerable information has been obtained concerning the distribution and localization of true and pseudo-cholinesterases in different tissues.

The erythrocytes of certain species and the brain had early been recognized as containing the true cholinesterase (Mendel & Rudney, 1943 ; Nachmansohn & Rothenberg, 1945). Pseudo-cholinesterase, which had been studied chiefly as a plasma enzyme, was next shown to be present in the pancreas (Mendel & Mundell, 1943); and Langemann (1944) found it also in human ovary, in contrast to human skeletal muscle, which contains only the true enzyme. The peripheral nerves of the cat, and the liver and certain glandular structures in the rat, were also shown to hydrolyze benzoylcholine (Sawyer & Hollinshead, 1945; Sawyer & Everett, 1947).

A more detailed study of the distribution of cholinesterase types in rat tissues was carried out by Ord & Thompson (1950), who were able to detect significant amounts of pseudo-cholinesterase in all the tissues studied except skeletal muscle. It appeared from their work that rat tissues could be classified into three groups.

Group A. Tissues containing largely or entirely the true cholinesterase—brain, skeletal muscle, and the adrenal glands.

Group B. Tissues containing both enzymes in amounts such that acetyl-methlcholine and benzoylcholine are hydrolyzed at about the same rate—stomach, liver, lung, and salivary gland.

Group C. Tissues containing chiefly pseudo-cholinesterase—heart, intestinal muscle and mucosa, harderian gland and skin

The pseudo-cholinesterase contents of the heart (both auricle and ventricle) and intestine were strikingly high. In the rat therefore it would appear that those tissues in which acetylcholine exerts a nicotine-like (*i. e.*,

it is concerned with transmission to another neurone or to a striated muscle cell) contain chiefly the true cholinesterase, while those in which it exerts a musculine like action (Brown, 1937) contain very largely the pseudo-cholinesterase; whether this apparent correlation is of physiological significance, however, is not yet known, and much more work is needed in other species before any final conclusion can be drawn. Koelle (1950), using a histochemical technique, has studied the distribution in the cat, and has found that pseudo-cholinesterase predominates in the liver and in the ileum. That pseudo-cholinesterase may be actively secreted by glands is suggested by the work of McCance *et al.* (1951), who have claimed that dog pancreatic juice and pig parotid saliva are rich in this enzyme.

True cholinesterase, although most plentiful in nerve and striated muscle, is not confined to these tissues. It is of interest that it is the chief cholinesterase perfused, blood-free placenta (Ord & Thompson, 1950b), since this tissue is usually regarded as being devoid of nerves. Turning to the nervous system, which Nachmansohn (1939) had studied in some detail as regards acetylcholine hydrolysis, it was concluded both by Mendel & Rudney (1943b) and by Nachmansohn & Rothenberg (1944) that brain contains only the true or "specific" cholinesterase. More recently it has been reported from a number of laboratories that brain hydrolyzes benzoylcholine, although at a relatively low rate (Augustinsson, 1948; Ord & Thompson, 1950). More detailed studies of different areas of the brain have been carried out by Burgen & Chipman (1951) and Ord & Thompson (1952), and it is now known that pseudo-cholinesterase is widely distributed in the central nervous system and that it appears to be particularly associated with the myelinated fibre tracts. In this connexion it is of interest that Koelle (1950) has shown the presence of pseudo-cholinesterase in glial cells and in the Schwann cells of myelinated nerves.

The effects proceed *in vivo* by inhibition of the cholinesterases have been extensively studied in connexion with the pharmacology of the new organophosphorus anticholine esterases. To take DFP as an example of these compounds, the effects produced by its administration to animals can in the main be interpreted as resulting from an accumulation of acetylcholine due to inhibition of cholinesterase. Thus, lacrimation and profuse salivation occur ; the sweat glands are stimulated, even after the rapeutic doses of DFP (Grob *et al.* 1947b ; Harvey *et al.* 1947); there is an incerase in the tone and motility of the musculature of the gastrointestinal tract (Modell *et al.* 1946; Grob, Lilienthal, & Harvey, 1947) ; in the lungs there is constriction of bronchial muscles and increased secretion of bronchial glands (Green, McKay & Krop, 1947), while with large doses of DFP there is a progressive fall of blood pressure.

In the eye an intense miosis is produced which may persist for weeks; application of DFP to the chronically denervated pupil, however, is not followed by constriction (Leopold & Comroe, 1946). In autonomic ganglia and neuromuscular junctions, the facilitating effects which are produced are also in accord with an inhibition of cholinesterase at these sites.

It has been repeatedly observed that a very considerable inhibition, of the order 80% of the true cholinesterase of the nervous system must be present before any abnormal signs develop which might be attributable to acetylcholine accumulation (Mazur & Bodansky, 1946; Koelle & Gilman, 1946; Nachmansohn & Feld, 1947). In the case of the pseudo-cholinesterase the level of this enzyme in the serum can be reduced almost to zero without the development of abnormal signs or symptoms (Mazur & Bodansky, 1946).

It is when we turn to considering the possible roles played by the cholinesterases inside the body that the suitability of the names true and pseudo-cholinesterase, in the light of our present knowledge concerning these enzymes, becomes apparent. For, although we have,on the whole, good evidence relating the true cholinesterase of synapses and neuromuscular junctions to the inactivation of physiologically released acetylcholine, the function of the pseudo-cholinesterase remains obscure, and much of the experimental evidence is interpreted by some workers in the field as arguing against any participation of this enzyme in acetylcholine metabolism at any rate in the brain.

With regard to the true cholinesterase, Nachmansohn and his collaborators (Bullock *et al.* 1947) have put forward a more comprehensive theory of its function in transmisson processes by suggesting that acetylcholine (and cholinesterase) is concerned also with conduction along the nerve fibre. This theory has, however, been extensively criticized.

It would certainly seem that, as pointed out above, no sign that could be referred to acetylcholine accumulation develop when the pseudo-cholinesterase of the plasma is virtually completely inactivated, and Hawkins & Gunter (1946) have claimed that this is so when the level of this enzyme in the pancreas and in the superior cervical ganglia is inhibited to the extent of 80—90%. On the other hand, it has recently been shown that certain late changes (demyelination of peripheral nerves and of certain tracts in the cord) can, under suitable conditions, be produced by certain selective inhibitors of pseudo-cholinesterase, including tri-*o*-cresyl phosphate, and that the level of the pseudo-cholinesterase in the spinal cord is profoundly lowered prior to the onset of the demyelination (Earl & Thompson, 1952). It cannot be concluded, however, that the demyelination is the result of this enzyme inhibition. Although it is known that, in the hen, DFP and bis (isopropylamino) fluorophosphine oxide can

cause demyelination, other organo-phosphorus anticholinesterases do not, even although considerable inhibition of pseudo-cholinesterase in the spinal cord is produced in animals poisoned by them (Thompson & Webster, unpublished). Further, there is evidence that, in the rat, inactivation of pseudo-cholinesterase by tri-*o*-cresyl phosphate in the central nervous system is not accompanied by any structure of functional changes

It has been proposed that pseudo-cholinesterase may act by supplementing the action of the true enzyme, since Koelle & Friedenwald (1950) have found that concentrations of DFP which have little effect on the true cholinesterase, but which cause a marked fall in pseudo-cholinesterase activity, produce an increase in the tone and motility of the cat's ileum. Feldberg (1950) has concluded that the acetylcholine metabolism of the intestine is in part independent of nervous activity and has suggested that it may be responsible for stimulating the spontaneous contractions of the intestinal wall and the secretion of the succus entericus; it is possible that the pseudo-cholinesterase, which is present in such high concentions in the wall of the intestine, may be concerned with such local actions.

Other observations, such as the rise in the level of pseudocholinesterase in the liver and in the plasma in animals fed on hypolipotrophic diets, and the return to normal on adding supplements of lipotrophic substances (Hawkins & Nishikawara, 1651), the fall in the level of the plasma cholinesterase in liver disease (Antopol, Tuchman, & Schifrin, 1937; McArdle, 1940) and in malnutrition (McCance, Widdowson, & Hutchinson, 1948; Waterlow, 1950; Hutchinson, McCance & Widdowson, 1951), together with the changes that have been reported in thyrotoxicosis (Antopol *et al.* 1937) and in alloxan diabetes (Saviano, Balbi, & De Francis, 1948), cannot at present be interpreted with anything but the utmost reserve, although certain of them suggest that the pseudocholinesterase in liver might be concerned with some aspect of choline or lipoid metabolism.

B. Preparation of enzyme solution from the electric organ

Electric organs are a suitable material for the preparation of active enzyme solutions, owing to the great amount of enzyme present and the low protein content. Very active solutions were obtained from the electric organ of *Narke japonica* and *Raja pulchra* respectively.

1. Slice and homogenized electric tissue

At first, the fresh electric organ of *R. pulchra* cut from the tail as soon as possible at low temperature (5°C), was washed and soaked with KRB's solution. Then one of the sample was used as a slice of electric tissue and cut about 0.2mm thick with the glass slicer, the other was used as its homogenized tissue:

0.5 mg of the tissue-piece in wet weight was grinded with the glass homogenizer of Potter-Elvehjem type. Although the pieces of tissue taken for grinding were small, the suspension had to be diluted to a large volume with KRB's solution, and the 3 ml put into Warburg vessel were only a small fraction of the total suspension. For instance, 3 ml of the suspension prepared from pieces of the electric tissue contained only 0.5 mg. fresh tissue. The Wet W. given in table indicates the fresh weight of tissue ground. As the amount account actually put into the vessels was approximately the same in each series of experiments, that for each determination is not specified separately but is indicated only for each table. The balance used permits rapid and precise weighting which is necessary for such small amount of fresh tissue.

2. Cell-free extract

Fresh electric tissue of *N.japonica* and *R. pulchra* has been used generally. The tissues were carefully washed with KRB's solutions and minced with an automatic machine. In a mortar 5 to 25g. was taken up with twice or four times as much KRB's solution. The mixture was shaken in shaking machine for half an hour, centrifuged at a constant speed of 3000 R. P. M., and fluid decanted. Very different time intervals were needed to produce as clear a fluid as possible. The buffered suspensions were kept in two or three days. The activity of these tissue suspensions were generally not changed remakably during two weeks, during which time all investigations were performed. Purification of the enzyme solution ; — the enzyme extracted from the electric organ of the fish, *N. japonica* and *Raja* sp. has been purified by fractional ammonium sulfate precipitation. After the ground tissue had been centrifuged and supernatant fluid removed 250 ml of 50 per cent ammonium sulfate solution were added to the extract tissue. The pH of this solution is lowered to about 4.5, when the enzyme is separated from the greater part of the albumins which are precipitated. AChE is then precipitated by increasing the salt concentrations to 70 per cent. The precipitate is dissolved in distilled water and the fluid dialyzed in collodion membranes against distilled water. The preparations thus obtained had been purified 20—25 times and yield was about 50 per cent.

The nitrogen contents of the enzyme solution has been determined by micro-Kjeldahl method and protein constitutes calculated. To 1 ml of the enzyme solution in a 3 ml centrifuge tube there was added 1 ml of 40 per cent trichloracetic acid. After 24 hours the precipitate was centrifuged for 30 minutes in a centrifuge at a rate of 4000 R. P. M. The precipitate was washed three times with distilled water. The centrifuged tube was put into a drying oven at 105°C, for several hours and the weight was then determined.

Table 6. Relationship between purification of acetylcholinesterase and its activity

preparation	activity*
Slice	40— 80
Homogenate	45— 80
Cell free extract	90—150
1st Amm. sulfate	200—300
2nd Amm. sulfate	300—500

* ACh hydrolyzed mg/mg protein/hr.

C. Activity of Acetylcholinesterase(AChE)in the electric organ of fish

1. Activity

In earier investigation, activities of AChE estimated with enzyme preparation from slice or homogenized electric tissue of *Raja pulchra*. The values obtained are given in Table 7 and 8. The electric slice is capable of hydrolyzing

Table 7. Acetylcholinesterase Activity in the Electric Organ of Common ray

Sample	Wet W. (mg)	Dry W. (mg)	CO_2 (μl/hr)	QACh (Wet)	QACh (Dry)
Slice	93	4.3	813.0	63.7	137.8
〃	46	2.3	421.5	66.4	133.5
〃	21	1.7	232.8	81.2	99.7
Homogenate	50	—	436.0	63.5	—
〃	50	—	454.0	66.1	—

amount of ACh ranking usually 70 —150 mg/g. fresh tissue/hour or 1400 mg/g. dry tissue/hour.* Namely the electric tissue can split as amount of ACh equivalent to 1—1.5 times as their own weight for 60 minutes. The same strong activity also obtained with its homogenized tissue. There is no difference in their activity between slice and homogenate as shown in Table 7. In spite of getting more enzyme activity with certain homogenized nervous tissue than that of the slice no different activity with both preparation of this tissue has been obtained. This is in close agreement with AChE activity of boar spermatozoa reported by Sekine (1953). The boar spermatozoa is capable of

* Nachmansohn's Q and A. A very useful unit of the cholinesterase (ChE) activity is Nachmansohn's Q (Nachmansohn and Lederer, 1936). This is mg. ACh hydrolyzed in 60 min. by 100 mg. tissue at 20°C. In later investigation Rothenberg and Nachmansohn (1947) have used A or mg. ACh hydrolyzed in 60 min. by 1 mg. protein Johnes & Tod (1935) assigned ml CO_2 evolved per min. by the effect of serum. Otherwise, the ChE activity is expressed as volume of CO_2 in μl. evolved in 60 min., or sometimes as the quantity of ACh in μM hydrolyzed during the same period. In this paper, AChE activity is usually symbolized by QACh which represents mg. ACh hydrolyzed per g. wet weight, dry weight and mg protein per hour at 37.5°C.

hydrolyzing amount of 70—80 mg ACh/g./hr., which is of the same order of magunitude as that estimated previously for some mammalian brains.

Table 8. Acetylcholinesterase Activity in the Slice and Homogenized electric Tissue of Common ray, *Raja pulchra Liu.* (Conditions: substrate. 0.01M ACh; gas, 95% N_2 and 5% CO_2)

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

Therefore, activity of AChE in the electric organ of the skate fish is more than 20 times as higher as those of brain and spermatozoa. Centrifuging 5 per cent homogenized electric tissue at a rate of 3000 R. P. M. for 10 minutes under low temperature (about 5° C), clear supernatant fluid was obtained. The results obtained are given in Table 9. This preparation splits 100—170 mg ACh/g. fresh

Table 9. Extraction of Acetylcholinesterase from the Homogenized Electric tissue of Common ray, *R. pulchra.*

Sample	Wet weight (mg)	CO_2 (μl/30 min.)	CO_2 (μl/mg/hr)
Homog.	50	237	8.90
Super.	40	145	7.23

Enzyme activity is represented μl CO_2 evolved per 100 mg wet tissue per 30 min. or 60 min. respectively. Homog. : 5% homogenized electric tissue with KRB's solution. Super. : cell free extract of 5% homogenized electric tissue centrifuged at a rate of 3000 R. P. M. for 10 min.

tissue/hr. The values show that the most of the enzymatic activity about 80 per cent may transmit to supernatant in the centrifuge of 5 per cent homogenate at a rate of 3000 R. P. M. Therefore, the ratio of the enzyme extraction is shown as the following table (Table 10).

Recently very active solutions were obtained from the electric organ of Japanese electric ray, *Narke japonica* with the powerful electric one.

Table 10. Rate of extraction of Acetylcholinesterase in the Electric Organ of Common Ray, *R. pulchra*.

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

Activity represents μl CO_2 evolved per 100 mg wet tissue per 10 min. Homogenate: 10% homogenized electric tissue with KRB's solution. Supernatant: supernatant of 10% homogenized electric tissue centrifuged at a rate of 3000 R. P. M. for 10 min.

It appeared interesting to compare the solutions which can be obtained from the electric organ of the electric ray with those of common ray, *Rajidae*. The preparation was carried out in the same way from the electric organ of *Rajidae*: about 500 mg of organ from the common ray were minced with an automatic machine. The minced tissue was ground with silicate and centrifuged for 30 minutes, about 500 ml clear supernatant fluid were obtained. One ml of this preparation split 325 mg, ACh in 60 minutes. The protein content was 13.6 mg per ml. One ml protein split therefore 25 mg ACh in 60 minutes.

In the preparations obtained in the same way from electric organ of *Narke japonica* 1 ml of the solution split 400—1500 mg ACh in 60 minutes; 1 mg protein split 100—200 mg. ACh per hour. After the ground tissue had been centrifuged and supernatant fluid removed 250 ml of 50 per cent ammonium sulfate solution were added to extract tissue. The tissue was ground again and

Table 11. Comparison with Acetylcholinesterase obtained from both Electric Organ of electric Ray and common Ray.

Fish	ACh hydrolyzed mg/mg. protein/hr	
	Cell-free extract	50% Amm. sulfate
Raja pulchra	23— 30	60—100
Raja tengu	40— 80	105—130
Narke japonica	100—200	200—300

centrifuged. The solution obtained in this way is more active per protein unit : 1 ml split 460 mg. ACh per hour and contained 3.85 mg. protein. One mg protein split therefore 130 mg ACh per hour. But solution prepared in this way from electric organ of *N. japonica* was also more active per protein unit :

1 mg protein hydrolyzed 2—300 mg ACh per hour. The electric organ of *N. japonica* appeared therefore to be more suitable material for the preparation of enzyme solution than that of *Raja*.

2. Concentration and Distribution

In addition to the high rate of its hydrolytic action, the enzyme must be present in concentrations adequate to account for significant amount of ACh split per unit time and unit tissue. Whatever the speed of the reaction, a very low concentration of the enzyme in muscle and nerve tissue would be prohibited before the assumption of its playing an essential role. Extensive studies on the concentration and distribution of AChE in electric tissue have been shown that the enzyme is not evenly distributed. Pieces of the slice were taken with the slicer successively at five positions from top to distal end of the organ, and activity of AChE was determined. The results obtained are given in Table 12.

Table 12. Activities of Acetylcholinesterase in slice of the Electric Organ of Common Ray, *Raja tengu*, taken successively from the top to distal end.
(Each value was determined on average of five cases)

D (cm)	Ww (mg)	Wd (mg)	CO_2 out put per hr. (μl)	QACh
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

Length of fish 85.0cm. Length of the electric organ 21.5cm. D: distance of section from the top of the organ in cm, Ww: mg. wet weight of slice, Wd: mg. dry weight of slice, QACh: mg. ACh hydrolyzed per mg. dry weigh: per hour

The highest activities of AChE are 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 (1955), the electric organ of the skate fish was microscopically observed consisting of five functional components of which electric plate, striated and alveolar layers were regarded as an electric disc. The electric plates are made of layer of protoplasm, containing numerous nerves which divide dichotomously as they pass backwards through a suporting connective tissue framework. Therefore, the greatest activities of AChE should be presumably at the electric plate which is at the innervated side of the disc. In order to get more information about the variations which may occur in a given piece due to uneven distribution of innervated surface, activities of AChE was

Table 13. Activities of Acetylcholinesterase in successive Slice of piece of the Electric Organ of Common Ray, *Raja tengu* at distance of 5 cm from top of the organ. About 100μ thickness of slice was prepared with the glass slicer.
(Each value was determined on average of five cases)

Slice No.	Wet Weight (mg)	Dry Weight (mg)	CO_2 output 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

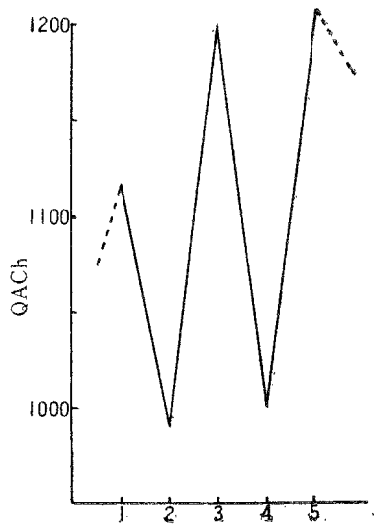


Fig. 1. Activity of Acetylcholinesterase in succesive Slice of piece of the Electric Organ of *Raja tengu* on the distance of 5cm from top of the organ. About 100 μ in the thickness of slices was prepared with glass slicer. Abscissae : QACh value, Ordinates: Number of the successive slice. (Each value was determined on average of five cases)

determined in successive slice of pieces of the electric tissue cut into about 100 μ thick with slicer at lower temperature. Pieces were taken at distance of about 5 cm from the top of the organ. The values obtained as shown in Table 13 and Fig. 1 were great varied from one slice to another in regular rhythm. It makes assumption that this rhythmical change is not incidental but should be corresponded to rhythmical change in active surface of the electric plate.

3. AChE activity in the caudal muscle connected with the electric organ

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

Since the time that Ewalt studied the ortogenetical developments of the electric organ of *Raja batis* there has been no doubt at the genetic relation between the two tissues, the organ and the *dorso-lateral* muscle of the tail. Especially the skate fish which is 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 the common skate. It was thought possible that AChE of the lateral muscle at the just front of the electric organ might be higher than that of

ventral- and *dorsal*-muscles located at the same portion. Therefore, AChE activities were determined in a few samples of the muscle at just front of the organ. The data obtained are given in Table 14. The relatively much higher

Table 14. Activities of acetylcholinesterase in the caudal muscle connected with the Electric Organ of Common ray, *Raja tengu*.

Muscle	W(w) (mg)	W(d) (mg)	CO_2 output hr μl	QACh
l	65	12.1	685.7	485
d	85	15.5	28.6	14
v	55	14.5	32.4	18

Each muscle preparation was taken from the portion at just front of the electric organ. l, lateral muscle; d, dorsal muscle; v, ventral muscle; W(w), wet weight; W(d), dry weight (Each value was determined on average of five cases)

activity of AChE, 485 mg ACh hydrolyzed per g. dry tissue per 60 minutes, was found in *"lateral"*. That is as higher as 35 times than that of other muscle. Conversely, in the *dorsal* and *ventral* muscle located at the same portion have lower activities than the former : QACh values were 14 and 18, at average of five cases, respectively.

4. Effect of addition of energy source

In order to give the enzyme solution the energy source, each sugar is added into the enzyme preparation. The values obtained are given in Table 15. There, however, is no effect for the activity of AChE in the electric tissue, even if

Table 15. Effect of addition of each sugar for Acetylcholinesterase in the electric organ of common ray, *Raja tengu*.
(ACh in the reaction mixture was determined colorimetorically by Hestrin's hydroxylamine method)

Sugars 10^{-2}M	Homog. concentration	CO_2 μl/mg/hr	QACh
Control	10%	64.3	1,762
Glucose		64.4	1,764
Control		64.8	1,766
Fructose	5%	61.4	1,741
Control		64.8	1,766
Glucose	5%	64.1	1,765
Fructose		64.0	1,761

the reaction solution had evoluted carbon dioxide and produced the lactic acid. It may be suggested that presence or absence of certain energy sources and change of metabolic pathway in the electric organ do not undergo for activity of AChE. Therefore, it makes assumption that the energy source for the formation of ACh

which is secondary component of ACh system (Nachmansohn) in the electric tissue should be concerned with such a catalysis that acetylkinase, acetyl coenzyme, choline acetylase and adenosinetriphosphate of these systems. The detailed description will be available in later references.

D. Specificity

1. AChE activity as function of enzyme concentration

As shown in Fig.2, the relation between concentration of the homogenized tissue and its esteratic activity is rightly demonstrated with accurate correspondence. Curves in the figure show that the more the enzyme concentration the lower its activity contrary. Therefore, an amount of ACh equivalent split by the homogenate decreases according to their concentrations. Enzymatic action gives usually direct proportinality between the rate of hydrolysis and the enzyme concentration. Such a relationship was found that the enzyme concentrations were not too high at low enzyme concentration, the proportionality was always obvious, when the substrate was continually in optimum concentration (Fig. 3).

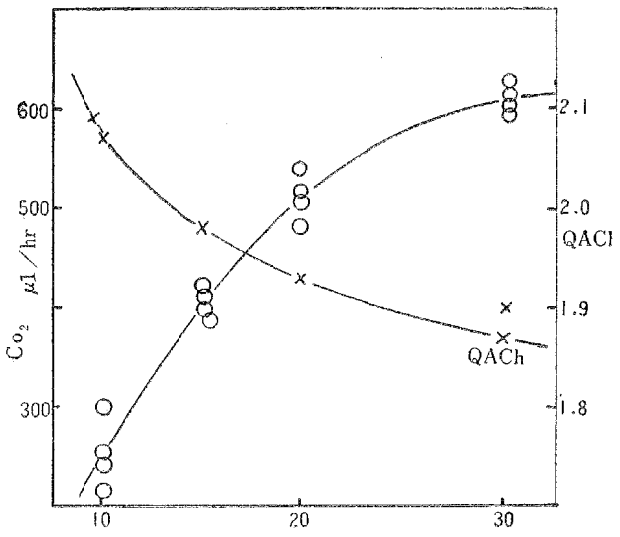


Fig. 2. Rate of enzymatic hydrolysis of acetylcholine by electric organ of *Raja pulchra* as function of enzyme concentration. Abscissae: Concentrations of homogenized electric tissue with KRB's solution(%). Ordinates: left, QACh, mg ACh hydrolyzed/g./hr. right, activity, mm^3 carbon dioxide out put/g/hr.

Previous investigator dealing with this relationship is, for example, those by Abdon and Uvnas (1937).

According to Augstinsson (1948), with increasing enzyme concentration a point was reached when no excess of substrate was present, when the enzyme concentration was increased further, the reaction rate would not be expected to change. Moreover, there may not be any direct proportinality between reaction rate and enzyme concentration, if the enzyme concentration contains impurities which related the action of enzyme. If such a case the reaction rate decreases gradually with increase in enzyme concentration. Such facts also may explain the deviation from proportinality. In most cases, however, it was difficult to draw conclusions as to whether the deviation was due to one or the other of these factors. Therefore the majority of reactions studied has shown

direct proportionality between reaction rate and enzyme concentration.

These phenomena obtained in Fig. 2 lead to the assumption that the stocks of CO_2 evoluted or chohesion of protein molecule inhibits the esteratic hydroysis by the enzyme.

2. AChE activity as function of substrate concentration

The study of the activity-substration relationship has contributed greately to our knowledge of AChE. This relationship was used as one of the first arguments for differentiating between two differnt types of ChE. This the specific ChE is said to be inhibited by excess of substrate which is not the case with the other type, the nonspecific.

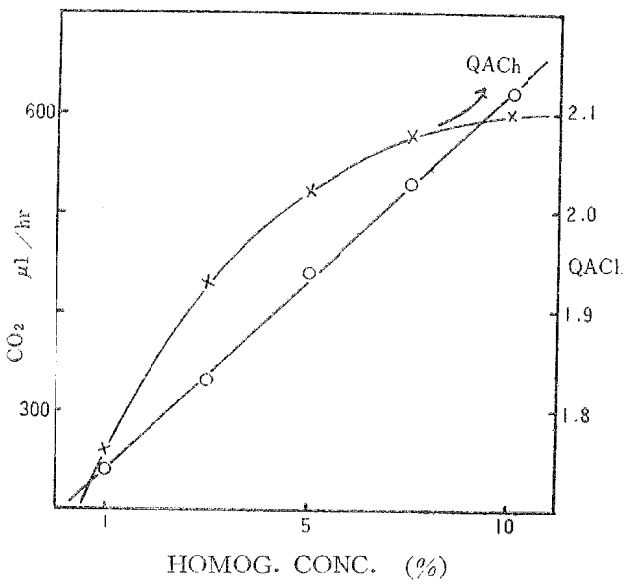


Fig. 3. Rate of enzymatic hydrolysis of acetylcholine by electric organ of *R. pulchra* as the function of enzyme concentration. Description and symbols are in Fig. 2.

Before research had gone so far, several investigations were carried out in order to find out how the reaction velocity is influenced by the concentration of the substrate. The widely accepted theory of Michaelis and Menten has also been applied to ACh-ChE system. The enzyme studied was the nonspecific ChE (blood, serum) in all cases. The dissociation constant (k_3) of the supposed intermediate compound between ChE and ACh was first determined by Glick (1937) who found $k_3 = 1.1 \times 10^{-3}$ (human serum).

According to Eadie (1942), this constant is 1.7×10^{-3} (dog serum), to Wright and Sabine (1943) 0.25×10^{-3} (dog serum) and to Goldstein (1944) 1.25×10^{-3} (dog serum). The constant has been determined also for the enzyme complexes of arsenocholine and butylcholine (Roepke, 1937). Since the observation by Alles and Hawes (1940) that the ChE activity of red blood cells is depressed by excess of ACh which is not the case with the serum activity, more studies were performed on the activity-substrate concentration of ChE. ACh only has been employed as substrate, except in an investigation by Augustinsson (1946), on the ChE of *Helix* blood.

Mendel's group indicated that this relationship is not a criterion by which ACh splitting enzymes can be differentiated. The shape of the activity-substrate concentration curve cannot serve as a basis for distinguishing specific from non-specific cholinesterase. As regards the optimum substrate (ACh) concentration, contradicting results have been obtained by various authors. Thus Alles and

Hawes (1944, '45) found that the erythrocyte ChE displayed its optimum activity at ACh concentration of pS 4.6 (pS = −log molar conc.). A similar value has been obtained by Mendel and Rudney (1943, '44) with the brain ChE in the absence of any salt other than $NaHCO_3$. These results contrast with the findings of Zeller and Bessegger (1943). The pS values at which optimum activity is displayed by the erythrocyte ChE were found by these authors to be 2.5 and 2.6 respectively; the values for the brain enzyme were 2.4, 2.0, and 2.5 respectively. In our opinion the difference between the high values of pS obtained by the former groups of workers and the low ones by the latter may be due to the different experimental conditions and to the methods used.

These disagreements have been discussed by Mendel and Rudney (1945) in a paper dealing with the effects of certain salts on the specific ChE (brain, erythrocytes). It was found that the relationship between ChE activity and substrate concentration was changed when salts, especially potassium chloride, were added to the medium. KCl in a concentration of 0.16 M caused a shift of optimum activity of the enzyme (brain) from pS 3.60 to pS 2.52. Moreover, these authors found that KCl reduced the activity at lower and increased it at higher concentrations, that is, the new optimum corresponded to higher activities than in that of any salt. This fact was said to bring "to light the cause of the discrepancy".

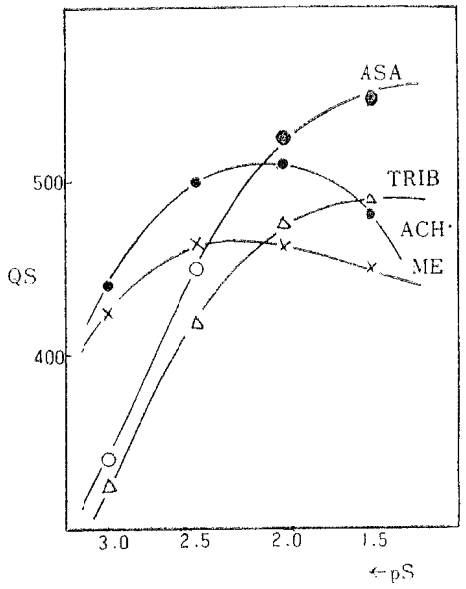


Fig. 4. Activity-pS curves for enzymatic hydrolysis of ACh, ASA, TRIB, and ME by a homogenized suspension of electric organ of *Narke japonica*. Abscissae: pS, negative log of molar concentration of substrate. Ordinates: QS, mm^3 carbon dioxide output/60 min.

Confirming previous findings, the system ChE-ACh gave an activity-pS curve of the familiar shape of a dissociation curve. In this type enzyme a specific ChE, the higher the substrate concentration is the more the activity increase, showing S-curve.

The activity-pS curve for AChE hydrolysis of MeCh showed a lower value of pS at very high substrate concentration the activity was decreased. Because of that, MeCh at high concentrations was hydrolyzed at a higher rate than ACh at the same high concentration, when the substrate concentration in both cases was 0.1 M, the ratio between the reaction velocities for ACh and MeCh was 0.57 in 0.001 M solution, on the other hand, this ratio 3.34. In comparison of the hydrolysis of ACh and others, therefore, it is important to consider these differences, previously not taken notice of.

It may explain the finding by Richards and Cutkomp (1945) and by Tabias, *et al.*, (1946) that insect nervous system is more active on MeCh than on ACh.

The activity-pS curve for the hydrolysis of AsaCh, as shown in Fig.5, has quite another shape than in cases of ACh or MeCh. The rate of reaction was not decreased by high substrate concentration and a familiar S-shaped curve was obtained. Electric organ hydrolyzed TB at a low rate. The enzyme responsible for this reaction is not the same as that catalyzing the hydrolysis of choline ester. It was established that the TB hydrolysis was not inhibited by excess of substrate (Fig. 4).

As shown in Fig. 6, the hydrolysis of ACh through the action of the electric tissue was decreased by high concentration of the substrate. The activity-pS curve is bell-shaped and is almost complete, thus indicating by Augustinsson, who is well fitted to this reaction.

The results obtained with electric organ extract were much same as these described for the enzyme activity of brain and blood cells (Fig. 4). All data favour the supposion that the ChE of electric organ and that of brain and erythrocytes are identical.

AchE activity of the organ was inhibited by high ACh concentration and bell-shaped activity-pS curve was symmetric (Fig. 6), indicating that Hadane's theory of complex pS is true for this AChE. The value of pSopt was 2.5 (0.0045 M ACh), very near value of erythrocyte enzyme(2.25)and brain (2.46)(Augustinsson, 1946) and is only higher than that of specific type of ChE in other animal tissue (*Sepia* liver 2.6, *Helix* blood

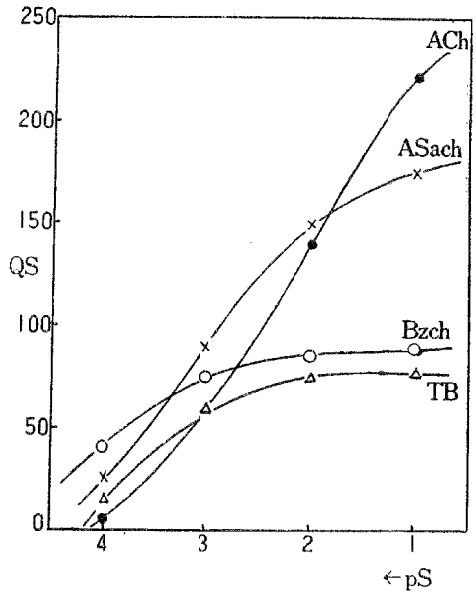


Fig. 5. Activity-pS curve of different esters by a purified ChE preparation from horse serum. Abscissae: pS (negative log of molar concentration of substrate). Ordinates: QS, mm^3 carbon dioxide output/30 min.

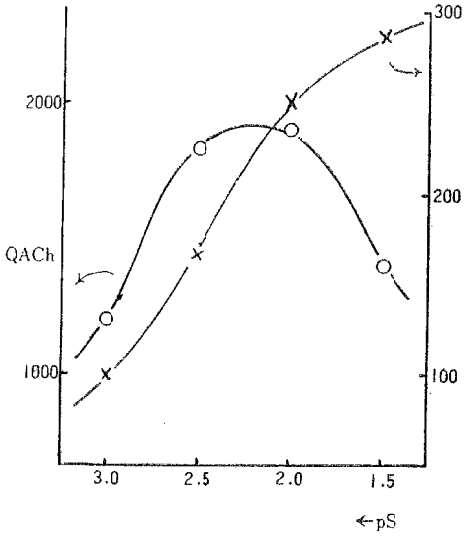


Fig. 6. Activity—pS curves for enzymatic hydrolysis of acetylcholine by electric organ of *Narke japonica* and horse serum respectively. Abscissae: pS (negative log of molar concentration of substrate). Ordinates: QACh, mg ACh hydrolyzed/mg. protein/hr.
○—○ homogenized suspension of electric tissue of *N. japonica*.
×—× purified cholinesterase from horse serum.

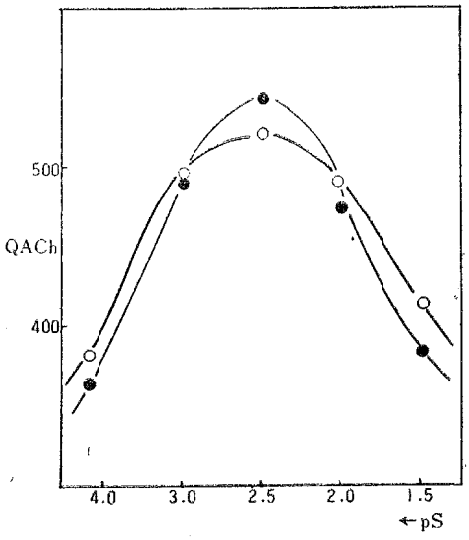


Fig. 7. Activity—pS curves for enzymatic hydrolysis for acetylcholine by electric organ of *Narke japonica*. As shown in the figure, optimum concentration of the substrate is pS 2.4 (0.0045M ACh) which is only higher than that (0.002M)of specific type pf cholinesterase in other animal tissue. QACh, mg ACh hydrolyzed/mg.protein/hr.
○—○ 5% homogenized suspension of electric tissue.
●—● 10% homogenized suspension of electic tissue.

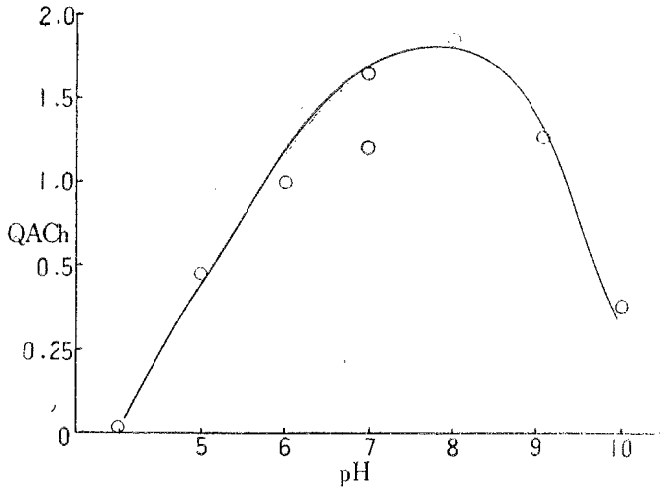


Fig. 8. Activity—pH curve from the enzymatic hydrolysis of acetylcholine by the enzyme preparation from the electric organ of commom ray, *Raja pulchra*.

2.65). Optimum pH-activity curve shown in Fig. 8 is about 8.

E. Comparison of QACh value Determined by both methods

Table 16 shows hydrolyzed ACh mg per g. per hr. determined by both the Warburg's manometric and Hestrin's colorimetric method. As soon as estimated manometrically, the rest of the

Table 16. Comparison with QACh value determined by both the manometric and the colorimetric method.

Hestrin's Hydroxylamic method		Warburg manometric method	
Sample	QACh	Sample	QACh
5% Homogenate	1,629	5% Homogenate	1,636
//	1,621	//	1,649
//	1,600	//	1,654
//	1,547	//	1,589
//	1,433	//	1,682
Ave.	1,497		1,636

reaction solutions were kept at lower temperature then soon determined colorimetrically. It is noticeable that the later shows lower QACh value than that with the former method, as if it were synthesized ACh, instead of

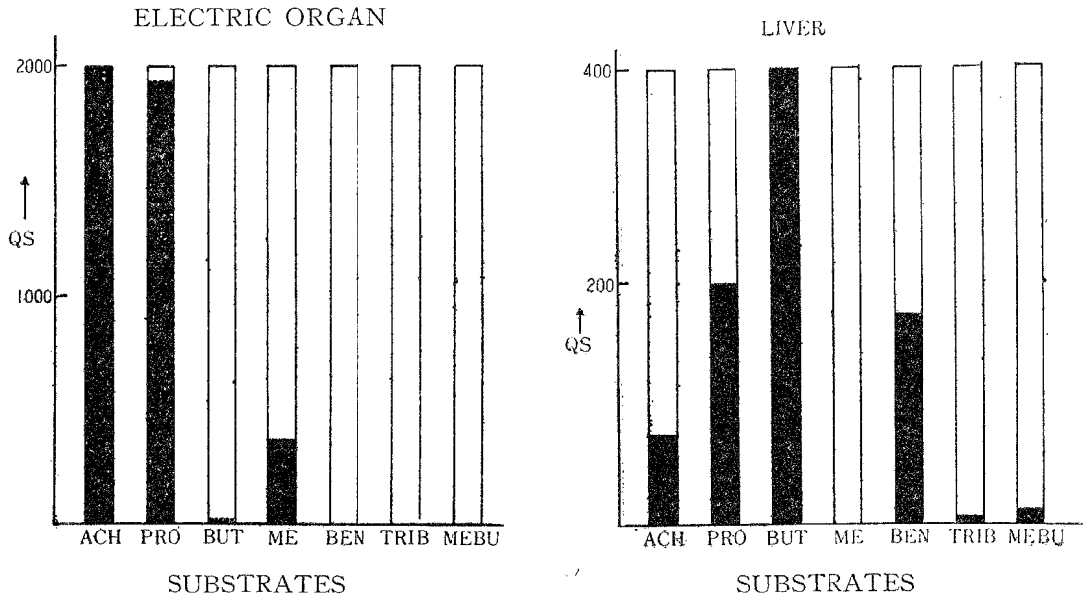


Fig. 9. Pattern of acetylcholinesterase (electric organ of *Narke japonica*) in presence of different substrates compared to that of an esterase (liver of *N. japonica*) no specific for acetylcholine, The columns represents QS, mg. substrate hydrolyzed g. dry tissue/hr. Abbreviation: ACh acetylcholine, PRO propionylcholine, but butyrylcholine, ME Acetyl-β-methylcholine, BEN benzoylcholine, TRIB tributyrin, MEBU methylbutyrate

hydrolyzing. It makes possible assumption that small amount of certain ester which may exist biological activity also reacts with hydroxylamic acid. Therefore, Hestrin's method may be not only the specific for ACh but for the related esters.

F. Consideration

AChE is present in all conductive tissues throughout the whole animal kingdom. It has been demonstrated in all types of nerve : vertebrate and invertebrate, motor and sensory, central and peripheral, so-called cholinergic and adrenergic, and in muscle free nerve. AChE occurs in *Tubularia*, the lowest Hydrozoa to possess neuromuscular tissue comparable to that of higher animals (Bullock, Grundfest, Nachmansohn, and Rothenberg, 1947).

Some properties of AChE in the electric organ were essentially similar to those described for that of nervous and muscle system. However, the QACh is about 20—50 times as higher as that of brain and spinal cord of the mammalia. Namely, these organs may split in 60 minutes as amount of ACh equivalent to 2—5 times as their own weight.

The outstanding feature of the enzyme from the physiological point of view is the high speed at which the enzyme hydrolyzed ACh. According to the brilliant investigation by Rothenberg and Nachmansohn, the turnover number is of the same order of magnitude of 200 millions per minute. The figure has been

obtained on the basis of experiments and enzyme solution prepared from electric tissue of *Electrophorus elctricus* which showed only one component in the ultracentrifuge. The molecular weight was estimated on the sedimentation rate. The data indicate that one molecule of AChE hydrolyzes one molecule of ACh in about few minutes.

Any mechanism of a living cell is, only all available evidence, connected with a whole chain of reaction. Several compounds have been associated with nerve activity and the extra oxygen uptake observed is sufficient evidence that the passage of the impulse must be associated with a great number of reactions. But no other chemical reaction known to be associated with nerve activity has a comparable speed been demonstrated. The extraordinary speed at which ACh may be metabolized is of paramount importance for evaluating its possible role in conduction. On the basis of the electrical signs it must be postulated the primary process at each point, where the impulse passes, occurs within less than 100 microseconds. The prequisite for any assumption is therefore a similar speed of the reaction. The high turnover number of AChE satisfies this postulate and makes it possible to associate this specific reaction with the primary event of conduction. The esterase-ACh system is, therefore, the only one which at present, can be associated directly with the electrical event.

III. BIOSYNTHESIS OF ACH IN THE ELECTRIC ORGAN OF THE FISH

As early as 1946 Nachmansohn and his associates found during the experiment which based on the energy transformation involved and on the thermodynamic considerations, that ACh was formed anaerobically. It appeared crucial to test whether or not energy-rich phosphate bonds were really the energy of the primary recovery process of conduction of the impulses is really used for the resynthesis of the compound which, by its release, supposedly initiates the impulse. It would, therefore, at the same time, constitute a new support for the assumption that the primary "excitatory disturbance" which produces a propagated impulse may, indeed, be the release of the ester.

In confirmation of this assumption, a new enzyme, choline acetylase, could be extracted from brain in cell free solution, which, under strictly anaerobic conditions, in presence of adenosine triphosphate (ATP), forms ACh. Phosphostigmine and fluoride must be present in order to inhibit the action of AChE and ATPase respectively.

A. Preliminary aspect of acetyl-activator

Previously the author described the specific type of ChE and AChE extracted from the electric organ of Japanese electric fish. This organ can split ACh more than 20 times as fast as boar spermatozoa and some types of mammalian brain (cf. Sekine, 1953). Recently, it has been noted that in the electric organ, AChE rapidly splits ACh into choline and acetate. Thus AChE catalyses the reaction:

$$\begin{pmatrix} CH_2O - COCH_3 \\ | \\ CH_2 - N(CH_3)_3 \end{pmatrix}^+ + HOH \longrightarrow \begin{pmatrix} CH_2OH \\ | \\ CH_2 - N(CH_3)_3 \end{pmatrix}^+ + CH_3COOH$$

It has now become clear that acetylation of aromatic *n*-acetylamine and acetoacetate are the same reaction throughout a coenzyme-enzyme system which is not reacted with "liberated" acetate but it reacts smoothly on "active" acetate, acetyl-coenzyme A.

After that, the author has been considering the acetylation of choline by the electric tissue should be activated by certain cofactor, such as acetylation of sulfanilamide by liver (Lipmann), and also biological oxidation of cozymase or flavin nucleotide.

As shown in Table 17, it was evident that ACh was clearly synthesized

Table 17. Acetylcholine synthesis by the extract of the electric organ of electric ray, *Narke japonica*.

Additions	ATP (μM/ml)	CoA (mg/ml)	ACh synthesized (μg/g/hr)
Acetate + Choline	0.33	2	0
Acetate + Enzyme	0.33	2	0
Choline + Enzyme	0.33	2	0
Acetate + Choline + Enzyme	0.33	—	0
Acetate + Choline + Enzyme	—	2	0
Acetate + Choline + Enzyme	0.33	2	160

All tubes contained, 0.0065M KCl, 0.00075M $MgCl_2$, 0.0017M $CaCl_2$, 0.0025M Na_2HPO_4, 0.0003M NaCl, 0.6M NaF, 0.26M *l*-cysteine, 10γ. eserinesalycilate and in addition sodium acetate 0.05M, sodium ATP 0.03M, choline chloride 0.002M, coenzyme A (0.5unit) and 0.9ml of the enzyme extract were added as indicated above. The final volume of the reaction mixture was made of to 3.0ml with water.

under the existence of coenzyme A and ATP as an energy source, whereas it was not synthesized by only the enzyme and substrates.

As mentioned above, it is necessary to form Ac-CoA that major condition of the acetylation of the substrates is performed.

Now, figures are given to explain of the correlation between pyruvate formate

exchange which is formed citrate into TCA cycle (Fig. 10). Present experimental data show following sparking effect under the presence of Mg and phosphoacetokinase, energy rich phosphate bond is produced between ATP and SH-bond of β-meracptoethylamine which is functional component of acetylation coenzyme.

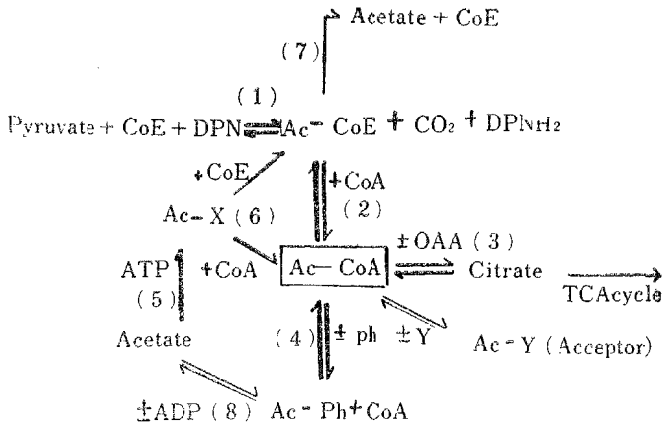


Fig. 10. Map of acetyl transfer "territory"

After that, changing the acetyl bond of citric acid, resulting of it, formation of acetyl coenzyme A (Ac—CoA) is performed (Fig. 10, (5) & (6)). This acetyl coenzyme A, acetyl receptor at the existence of cholineacetylase and acetyl-acceptor of choline was biosynthesized into ACh.

In these experimental facts that second components of ACh cycle (Nachmansohn) and chiefly concerned with the biosynthesis of both substrates, choline and acetate, which are productive substance of hydrolysis of ACh in the presence of high concentration of AChE in the organ.

So-called "choline acetylase" extracted from the nervous tissue by Nachmansohn (1943) catalyzed ACh formation :

$$\text{Acetate} + \text{CoA} \xrightarrow[(\text{ATP})]{} \text{Acetyl-CoA}$$

In this reaction ATP is necessary to only energy source for biosynthesis of ACh, separating from AChE system.

In last steps of the resynthesizing reaction of ACh, thus the cholineacetokinase catalyzes the reaction ;

$$\text{Acetyl CoA} + \text{Choline} \longrightarrow \text{ACh} + \text{CoA} \quad \cdots\cdots (1)$$

In former steps of this reaction, thus phosphoacetokinase catalyzes the reaction ;

$$\text{Substrate} + \text{CoA} \longrightarrow \text{Acetyl—CoA} \quad \cdots\cdots (2)$$

The results obtained in the present experiment (Table 17) show both activity combined the reaction (1) catalyzed by phosphoacetokinase and (2) by choline acetokinase. Therefore it may be said "choline acetylase" of the electric organ, in wide sence. Hence, it became clear that acetylation coenzyme closely connected with acetylation of choline was investigated. The results obtainted

are shown in Table 18, that reversible inactivation of choline acetylation system of the electric organ throughout autolysis. Namely as acetylation of choline in

Table 18. Reversible inactivation of choline acetylating system in the electric organ of the fish, throughout autolysis.

Treatment of extract	Filtrate of boiled organ added, corres. to g.	Choline conjugated (γ)	Fish
untreated		142	
kept 10 hrs, 24°C		0	
	200 mg. cat liver	111	*Raja kenojei*
untreated		125	
kept 10 hrs, 24°C		0	
	200 mg. electric organ	119	
untreated		125	
kept 10 hrs, 24°C		0	
	200 mg. cat liver	89	*Narke japonica*
untreated		128	
kept 10 hrs, 24°C		0	
	200mg. electric organ	98	

The electric organ extract, treated or untreated, was incubated in Warburg's vessels in water bath at 37.5°C. The vessel contains 0.9ml of extract in a total volume 3ml; salts buffer and sodium acetate were presented 0.005M concentration.

The extract was combined with activator, and, after adaptation to the bath temperature, the experiment was started through addition of 0.33M of ATP. 0.002M of choline chloride, 10γ/ml of eserine salycilate, 0.22M of cysteine, and 0.01M NaF.

the organ is inactivated through aging enzyme, being regained by the additions of filrate of boiled organ, this appearently indicates the properties of coenzyme activating factor of the choline acetylation system in the organ.

In 1948, during the study on sulfonamide acetylation in pigeon liver extract Lipmann and his co-workers noted that towards a solution of puzzling problem of metabolic acetate utilization. It appeared that a coenzyme was involved in this process. On dialysis as well as on aging, the enzyme solution lost ability to acetylate, which was regained on the isolation of this, appearently new coenzyme was therefore attempted. On purification it appeared that new coenzyme, CoA, was pantothenic acid derivative (Lipmann *et al*, 1948). In order to certify how to react between the phosphoacetokinase prepared from the rat liver and acetylating activator from the electric organ of *Narke japonica* respectively, experiments of the effect of crude activator extract on aged electric organ for both choline and sulfanilamide acetylation, aged rat liver enzyme was used. Results obtained are shown in Fig. 11.

Therefore, acetylating co-factors which extracted from the electric organ should be identical with acetylation coenzyme commonly such as in all the living

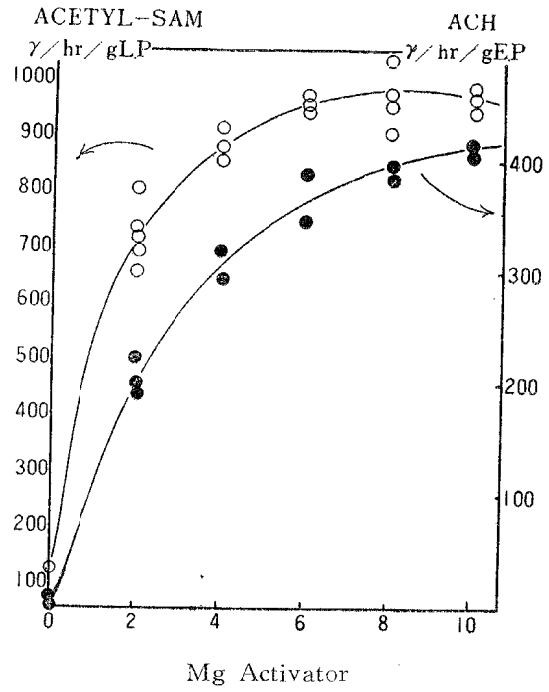


Fig. 11. Comparison of the effect of same activator preparation on aged electric organ extract for choline acetylation and aged cat liver extract for sulfonamide acetylation. Abbreviation: ACETL-SAM acetyl sulfonamide, ACH acetylcholine, LP cat liver powder, EP electric organ powder

organisms. This makes possible assumption that acetylation coenzyme is not only to play the part of pathway on TCA cycle, which is contained great important metabolism of the living organisms, but also closely connected with biosynthesis of ACh and other choline ester which hold an important position of the activity of cell and tissue. Therefore, further research of these problems must be pursued, that not only problems of the energy source to TCA cycle but also controling mechanisms contained acetylation coenzyme such as regulating the pathway on their metabolism and function.

The present experiments were pursued preliminarily under the assumption that choline should be readily acetylated by the action of that coenzyme linked enzyme system in the electric organ of the fish which has such role of the condensing enzyme (Krebs) that pyruvate-citrate reaction in TCA cycle, in which ATP serves as an energy source.

B. Assay and distribution of Coenzyme A in the electric organ.

The discovery of an acetyl-activator which the longer the dialysis is carried on, the weaker is the reactivation by the preparation mentioned has revealed the position of ACh in general metabolism. The experiment has been suggested that these enzymatic reactions should be involved acetylation coenzyme (CoA), a pantothenic acid derivative. This observation also characterized the enzymatic acetylation of aromatic amide(Lipmann, 1948), which ATP as a condensing agent, as being due to an apoenzyme-coenzyme system.

1. Preparation of the electric organ extract.

For the preparation of the electric organ extract of *Narke japonica*, fish was killed as completely as possible, homogenizing the organ at lower temperature (4°C), and making the powder of acetone dried organ. The powder is

eventually dried in a vacuum desiccator over phosphorus. Passing the powder through a sieve, from 0.5 to 1.0 g. of white-yellow powder are obtained from 100 g. of fresh electric organ, corresponding to one to two electric fishes.

In these experiments, the white color is a helpful index for the activity of the preparation. A brown-yellowish acetone powder results which invariably has poor or no activity, although cysteine reactivates and stabilizes the acetylation enzyme.

2. Preparation of Aging extracts.

The electric organ extract is prepared by carefully rubbing up 10 g. of the organ powder with 100 ml. of salts buffer (0.06M KCl, 0.00075 $MgCl_2$, 0.0017M $CaCl_2$, 0,0025M Na_2HPO_3 and 0.0065M KCl). About 50ml of white-yellowish obtained have now to be kept frozen overnight by deep freezing. The insoluble was removed by centrifugation at a rate of 3000 R.P.M. for 15 minutes. In crder to inactivate the extract, let it alone at 25°C for 4 hours. More complete inactivation is obtained by autolysis for 4 hours at room temperature. After aging, the extract is recentrifuged, and insoluble material discarded, then kept again by deep freezing to precipitate a large amount of ATP-ase.

3. Determination of Coenzyme A Lipmann Unit

i) Coenzyme A Lipmann Unit

Lipmann and his associates (1948) determinated CoA unit by his sulfanilamide-acetylation system *"Such an amouut, therefore, as well activate our system the maximum activity is defined as 1 unit of coenzyme A"*. The unit is largely independent of the individual enzyme preparation, even if the absolute amount of acetylated sulfanilamide may vary. The CoA unit *contains 0.7γ bound pantothenic acid* and is independent of the purity of preparation.

ii) Preparation of assay

For the assay of CoA in the organ, the solution was prepared by quick boiling fresh minced organ of the fish,(*Narke japonica*)with 3 volume of water. To make a dry preparation of CoA, this solution is brought to *pH* 2 with 0.1 N hydrochloric acid and then was precipitated with 1 volume of acetone. This preparation was washed with acetone and ether and dried. This preparation was very hygroscopic in air and deteriorates on wetting, and then kept in desiccator or in a freezer.

4. Assy procedure

In order to determine the activity of CoA prepared by the electric, it is necessary to test coenzyme A unit under the Lipmann's acetyl-sulfanilamide system.

Namely, for the routine assay of the coenzyme A, the following assay reaction mixture were prepared : 10 ml. of 0.04 M sulfanilamide,

2.5 ml. of M sodium acetate

8.0 ml. of ATP approximately 0,05 M

10 ml of 0.2 M sodium citrate.

(The *pH* of the mixture should be around 7).

To each Warburg vessel there are added (1) the solution to be tested coenzyme activity 0.2 ml., (2) 0.3 ml. of the assay reaction mixture, (3) 0.08 ml. of a freshly prepared M sodium bicarbonate solution (4) 0.1 ml of 0.1 M cysteine hydrochloride (5) 0.25 ml. of the aged enzyme (6) 0.4 ml. of 10*r*/ml. of eserine salicylate (7) volume of water.

A blank is prepared by adding all the constituents except coenzyme A. The experiments were carried out in a gas mixture of 95 per cent N_2 and 5 per cent CO_2. The vessels are shaked, and incubated for 2 hours in a water bath of 37°C. The reaction is halted by adding 4 ml. 5 per cent trichloroacetic acid. After centrifugation, aliquots of 1 ml. are taken for sulfanilamide determinations, and carried out by the procedure of Bratton & Marshall. Reading was made on the photoelectric colorimeter, with filter 540 mμ. The difference in sulfanilamide between the blank and the tubes containing coenzyme represents the amount of sulfanilamide acetylated. In these systems, such crude coenzyme preparations contain about 0.26 unit per mg. of dry matter. The results obtained in analysis of the electric organ were in remarkable agreement with the Lipmann's standard coenzyme A by pigeon liver preparations which contain 0.25—0.5 unit per mg. of dry matter.

5). Isolation of Coenzyme A from the Electric Organ

The isolation of CoA from the electric organ of *Narke japonica* was followed throughout the purification by the assay method described by decapitation and the organs quickly removed and chilled. The organs are minced or homogenized with the appropriate volumes of water and rapidly boiled. The temperature was not allowed to drop below 80°C. After completion, the mixture was simmering for 15 minutes more. The juice was passed through muslin.

i) Deproteinzation and mercury precipitation———

The juice was cooled and deproteinized with 5 per cent trichloroacetic acid. The filtrate was, as quickly as possible, neutralized to pH 5 and the coenzyme precipitated with a slight excess of mercuric acetate. The precipitate was collected after settling overnight, and decomposed with hydrogen sulfide.

ii) Aceton fractination and barium precipitation.

The aerated filtrate from mercuric sulfide was neutralized to pH 4 to 5,2 volumes of acetone were added, and the precipitate discarded. Another 7 volumes of acetone were added, and after being kept overnight in the cold, the oily yellow deposit was collected by decantation. The oily deposit was dissolved in water, neutralized to pH 8.4 to 9, and barium acetate was added, followed by 2 volumes of acetone. The precipitate was dried with alcohol and ether. one hundred mg. of electric organ gave *800* mg of barium salt of *0.8* units per mg.

iii) Lead, silver, and second barium treatment——

The barium salt was dissolved by addition of N acetic acid to about pH 4, a dirty residure was discarded, and a slight excess of lead acetate was added. The lead precipitate was decomposed with hydrogen sulfide reextracted with addition of sodium acetate to bring it to pH 4.5. The combined filtrates were brought to pH 4.5 and silver nitrate was added. The silver was removed with hydrogen sulfide and the filtrate brought to pH 8.5 to 9. Barium acetate was added, followed by half the total volume of alcohol. The precipitate was collected and dried with ether. From about 1000 g. electric organ were obtained 950 mg of this barium salt of 10 units per mg.

Table 19. Coenzyme A in tissues of electric ray, *Narke japonica*.
(All values are given in unit of CoA per g. of fresh tissue).

Organ	CoA per g. fresh weight
Liver	180
Adrenal	83
〃 demedullated	70
Kidney	95
Brain	83
Heart	51
Testis	23
Intestine	23
Thymus	25
Skeletal muscle	6
Plasma	0—1
Electric organ	98
Red blood cells	0

iv) Coprecipitation with barium sulfate and elution.

The barium salt was dissolved to make one per cent solution with N acetic acid and a small residure discarded. For every 100 ml., 6 ml. of 6 per cent barium acetate were added and 6 ml. of 0.2 M sodium sulfate dropped slowly in with agitation. Barium sulfate was thus precipitated in the presence of a considerable excess of barium ion. The combined barium sulfate precipitates were extracted with 0.2 M sodium sulfate in 0.1 N acetic acid. Approximately 25 ml. are used per g. of original barium salt. The extraction was carried out at 50°C with vigorous stirring, and repeated three times. The combined eluates were cooled, the solution made 0.8 N with concentrated hydrochloric acid, and an excess of phosphatungstic acid was added. The precipitate was washed carefully three times with acidified acetone and dried with ether. The activity of this compound averaged close to 30 units per mg.

v) Third barium treatment and precipitation with acetone HCl——

One per cent solution of the compound was brought to *pH* 8.5, and the CoA

precipitated with barium acetate and an equal volume of alcohol. Eight hundred and fifty mg. of barium salt were obtained with an activity of *55* units per mg. The barium salt was dissolved with a comfortable excess of hydrochloric acid, and 5 ml. of N sulforic acid were added. After removal of barium sulfate, 9 volumes of cold acetone were added, and the white precipitate was dried with acetone and ether. This preparation was whitish. The total yield from *5000* g. of electric organ was *850* mg., and activity of *85* units per mg.

vi) Unit activity of coenzyme A compared with pantothenic acid content—

According to Cheldelin's observation which PTA value by test with *Lactobocillus arabinosus* increased slightly on digesting with clarase and papain

Table 20. The culture base of *Lactobacillus arabinosus** for the quantitative analysis of pantotheric acid.
**L. arabinosus* was kindly supplied by Mr. Maeda.

Component	*L. arabinosus**
Casein hydrolysate	1g
Glucose	49g
Na-acetate	1.2g
Tryptophane	20mg
Cystine	20mg
Adenine sulfate	1mg
Guanine HCl	1mg
Xanthine	1mg
Salt	2cc.
Thiamine	200γ
Riboflavin	200γ
Pyridoxine	400γ
Nicotinic acid	200γ
Biotin	0.5γ
P-Amino-benzoic acid	20γ
pH	6.6—6.8
H_2O	100cc.

suggested the possibility that the enzymatic treatment with clarase and papain, as used in the routine assay for pantothenic acid (PTA), might liberate only very slowly the PTA from CoA. Therefore, new assay proposed by Lipmann's group was : The sample was subjected to acid hydrolysis and then tested for the presence of β— alanine, with yeast as the test organism. The yeast assay revealed then the presence of amounts of β—alanine in CoA corresponding to a pantothenic acid content of 11 per cent.

As to the purification of CoA, it is necessary to determine not only CoA unit activity but also the pantothenic acid (PTA) value tested with *L. arabinosus*:

$$\text{PTA (Degree of Purity)} = \frac{\text{CoA-Bound PTA}}{\text{Sample (Dry Weight)}} \times 100 \text{ (PTA\%)}$$

or 1 Lip. Unit/mg=0.065 PTA%

1 M CoA=800±50 (766?)

PTA=1/4M CoA

$$\text{CoA (Degree of Purity)} = \frac{\text{CoA}}{\text{Sample (Dry Weight)}} = 4 \times \text{PTA (Degree of Purity)}$$

Lip. Unit/mg=0.26 CoA (Degree of Purity) (%).

Each CoA preparation, ranging in potency from *0.5* to *85* units per mg., was subjected to enzymatic treatment and PTA was determined microbiologically by the method of Skeggs & Wright (1944). Table 19 shows the Unit activity of CoA compared with PTA content.

C. Cholineacetylase

As early as 1926 Abderhalden & Paffrath found during experiments with the small intestine of pig that the ACh-hydrolyzing enzyme has a synthesizing effect strongest at *pH* 6, as opposed to a hydrclyzing one. These results have been later confirmed in experiments with pig serum (Shaw, 1935), embryonic extracts (Ammon & Kwiatkowski, 1934), leech extracts, and humann serum (Kwiatkowski, 1936). In respiration experiments with brain slices, (Quastel, Jennenbaum, & Wheatly 1936) obtained a substance which was supposed to be a ChE ; they did not find any connection between the ChE concentration of various tissues and the ability of synthesizing choline esters. In all these instances, the synthesis took place aerobically. It is certain that ACh is synthesized during the electric stimulation, for example of the superior cervial ganglion, but it is as yet not clear whether the ACh formed is identical with that liberated at the nerve endings or synapses. This synthesis at the cervial ganglion is not inhibited by physostigmine ; therefore it is doubtful whether this process is catalyzed by ChE. The formation of ACh is increased by adding glucose, lactic acid, and pyruvic acid. These substances of carbohydrate metabolism have no influence on the ChE activity, but they are sources of the acetyl group needed for the synthesis of ACh. The phospholipids are the origin of choline. Nachmansohn & Machado (1943) have shown that ACh is also formed anaerobically. They have extracted from brain a new enzyme, choline acetylase, which, under strictly anaerobical conditions in the presence of ATP and K, forms ACh in cell-free solution. Physostigmine and fluoride must be present in order to inhibit the action of ChE and ATP-ase respectively.

Ochoa (1941) has shown that fluoride inhibits the activity of ATPase without interfering with the transfer of phosphate to phosphate acceptor. This may be

due to the precipitation of calcium, since it is known that calcium activates.

Extraction prepared from fresh tissue is very unstable and the rate of formation decreases rapidly. When, however, extracts were prepared from acetone-dried powder, a much more stable solution was obtained moreover, in this way, choline acetylase is separated from most of the ACh and all of the ATPase, since treatment with ACh inactivates these two enzymes.

1. Preparation of Choline acetylase in the electric organ of *N. japonica*.

i) Slice or homogenized electric tissue

Working first, the preparation of ChAcE in the electric organ with slice or homogenized electric tissue were used. In addition to ATP as energy source, and choline and acetate as substrates, fluoride and eserine must be present in order to inhibit the strong activity of AChE or ATPase, eserine and fluoride should be added respectively to its reaction system. Ochoa has shown that fluoride inhibits the action of ATPase without interfering the transfer of phosphate to a phosphate acceptor, since it is known that fluoride promotes the precipitations of calcium which activates ATPase. In spite of these conditions, the slice or homogenized tissue preparation found no synthesizing action of ACh at all.

ii) Cell-free extraction

But, under the same condition, when they prepared cell free-extract (supernatant in the centrifuging of the homogenized tissue at a rate of 3000 R.P.M. for about 10 minutes at 5°C), about 210 μg ACh/mg. dry weight/hour was synthesized. This extraction prepared from fresh electric tissue is very unstable and rate of formation decreased rapidly (Table 22).

iii) Acetone dried powder

These very unstablity and the rate of decrease of ChAcE activity which are due to the strong action of ATPase and AChE that inhibits the synthesizing ACh, therefore, it must be prevented by preparing from acetone-dried powder and may be obtained much more stable solution. However, in the first acetone powder, about 200 mg. ACh was formed g powder/hr. (as shown in Table *21*), which is less than that of cell-free extract.

Indeed, acetone dried powder is a convenient source for ChAcE preparation, if

Table 21. ACh formation by choline acetylase prepared from the electric organ of *Narke japonica*

Substrate	ATP μM/ml	ACh μg/g/hr	Preparation used for ChAcE
Acetate + Choline	0.03	0	Slice
Acetate + Choline	0.03	0	Homogenate
Acetate + Choline	0.03	210	Cell-free extract
Acetate + Choline	0.03	200	acetone-dried powder

it is suitable for some optimum condition, since it is readly prepared and preserved ; besides AChE and ATPase content would be very low. The reasons for this superiority of cell-free extract are able to adequately explain by the consideration of acetylation problem mentioned above, that coenzyme in ACh synthesyzing system should be easily inactivated by acetone treatment.

2. Purification of ChAcE

i) sulfhydryl (−SH) groups

As mentioned above, extractions prepared from fresh electric tissue are very unstable and rate of formation decreases rapidly, and also, on acetone treatment the coenzyme rapidly loses its activity, these findings support the view that ChAcE belongs to the class of enzymes containing sulfhydryl group (−SH) which are easily oxidized. The significance of −SH groups present in so many enzyme proteins has been stressed by the work of Barron. Monoiodoacetic acid is strong inhibitor of −SH groups. It is interesting to test that the formation of ACh by the choline acetylating system added to iodoacetic acid which reacts with -SH groups. The results obtained from these relationships are given in Fig. 12.

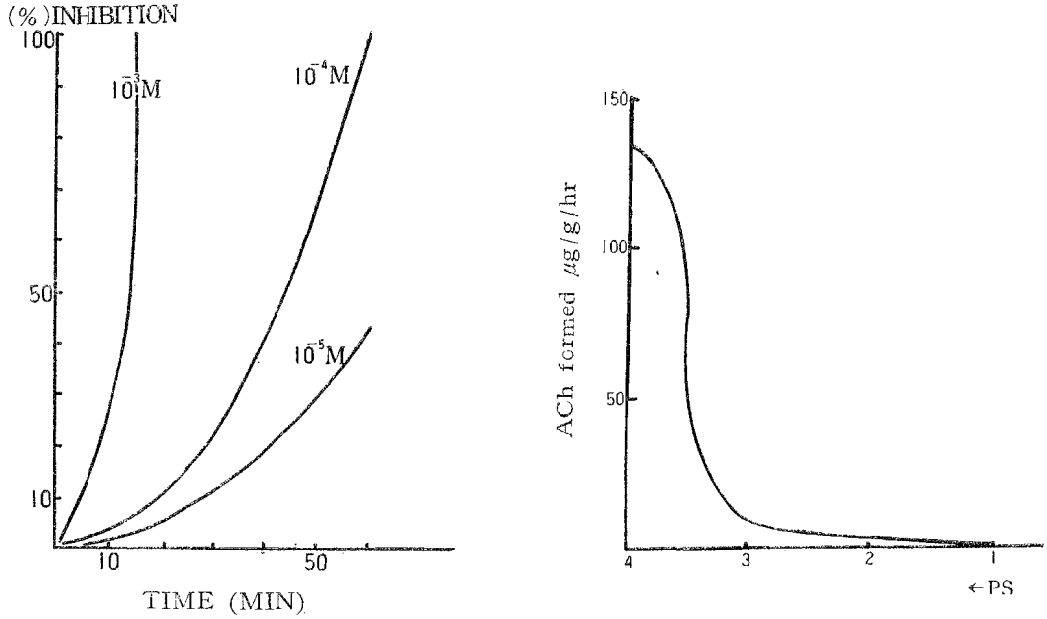


Fig. 12. Inhibitory effort of monoiodoacetic acid on choline acetylase in the electric organ of *Narke japonica*.

In 10^{-3} M concentration, iodoacetate completely inactivated ChAcE within 15 minutes, also in 10^{-4} M conc. have a half inhibitory effort on enzyme activity for 60 minutes. The results obtained in the analysis of this effect to the enzyme activity were essentially similar to those described for inhibition of ChAcE by copper reported by Nachmansohn. Formation of ACh in fresh brain extract was virtually completely inhibited by 3×10 M^{-5} concentration of copper ions and the inhibition was still 66 per cent at 1.5×10 M^{-5} conc., since SH groups are also

high sensitive to copper.

ii) Cysteine effort

Further support for the high sensitivity of −SH groups of ChAcE in electric tissue may be seen in the fact, in fresh extract, and the yield is considerable decreased by incubation in air instead of under strictly anaerobic conditions

Table 22. Decrease of activities of choline acetylase in electric tissue extract

incubation	per cent decrease	
	25°C	4°C
hrs.		
1	55	
2	80	
3	95	15
4	100	
8		
16	70	
24	90	

(Table 22). Presence of cysteine improves the yield considerably and, in sufficiently high concentration, may prevent any effect of oxygen. In extracts which have been dialyzed or prepared from acetone-dried powder, presence of cysteine in high concentrations may increase the rate of formation, up to 2—3

Table 23 a. Effect of *l*-cysteine on choline acetylase activity in each preparation from the electric organ of *Narke japonica*.

Condition	ATP μM/ml	Cysteine μM/ml	ACh μg/g/hr	Preparation
Acetate + Choline	0.03	none	230	Cell-free Ext.
Acetate + Choline	0.03	0.026	500	〃
Acetate + Choline	0.03	none	0	Acetone powder
Acetate + Choline	0.03	0.026	8.9*	〃

(*ACh mg/g/hr)

mg/g powder/hour (Table 23,a). Observations as to effort of cysteine on ChAcE activity apparently indicate that cysteine activates easily oxidizable −SH groups during acetone treatments or effort of oxygen.

iii) Coenzyme A

Only a brief summary of this work is given here, since the detail was already described above. The longer the dialysis is carried on, the weaker is the reactivation by the compounds mentioned. The experiments suggest that choline acetylase requires a coenzyme for its activity. The coenzyme has been found. In contrast to enzyme which occurs only electric tissue, coenzyme A has been extracted from brain, liver, nerve tissue, and skeletal muscle. Coenzyme A has

been purified to a certain degree by treatment with barium salt, which precipitates the coenzyme A. It is well established that the first step of acetylation in animal cell is the formation of acetyl CoA by an enzyme referred to acetylkinase, using the energy of ATP. The mechanism of this formation of acetyl CoA has been recently investigated jointly by Lynen & Lipmann. The acetyl group is then transferred from acetyl CoA to acceptor through the action of enzyme more or less specific for the acceptor. Choline acetylase transfers the acetyl group from acetyl CoA to choline. CoA mediates a great number of key reactions in intermediate metabolism, as for instance the condensation of oxaloacetic and acetic acid to citric acid, or various steps in the fatty acid cycle investigated mainly by Lynen, Ochoa, Green, and their associates.

iv) Purification by fractional ammonium sulfate precipitation

The choline-acetylating system of electric organ was purified and concentrated by fractional ammonium sulfate precipitation. The procedure used is as follows : Acetone-dried powder prepared from electric organ of *N. japonica* was extracted with 0.05 M K_2HPO_4 buffer of *pH* 7.4 and was centrifuged in the preparatory ultracentrifuge. The proteins of the supernatant solution were precipitated with an equal volume of 50% ammonium sulfate and centrifuged. The precipitate was dissolved in 0.1 M K_2HPO_4 buffer of *pH* 7.8. The ammonium sulfate was removed by dialysis against 0.05 M K_2HPO_4 buffer of *pH* 7.4.

Protamine sulfate, in a 2% solution was added (1 mg. per 10 mg. protein) and the precipitate formed was removed by centrifugation. When large amounts of materials were used, an ammonium sulfate fractionation was carried out at this stage and the protein fractionated between 16 and 28% (g/dl). With smaller amounts the next step was carried out directly, consisting of treatment with 3.3 mg.

Table 23 b. Purification of choline acetylase in the electric organ of *Narke japonica*.

Enzyme Preparation	ACh formed (mg/g protein/hr)
Crude extract	12.8—13.0
Ist Amm. sulfate	25.0—30.0
Protamine sulfate	25.1—26.0
Ca gel	100—110
2nd Amm. sulfate	150—160

of calcium PO_4 gel per mg. of protein at *pH* 6.2. The gel was centrifuged and eluted with 0.05 M phosphate buffer of *pH* 7.5 and the elute discarded. Further elution with 0.2 M phosphate buffer of *pH* 8.2 contained most of the enzyme. The elute was again fractionated with ammonium sulfate, between 16 and 32%. The data of preparation are given in Table 23 b.

3. Optimum condition to choline acetylating system

The following condition was found to be optimum for the activity of choline

acetylating system in the electric organ of *N. japonica*, homogenizing the electric organ of the fish at lower temperature and then making the acetone dried powder of the tissue. Enzyme solutions were prepared with supernatant, which the acetone dried powder of this tissue was extracted by the salts buffer solution contained KCl and was centrifugated at the rate of 3000 R.P.M. for 3 min.

Nine-tenths ml. of the enzyme solution are added with cysteine in order to activate it, and then are added with 1.2 ml. of the salts buffer containted eserine and NaF as to the inhibitor of AChE and ATPase respectively. More added to it 0.3 ml. of choline chloride, sodium acetate and ATP respectively, resulting of it, making 3.0ml. of the total volume. The reaction solutions which are added 6 mg. of CoA (5 unit) are incuvated at 37.5°C for 60 minutes. The amount of formed ACh was measured by Hestrin's hydroxylamine method. As shown in Table 22, it was evident that at the presence of CoA and ATP, when an essential component prepared with acetate, choline, and enzyme a high rate of formation, up to ACh 2.5 mg./g./hr. was obtained. Whereas, on other condition, only enzyme and substrate were found only a small synthesis of ACh, or none at all. Under such a optimum condition, synthesized ACh mg./g. powder/hr. was about 2.0 which is of the same order of the magnitude as that estimated in some mammalian brain and squid ganglion. From the results obtained in this analysis at the present experiments it was clearly evident that the electric organ of Japanese electric ray might hold the exciting system contained ACh metabolism as in the case of the neuromuscle junctions.

4. Problem on acetyl donor as to ChAcE activity

i) Acetyl donor

Recently, it has been assumed that during synthesis of ACh, what has become of acetyl donor is one of the key point of considerable problem on ChAcE. Nachmansohn considered that the unique substrate of acetyl-groups was acetic acid, on the other hand, citrate, glutamate and *l*-cysteine had only effective force or not at all, on its acetylating system. Other workers, however, have been apt to admit the citrate as a chief acetyl donor (Fulton 1948, Feldberg *et al.* 1946). An interesting observation, revenant in this problem, has been recently reported by Ebashi. The results showed that citrate was an excellent acetyl donor, while acetate was also utilized by guinea pig brain.

Before discussing the acetyl donor as to ChAcE activity by the electric organ of *N. japonica* it was pursued on the amount of ACh synthesized by enzyme preparations in connection with certain acetyl-donors.

In ACh synthesizing system, it is possible to support that both acetate and citrate are typical acetyl donor and, furthermore, *l*-glutamic acid, and keto acid would have some effect as activator or inhibitor for this system. Table 24 illustrates the amount of synthesized ACh by ChAcE in the electric tissue in

Table 24. The amount of synthesized ACh by choline acetylase in the electric organ of electric ray, *Narke japonica* in connection with certain acetyl donor.
(Each value illustrated is determined on average of five cases)

Preparation	ACh formed, mg./g./hr.			
	Acetic acid	Citric acid	*l*-Glutamic acid	Keto acid
A	250±15	132±32	191±21	231±12
B	600±50	180±43	291±21	156±10
C	2000±40	135±43	130±21	50± 6

A: fresh organ extract, B: acetone dried powder, C: acetone dried powder more active complete system

connection with certain acetyl donor.

In the electric organ, the added acetate was found to be an essential component, and was a unique acetyl donor. Citric acid has an effect nearly as strong as glutamic acid, whereas the oxidation product of amino acid and keto acids have a strong inhibitory effect on the formation of ACh when present in concentration of 10^{-4} to 10^{-5} M. The observations show that the activity or inhibitory effects of some intermediate compounds on the enzyme activity may be upon the degree of purity and other accessory conditions of the enzyme preparation.

ii) The mechanism of the ATP-CoA-Acetate reaction

As mentioned above, in the electric organ the added acetate was found to be an essential component and was a unique acetyl donor. If we accept this general acetylation problem, it becomes considerably easier to understand the reaction ATP-acetate reaction which we think reveal a rather unexpected mechanism. This conversion of the phosphate bond in ATP into acetyl bond of acetyl CoA—excluding acetyl phosphate as an intermediary has so far been poorly understood. Quite a while age, during Chou's work on this reaction with hydroxylamine as acetate acceptor(Chou and Lipmann, 1952), Lipmann and his associates were puzzled by the observation that hydroxamic acid formation was balanced by too little inorganic phosphate. On synthesis of ACh similar results were recently obtained using active electric tissue.

It was mainly this observation which gave a way the explanation of the mechanism, namely, that not phosphate but pyrophosphate was liberated in the ATP-CoA-acetate reaction. It appeared that the author had overlooked the relative abundance of pyrophosphatase of ChAcE in the electric tissue. Pyrophosphatase in the organ is strongly inhibited by fluoride and, for this reason, fluoride preserves the pyrophosphate. On the other hand, without the fluoride, initially formed pyrophosphate is split ; this explains the appearance of two moles of inorganic-phosphate for every mole ACh synthesized and ATP used, in contrast to a nonformation of orthophosphate with fluoride present.

The reaction therefore involves a pyrophosphate split of ATP, leaving AMP as

the product of reaction :

$$ATP + CoA + Acetyl \sim \rightleftharpoons AMP + PP + Acetyl \sim CoA \quad (1)$$

In present experiment with choline as acetyl acceptor, acts catalytically, being recovered in the second and irreversible reaction :

$$Acetyl \sim CoA + Choline \rightleftharpoons CoA + Acetyl \sim Choline \quad (2)$$

This formulation was borne out by the results of the experiment by Lipmman. There, with fluoride present ATP disappearance and choline acetylation are balanced by a formation of AMP and pyrophosphate. Pyrophosphate was determined by the manganese precipitation of Kornberg (1950) and more rigorously identified by use of a crystalline preparation of pyrophosphatase (Kunitz 1952). AMP was determined spectrophotometrically (Kalckar 1947) using Schmidt's deaminase. ATP was assayed enzymatically according to Kornberg's technique (1950)(to detect the disappearance of ATP, the enzymatic method had to be used). The easily hydrolyzable phosphate remained uncharged as was to be expected for a decomposition of ATP to inorganic pyrophosphate.

Analogous experiments were carried out with electric organ extract of *N. japonica* where likewise, with the system ATP-CoA acetate-hylomine by Lipmann, equivalence of AMP and pyrophosphate formation with disappearance of ATP and ACh formation were observed.

In order to further clarify the mechanism, the possibility of reactions between (a) ATP and CoA and (B) between acetyl CoA and pyrophosphate of CoA-pyrophosphate as an intermediary and make more cautions and leave still open the alternative of an enzyme-bone intermediately.

The reversibility of over-all effects could be shown most clearly in following manner. Acetyl phosphate was used with transacetylase as acetyl feeder to CoA. The thus-formed acetyl~CoA reacted with AMP and pyrophosphate to form ATP, while orthophosphate in place of pyrophosphate showed no reaction :

$$Acetyl \sim ph + CoA \xrightarrow[\text{transacetylase}]{} acetyl \sim CoA + ph \quad (3)$$

$$Acetyl \sim CoA + PP + ATP \rightleftharpoons ATP + CoA + Acetyl \quad (4)$$

Using the organ enzyme and transacetylase and applying methods similar to those described, the disappearance of 5μM of acetyl phosphate was balanced by the appearance of 6.5 μM of ATP, while at the same time, 7.7 μM of AMP disappeared. The experiment shows that, by way of acetyl CoA and pyrophosphate, energy-rich acyl bonds may rather easily be converted to energy rich phosphate bonds of ATP.

D. Historical considerations of acetylation problem relating to acetylcholine synthesis.

The second line of investigations, in which enzyme activity could be correlated

with events in the living cell recorded by physical methods, is based on the energy transformations involved and on thermodynamic considerations. If the release and removal of ACh are associated with the primary alternations of the nerve membrane during the passage of the impulse, then the primary source of the chemical energy released during the recovery process should be used for the resynthesis of ACh.

The most readily available source of energy in living cells is that released by energy-rich phosphate bonds. Phosphocreatine, the main "storehouse" of energy-rich phosphate bonds in muscle, is also present in nerves. The electric organ offers a suitable material for investigation on the chemical reactions which supply the energy for the action potential. Both electric and chemical energy are within the range of measurment, whereas, in ordinary nerves such an analysis is difficult. It is generally accepted that the nature of the discharge is basically identical with the action potential of nerve and muscle. The cellular units of the electric organ are the electric plates. The potential difference developed by a single plate is about 0.1 volt, which is of the same order of magnitude as that found in ordinary nerves. All other electrical characteristics show also similar typical features. However, the plates are arranged in series. It is this special arrangement of the units in series like in voltaic pile by which these organs are distinguished from ordinary nerves and are capable of producing the high voltage.

Before discussing about sequence of energy transformations and correlation between electrical events and chemical reactions, and also, integrations of acetylcholine into metabolic cycle of the nerve cell, I would like to consider historical aspects of acetylation problem, which closely associated with the biosynthesis of acetylcholine.

The most important event during the development of acetylation problem, by Lipmann's brilliant work, was the accidental observation that, in the *L. delbrueckii* system, (1936), pyruvic acid oxidation was completely dependent on the presence of inorganic phosphate. This observation was made in the course of attempts to replace oxygen by methylene blue. To measure the methylene blue reduction manometrically, Lipmann had to switch to a bicarbonate buffer instead of otherwise routinely used phosphate. In bicarbonate, to his surprise, pyruvate oxidation was very slow, but the addition of a little phosphate caused a remarkable increase in rate. Then it appeared that the reaction was really fully dependent on phosphate.

In spite of such a phosphate dependence, the phosphate balance measured by the ordinary Fiske-Subbarow procedure did not at first indicate any phosphorylative step. Nevertheless, the suspicion remained that phosphate in some manner was entering into the reaction and that a phosphorylated intermediary was formed. As a first approximation, a coupling of this pyruvate oxidation with adenylic acid phosphorylation was attempted. And, indeed, addition of adenylic acid to the

pyruvic oxidation system brought out a net disappearance of inorganic phosphate, accounted for as adenosine triphosphate. In parallel with the just developing fermentation picture, Lipmann concluded that the missing link in the reaction chain was acetyl phosphate. In partial confirmation it was shown that crude preparation of acetyl phosphate, synthesized by the old method of Kammerer & Carius (1864) would transfer phosphate to adenylic acid. However, it still took quite some time from them on to identify acetyl phosphate definitely as the initial product of pyruvic oxidation in this system (Lipmann, 1940, 1944). Most important during this and later work was the development of procedures (Lipmann & Tuttle, 1944) and in particular of the very handy hydroxamic acid method (Lipmann & Tuttle, 1944) for the determination of acyl phosphates and other reactive acyl derivatives.

At the time these observations were made, about a dozen years ago, there was, to say the least, a tendency to believe that phosphorylation was rather specifically coupled with the glycolytic reaction. Here, however, Lipmann and his associates had found a coupling of phosphorylation with a respiratory system. This observation immediately suggested a rather sweeping biochemical significance, of transformations of electron transfer potential, respiratory or fermentative, to phosphate bond energy, and therefrom to a wide range of biosynthetic reactions (Bentley, 1948).

There was further unusual feature in this pyruvate oxidation system in that the product emerging from the process not only carried an energy-rich phosphoryl radical such as was already known, but the acetyl phosphate was even more impressive through its energy-rich acetyl. It rather naturally became a contender for the role of "active" acetate, for the wide-spread existence of which the isotope experience had already furnished extensive evidence. It became, therefore, quite attracted by the possibility that acetyl phosphate could serve two rather different purposes, either to transfer its phosphoryl group into the phosphate pool, or to supply its acetyl donor as well as phosphoryl donor, such as indicated by Bentley's early experiments on cleavage of acetyl phosphate in H_2O (Bentley, 1948).

These two novel aspects of the energy problem, namely (i) the emergence of an energy-rich phosphate bond from a purely respiratory reaction, and (ii) the presumed derivation of a metabolic building-block through this same reaction, prompted Lipmann's group not only to propose the generalization of the phosphate bond as a versatile energy-distributing system, but also to aim from there toward a general concept of transfer of activated groupings by carrier as the fundamental reaction in biosynthesis (Lipmann, 1946). Although in the related manner the appearance of acetyl phosphate as a metabolic intermediary first focused attention to possible mechanisms for the metabolic elaboration of group activation, it soon turned them that the relationship between acetyl phosphate and acetyl transfer was

much more complicated than anticipated.

Since a better understanding of the mechanisms of group activation seemed to become a most urgent problem in biosynthesis, Lipmann now set out to find a suitable system to check on the assumption that acetyl phosphate represented active acetate. After working out a relatively easy method to prepare the compound (Lipmann, 1944, Stadtman & Lipmann, 1950), a first unexpected difficulty arose when it appeared that animal tissues contained rather generally a very active, specific, and heat-stable acetyl phosphatase (Lipmann, 1945, Lipmann, 1946). In crude preparations of muscle, liver, and other tissues, the half-life of acetyl phosphate is only a few minutes.

This strange activity in animal tissues made tests with this substance very difficult. In looking for a sensitive method to study acetyl transfer, the acetylation of aromatic amines was chosen eventually as a most promising and technically easy procedure. Lipmann and his coworkers were furthermore, quite confident that any result obtained with this method could be generalized over the whole metabolic territory concerning the transfer of active acetate, including such reactions as citrate, acetoacetate, and lipid synthesis. Acetylation of sulfonamide had been found to occur in rabbit liver slices (Sykes, 1944, Klein & Harris, 1938). However, for their purposes, they had to eliminate cell membrane barriers to test for the activity of complex intermediary metabolites. Although acetylation was found with rabbit liver homogenate, the reaction was rather weak. In search of a more active system, pigeon liver homogenate was tried and found to harbor an exceedingly potent acetylation system (Lipmann, 1945, Sykes, 1944). This finding of a particularly active acetylation in cell-free pigeon liver preparations was most fortunate and played quite an important part in the development of the acetylation problem.

Lipmann and his associates eventually arrived at the point where the desired test for acetyl phosphate as an acetyl precursor could be performed. Although the acetyl phosphatase activity of the pigeon liver homogenate was considerable and, to some extent, obscured the test with acetyl phosphate, it became, nevertheless, clear to them that in this preparation, acetyl phosphate did not furnish active acetate (Lipmann, 1945). Under anaerobic conditions the massive concentrations of acetyl groups for the acetylations of sulfonamide could be derived under conditions where an easy acetylation occurred with a respiring homogenate.

It furthermore appeared that, as an energy source, the particle-bound oxidative phosphorylation of the kind observed first by Kalckar (1938) could be replaced by ATP, as had first been observed with the acetylation of choline in brain preparation by Nachmansohn and his groups (1943). Using ATP and acetate as precursors, it was possible to set up a homogenous particle-free acetylation system obtained by extraction of acetone pigeon liver. In this extract likewise acetyl phosphate was

unable to replace the ATP-acetate as acetyl precursor.

In spite of this disappointment with acetyl phosphate, its decision to turn to a study of acetylation started then to be rewarding in another way. During these studies he became aware of the participation of a heat-stable factor which disappeared from his enzyme extracts on aging or dialysis. This cofactor was present in boiled extracts of all organs as well as in micro-organisms and yeast. It could not be replaced by any other known cofactor. Therefore, it was suspected that they were dealing with a new coenzyme. From then on, for a number of years, the isolation and identification of this coenzyme came the prominent task of Lipmann's laboratory. The problem now increased in volume, and it was his fortune that a group of exceedingly able people were attracted to the laboratroy ; first Tuttle, then Kaplan, and shortly afterward Novelli. More recently, Soodak and Gregory and quite a few others have made here most important contributions to the advance of this problem.

Firding pig liver a good source for the coenzyme, Lipmann set out to collect a reasonably large quantity of a highly purified preparation and then to concentrate on the chemistry with this material. In this analysis Lipmann paid particular attention to the possibility of finding in this obviously novel cofactor one to the vitamins, then not as yet metabolically identified. In this task they were very fortunate to have the help of the great experience of Willams's laboratory. The appearance of a vitamin B in the preparation was, of course, a most exciting event for this study and gave it further confidence. It was still felt, however, slightly dissatisfied with the proof for pantothenic acid. Therefore, to liberate the chemically rather unstable pantothenic acid from CoA, they made use of observations on enzymatic cleavage of the coenzyme. Two enzyme preparations, intestinal phosphatase and an enzyme in pigeon liver extract, had caused independent inactivation. It then was found that through combined action of these two enzymes pantothenic acid was liberated (Kaplan & Lipmann, 1949).

The two independent enzymatic cleavages indicated early that in CoA a phosphate link, linking presumably to one of the hydroxyl groups in pantothenic acid. The other moiety attached to pantothenic acid, which cleaved off by liver enzyme, remained unidentified for a long time. In addition to pantothenic acid, the sample of 40 percent purity had been found to contain about 2% sulfur by elementary analysis and identified by cyanide-nitroprusside test as a potential SH-grouping (Novelli and Lipmann, 1949). Furthermore, the coenzyme preparation contained large amounts of adenylic acid (Novelli, 1949). In the subsequent elaboration of the structure, the indications by enzyme analysis for the two sites of attachment to pantothenic acid have been most hopful. The phosphate link was soon identified as a pyrophosphate bridge (Novelli, Kaplan, & Lipmann, 1950). 5—Adenylic acid was identified by Novelli (1953) as an enzymatic split product

and by Baddiley (1951), through chemical cleavage. At the same time, Novelli made observation that indicated the presence of a third phosphate in addition to the pyrophosphate bridge. These indications were confirmed by analysis of a nearly preparation that was obtained by Gregory (1952) from *Streptomyces*

Table 25. Coenzyme A-catalyzed acetyl transfer reactions

TYPE OF REACTION	ENZYME SYSTEMS, EXTRACTS
Donor Systems :	
ATP—acetate—CoA—acetyl—CoA	pigeon liver, yeast
phospho—transacetylation, ac—P—CoA⇄ac—CoA→P	*Clostridium kluyveri, Escheriehia coli*
formo—transacetylation, pyruvate—CoA⇄ac—CoA→formate	*Escherichia coli*
transacetylase :	
ac—CoA + — butyrate⇄butyryl—CoA + acetate	*Clostridium klutveri*
acetoacetate	pigeon liver
citrate in reverse	pigeon liver
acetaldehyde + CoA + DPN⁻ → ac—CoA + DPNH + H	glyceraldehyde dehydrogenase
	Escherichia coli
	Clostridium kluyveri
pyruvate + CoA + DPN→ac—CoA + DPNH + CO_2 + H	*Escherichia coli, Streptococus faecalis*
	heart and muscle
Acceptor Systems, Acetokinases:	
aromatic amines	pigeon liver
choline	rat brain
histamine	pigeon liver
amino acids	*Clostridium kluyveri*
glucosamine	pigeon liver
Condensation :	
acetoacetate	pigeon liver
citric acid	pigeon liver, yeast, heart
pyruvate	*Escherichia coli*
Succinyl Transfer	
Donor System :	
ketoglutarate + DPN + CoA→succiny—CoA + DPNH + H⁻	heart muscle
ATP—succiinate	*Escherichia coli*
Acceptor System :	
heme synthesis	red cells hemolysate
Benzoyl Transfer hippuric synthesis	rat liver
Stearyl Transfer phospholipid synthesis	rat liver
Complex Reaction Systems :	
fatty acid synthesis	*Clostridium kluyveri* extracts
butyrate oxidation	*Clostridium kluyveri* extracts
fatty acid oxidation	liver homogenate
steroid and fat synthesis	resting yeast cells
	liver slices

fradias in collaboration with the research group at the Upjohn Co. (1950). The generous help of the Upjohn Laboratories has been of great importance for the final identification of the structure of CoA.

Through analysis and synthesis, Baddiley now identified the point of attachment of the phosphate bridge to pantothenic acid in 4—position (1951), and Novelli *et al.* (1953) completed the structure anaylsis by enzymatic synthesis of "dephospho-CoA" from pantotheine—4' —phosphate and ATP. Furthermore, the attachment of the third phosphate was identified by Kaplan (1953) to attach in 3—position on the ribose of the 5—adenylic acid. Therefore, the structure was established.

Parallel with this slow but steady elaboration of the structure, all the time Lipmann's group explored intensively metabolic mechanisms in the acetylation field. By use of enzymatic assay, CoA was found to be present in all living cells, animals, plants, and micro-organisms (1948), made it clear that CoA represented the only functional form of this vitamin.

The first attempts to explore further the function of CoA were made with pantothenic acid-deficient cells and tissues. A deficiency of pyruvate oxidation in pantothenic acid-deficient *Proteus morganii*, and early isolated observation by Dorfman (1942), and Hill (1943), now fitted rather well into picture.

A parallel between CoA levels and pyruvate oxidation in *P. morganii* was demonstrated (Novelli & Lipmann, 1947). Using pantothenic acid-deficient yeast Novelli *et al.* (1948) demonstrated a CoA—dependence of acetate oxidation, and Olson & Kaplan (1948) found with duck liver a striking parallel between CoA content and pyruvic utilization.

But more important information was being gathered on the enzymatic level. The first example of a generality of function was obtained by comparing the activation of apoenzymes for choline and sulfonamide acetylation respectively, using Lipmann's highly purified preparations of CoA (1949). Similar activation curves were obtained for the two respective enzymes. Through these experiments, the heat stable factor for choline acetylation that had been found by Nachmansohn & Behman (1946) and by Feldberg & Mann (1946) and Kuwabara (1954) was identified with CoA.

The next most significant step toward a generalization of CoA function for acetyl transfer was made by demonstrating its functioning in the enzymatic synthesis of acetoacetate. The CoA effect in acetoacetate synthesis was studied by Soodak (1948), who obtained for this reaction a reactivation curve quite similar to the curves for enzymatic acetylation. Soon afterwards Stern & Ochoa (1949) showed a CoA—dependent citrate synthesis with a pigeon liver fraction similar to the one used by Soodak for acetoacetate synthesis. In Lipmann's laboratroy, Novelli *et al.* conformed and extended this observation with extracts of *Escherichia coli* (1950).

In the course of this work, which more and more clearly defined the acetyl transfer function of CoA, Novelli once more tried acetyl phosphate. That in *E. coli* extracts, in contrast to the animal tissue, acetyl phosphate as acetyl donor for citrate synthesis was more than twice as active as ATP-acetate (Novelli & Lipmann, 1950) Acetyl phosphate, therefore, functioned as a potent microbial acetyl donor. Acetyl transfer from acetyl phosphate, like that from ATP-acetate, was CoA-dependent. Furthermore, a small amount of "microbial" conversion systems (Stadtmar, Novelli & Lipmann, 1950). Eventually the microbial conversion factor was identified by Stadtman *et al.* (1950) with transacetylase first encountered by Stadtman & Barker in extracts of *Closiridium kluyveri* (1950) and likewise, although not clearly defined as such, in extracts of *E. Coli* and *C. butylicum* by Lipmann & Tuttle (1945).

The definition of such a function was based on the work of Doudoroff *et al.* (1950) on transglucosidation with sucrose phosphorylase. Their imaginative use of isotope exchange for closer definition of enzyme mechanisms has been most influential. Like glucose-phosphate with sucrose phosphorylase, acetyl phosphate with these various microbial preparations equilibrates its phosphate rapidly with the inorganic phosphate of the solution. As in the Doudoroff *et al.* experiments, first a covalent substrate enzyme derivative had been proposed (1947). However, then Stadtman *et al.* (1950), with the new experience of CoA-depenent acetyl transfer, could implicate CoA in this equilibration between acetyl- and inoganic phosphate and thus could define the transacetylase as an enzyme equilibrating acetyl between phosphate and CoA :

$$\text{Acetyl} \sim \text{OPO}_3^{=} + \text{CoASH} \xleftarrow{\text{transacetylase}} \text{acetyl} \sim \text{SCoA} + \text{HOPO}_3^{=}$$

In the course of these various observations, it became quite clear that there existed in cellular metabolism an acetyl distribution system centering around CoA as the acetyl carrier that was rather similar to the ATP-centered phosphoryl distribution system. The general pattern of group transfer became recognizable, with donor and acceptor enzymes being connected through the CoA $\rightleftharpoons$ acetyl CoA shuttle. A clearer definition of the donor-acceptor enzyme scheme was obtained through acetone fractionation of Lipmann's standard system for acetylation of sulfonamide into two separate enzyme fractions, which were inactive separately but showed the acetylation effect when combined. A fraction, A—40, separating out with 40% acetone, was shown by Ochoa (1952) to contain the donor enzyme responsible for the ATP—CoA-acetate reaction, whereas with more acetone, the acceptor function, A—60 precipitated, the acetoarylaminekinase. The need for a combination of the two for over-all acetyl transfer. This showed that a separate system was responsible for acetyl CoA formation through interaction of ATP, CoA,

and acetate and that the over-all acetylation was a two step reaction :

$$ATP+CoA+Acetate \rightleftharpoons Aceyl\sim CcA+AMP+Pyrophosphate \quad (1)$$

$$Acetyl\sim CoA+Arylamine \rightarrow Acetyl\sim Arylamine+CoA \quad (2)$$

These observations crystallized into the definition of a metabolic acetyl transfer territory as pictured in Fig. 10. This picture had developed from the growing understanding of enzymatic interplay involving metabolic generation of acyl CoA and transfer of the active acyl to various acceptor systems. A most important, then still missing link in the picture was supplied through the work of Lynen (1952) who chemically identified acetyl CoA as the thioester of CoA. There with the thioester link was introduced as a new energy-rich bond, and this discovery added a very novel fact to understanding of mechanism of metabolic energy transformation.

In spite of many similarities between the general aspects of group transferring phosphoryl and acetyl groupings, there is a considerable difference in so far as the grouping transferred in the acetyl territory is an organic grouping and displays a quite different versatility for condensation reactions, yielding eventually large and complex carbon structures. There is one feature in this picture that always has attracted Limpann's groups particular attention : the two fold type of activation involving (1) and carboxyl end, or the "head," of the acetyl and (2) its methyl, or "tail", end.

The definition of the head reaction is relatively simple, acetylation of choline is a typical head reaction. There is to be mentioned, furthermore, the observation by Chantrene (1951), introducing CoA as a rather general catalyst of acyl activation. He demonstrated the activity of CoA in benzoyl transfer such as hippuric acid synthesis. The mechanism of this synthesis was elaborated recently by Taggart (1953), introducing CoA as a rather general catalyst of acyl activation. The mechanism of this synthesis was elaborated recently by Taggart (1953), who clearly defined benzoyl CoA as the benzoyl donor in this reaction. As even greater and more prominent generalization is offered through the more and more developing importance of succinyl CoA in intermediary metabolism.

The second type, the methyl end engages in an aldolase type of condensation with the carbonyl group of the oxaloacetate as acceptor. This condensation requires an energy input that must be derived from the thioester link, and at the end of the reaction CoA appears to be liberated in some manner.

The complexity of the citrate condensation is emphasized through the existence of an ATP-CoA-citrate reaction recently observed by Srere *et al.* (1953), which results in the disruption of citrate to oxaloacetate and acetyl CoA.

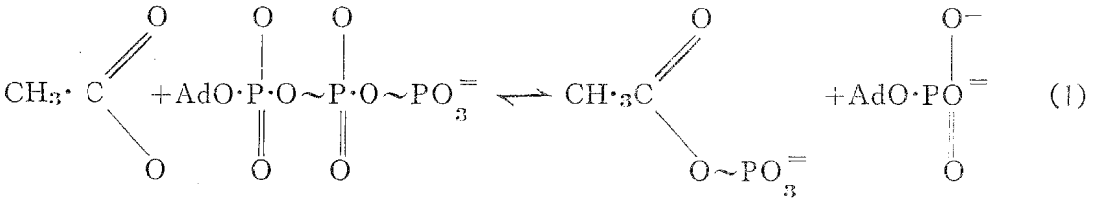
$$CoA+ATP+citrate \rightleftharpoons acetyl.\ CoA+oxaloacetate+ADP+P$$

The mechanism remains still to be understood in greater detail. The reaction is mentioned here because it introduced the new variety into the citric acid cycle

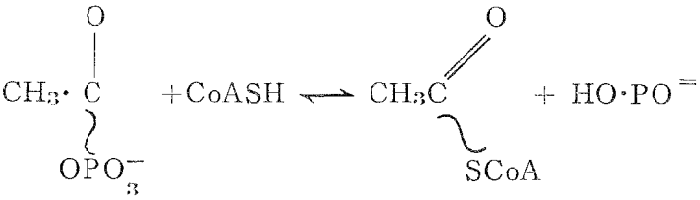
through a conversion of phosphoryl via citrate into acetyl.

For a long time the citrate reaction was the only known tail condensation. however, recently another interesting example has developed in the study of the precursors in steroid and isoprene synthesis. The initial condensation product in this series appears to be β-methyl, β-hydroxyglutarate (dicrotonic acid), formed through condensation of acetoacetate with acetyl CoA. This initial condensation seems to be followed by decarboxylation and dehydration to β-methyl crotonic acid first demonstrated by Bonner *et al.* (1949) as intermediary in rubber synthesis.

In the recent past Lipmann and his associates have been mostly occupied with the mechanism through which the phosphate bond in ATP converts to acetyl bond in acetyl CoA. In animal tissue where acetyl phosphate appeared not to be an intermediate, the conversion mechanism remained very puzzling for a long time. Before considering this reaction, it will be advantageous to review first the microbiological mechanism of such interconversion and in particular the role of acetyl phosphate as an intermediary. This transformations is rather straight forward: A sequence of two independent enzymatic reactions, the first a transphospharylation from ATP to acetate and the second, discussed, a transacylation from acetyl phosphate to CoA :



(1)



(2)

It should be noted that in the first transphosphorylation step the acetyl phosphate cleaves and condenses between O and P. In the second transacylation reaction, however, acetyl phosphate cleaves and condenses between C and O., Thus the same molecule reacts on each side of the oxygen bridge between the carbon and the phosphorus. This shift of the site of cleavage in the sequence is significant. This possibility attracted Lipmann's group early attention and was one of the reason that prompted them into this whole exploration. A shift from P—O to PO—C should actually be a feature of many condensations initiated by a phosphoryl split from ATP. These become increasingly numerous, such as in glutamine, glutathione, pantothenate, and seemingly in protein synthesis. The finer mechanism generally is obscured by enzyme-bound steps. In all the reactions, however, somewhere

along the line a shift from transphosphorylation to transacylation seems to be inherent. This shift stands out very clearly in the microbial two-step reaction. But in animal tissue, the energy transmission from phosphoryl to acetyl occurs through a continuous enzyme bound reaction which is more difficult to elucidate. Nevertheless, some progress has been made, which also starts to reflect on other mechanisms of this type.

CONCLUSION

If ACh is responsible for the transient change in permeability, it must be rapidly inactivated. This inactivation is performed through hydrolysis by enzyme, AChE. Intimate knowledge of the properties of this enzyme is therefore fundamental.

Several features of the AChE appear to be of physiological significance. The type of esterase present in conductive tissue is distinctly different from other esterases occurring in the tissue. The enzyme in present in every conductive tissues throughout the whole animal kingdom, shows some properties. It has been demonstrated in all types of nerve, central and peripheral, motor and sensory, so-called cholinergic and adrenergic, vertebrate and invertebrate, and in muscle tissue free of nerves. Recently it has been demonstrated in the *Tetrahymena geleii*, a unicellar organism with ciliary movements. (Seaman 1951) The outstanding of physiological feature of the enzyme is the high speed of hydrolysis. The turnover number is of the same order of magnitude of 20 millions, that means that 1 molecule of enzyme may split 1 molecule of ACh in about 3 to 4 microseconds. On the basis of electrical sign it must be postulated that the elementary process occurs within less than 100 microsconds. The prequisite for connection a chemical reaction directly with the electrical manifestation is a similar speed of the reaction. The high turnover number of AChE satisfies this postulate and makes it possible to associate this specific reaction with the elementary process of conduction. Although several other compounds have been associated with nerve activity, for none of them a chemical reaction of a comparable speed has been demonstrated. This system is therefore, the only one which at present can be associated directly with the electrical events. Another interesting feature is the high concentration of the enzyme and its localization in the surface. No activity is detectable in the axoplasm (Boell & Nachmansohn, 1944). Since bioelectrical potentials are surface phenomena, this localization is pertinent, especially since in contrast to AChE most respiratory enzymes are located in the axoplasm.

The presence in conductive tissue of a special type of esterase AChE, which has a high affinity to ACh, the ubiquity of this enzyme in all types of conductive tissue through out the whole animal kingdom, the extraordinary high speed of the

enzyme reaction and the exclusive localization in the axonal surface, all these properties are suggestive and to make possible the assumption that the enzyme and its substrate are important for conduction, but they do not prove such a role. For necessary to demonstrate a direct relationship with this function. A frequent method of testing the necessity of a chemical reaction for a cellular function is block of this reaction by specific inhibitions. The well known classical example which profoundly influenced the development of muscular physiology is Lundsgard's demonstration that lactic acid formation may be blocked by monoiodoacetate while contraction is still continuing.

If the release and removal of ACh are essential for conduction, inhibition of AChE should block this function. In 1945 it has been demonstrated that eserine, a specific inhibitor of AChE, blocks conduction when applied to the nerve (Bullock, Nachmansohn, & Rothenberg, 1946)

The effect is readily reversible, as should be expected in view of the reversibility of the enzyme inhibition. Discovery of a group of compounds, the alkyl phosphates, which inactivate AChE irreversibly, initiated a dramatic development. Observations were described, in 1946, which seemed to contradict the assumption of the essentiality for AChE in conduction. Some investigators thought that it was possible to inactivate the enzyme completely when the nerve was still able to conduct (Nachmansohn, & Jhone, 1949). These results did not withstand a more critical approach. The most widely used compound for these studies testing the function of the enzyme in conduction was diisopropyl fluorophosphate (DFP).

The inhibition of AChE by this compound is a slowly progressive process. A most striking parallelism between the rate of inhibition of AChE and the progressive rate of abolition of conduction has been demonstrated as a function of time as well as a function of temperature (Bullock *et al.*, Grundfest, *et al.* 1946, '47). Under no condition is it possible to dissociate conduction from AChE activity. The failure of earlier investigation to detect enzyme activity in nerves exposed to DFP may be attributed to the small absolute amounts of enzyme required for function. In view of the small amount of energy involved this is not surprising. The initial enzyme concentration is in considerable excess of the minimum required, as may be expected on the basis of experience with other enzymes. In recent experiments of Nachmansohn's laboratory, AChE activity has been determined on the intact nerve and conduction measured simultaneously. Conduction failed long before AChE activity stopped (Wilson, 1953).

The inhibition of AChE leads to a block of conduction in all types of nerves, motor and sensory nerves, adrenergic as well as cholinergic, vertebrate and invertebrate. The motility of unicellular organisms (*Tetrahymena geleii* S) is also blocked by specific inhibition of AChE (Seaman & Houlihan, 1951).

The use of inhibitors of the enzyme and especially the use of DFP has given

an unequivocal answer to the question of the especially the use of DFP has given for conduction. Whereas the first observations with DFP appeared to be a challenge role of the enzyme, the further development has conclusively established that conduction is impossible if the enzyme is inactivated.

The next step was the demonstrated that energy released by the breakdown of phosphocreatine is adequate to account for the total electric energy released by the discharge. It is known from the studies on the intermediate metabolism with muscle contraction that energy certain phosphorylated compounds is the most readily available source of energy for endergonic processes. The work of Meyerhof and Lohman suggested that ATP might be the primary source of energy for muscle contraction and that the ADP formed was rephosphorylated by phosphate of phosphocreatine,a transfer which occurred without loss of eneryg. Phosphocreatine thus acts as a kind of storehouse for supplying phosphate for rapidly building up ADP to ATP. Although the breakdown of phosphocreatine would provide enough to account for the total energy released by the discharge, there is addition, energy released to the simultaneous formation of lactic acid. It may be assumed that energy release during lactic acid formation is used as in muscle for rephosphorylating creatine. The sum of the two reactions of the energy supplied by phosphorylated compounds rich in energy ; this amount is greater than required to account for total electric energy released. It is, however, possible and indeed possible, that sort of the energy released is used not for the intermediate process of recovery, but for the restration of the ionic concentration gradient which provides the primary energy source of the action potential.

It is safe to assume that, as in muscle, the break down of ATP during nerve activity preceded that of phosphocreatine. It is today generally accepted that ATP reacts directly with the structural muscle protein in the elementary process of contraction. However, it appeared mostly unlikely for many reason that ATP was responsible for the change in permeability postulated in the elementary process of conductions. It would be indeed suprising that the same compound and nerve impulse conduction, two functions of so entirely different as order of magnitude in regard to energy requirements and speed involved. On the losses of the available evidence it appeared more likely that the action of ACh was directly responsible for the alternations of the membrane required for the generation of the actions potential and that these reactions occurred prior to breakdown of ATP. The latter would then be a recovery process supplying the energy for the resynthesis of ACh hydrolyzed during activity, in other words, provides the energy for acetylation. It was shown that cell free extract of electric tissue might acetylate choline in presence of ATP. These experiments were the first demonstration that ATP provided energy for synthetic reaction outside the glycolytic cycle in addition of its role in muscle contraction. It initiated intensive studies in many laboratories on the

mechanism of acetylation generally.

The acetylating enzyme requires a coenzyme, later called CoA. It is well established that the first step of acetylation in the electric tissue is the formation of acetyl CoA by an enzyme referred to acetylkinase using energy of ATP. The mechanisms on this formation of acetyl CoA has been recently investigated jointly by Lynen and Lipmann. In bacterial cell acetyl CoA is formed from acetyl phosphate by enzyme phosphotransacetylase. The acetyl group is then transferred from acetyl CoA to acceptors through the action of enzyme more or less specific for acceptor. Cholineacetylase transfers the acetyl group from acetyl CoA to choline. CoA mediates a great number of key reactions in intermediate metabolism, as for instance the condensation of oxaloacetic and acetic acid to citric acid shown by Ochoa and his associates, or various steps in fatty acid cycle investigated mainly by Lynen, Ochoa, Green, and their associates.

It may be of interest to discuss the differnce between rates of ACh formation and hydrolysis. For an understanding of the problem, the decisive difference which has to be considered is not the difference of rates, but the difference of function. There is a fundamental difference between the function of AChE and ChAcE. If the release of ACh is an essential event in the alternations of the membrane during the passage of the impulse, then the active ester has to be destroyed within a millisecond so that the resting condition may be restored. Therefore, the enzyme which removes the active ester, AChE, has to be very active, but only during this brief period, and may then be inactive until the passage of the next impulse. The formation of ACh, on the other hand, need not be such a rapid process. It is generally assumed that the active ester is released from an inactive form.

This is supported by the fact that the primary energy released during recovery is used for the synthesis of ACh, thus impling that the synthesis is a slow recovery process. It is, therefore, not difficult to assume that the period required for the formation is longer than that for the hydrolysis of the same amount, according to the kind of nerve, its condition, temperature, and so on.

From all these investigations emerged the following picture, which integrates ACh into the metabolic cycle of the electric tissue and indicates the sequence of energy transformations. The ionic concentration gradient is the primary energy source of the action potential. ACh acts as the trigger which opens the barrier to the entrance of Na and makes the potential source of energy effective. Our group must postulate again that at least different proteins react with ACh and are thus involved in the elementary process. ACh must be, in resting condition, present in an inactive form and bound to a protein or lipoprotein, protected hereby against the action of AChE. Even for ATP, which is much less active compound, it is today generally assumed that it is present in the cell in a bound form. When the

stimulus reaches the active membrane, ACh is released. This release may easily be effected by ions, possibly hydrogen ions activated in their movement by the current.

The protein to which ACh is bound in the resting state is one of the three proteins involved. Once release, the free ester must act on a second protein, the so called receptor protein. It is the change of this second protein effected by the ester which opens the barrier. Having produced this effect the ester which in its free form is suspectable to attack by the third protein, the AChE, is hydrolyzed by the enzyme. The rapid inactivation of the ester by the enzyme makes possible the restitution of the membrane to its initial state. It is only in the recovery period that the other chemical reactions indicated in the figure take place. Except for the first steps associated with the primary reactions of the ester with the proteins of the membrane and later its resynthesis, the metabolic cycle in the conductive process resembles that accepted for muscular contraction.

It is not surprising that the first differs so strongly in view of the fundamental difference between conduction and contraction : The first gear driving the primary process of muscle contraction must be more powerful but may be slower than that driving the primary process of the impulse conduction. In the sequence of all the energy transformations, that linked to ACh is by far the fastest, but releases a relatively small amount of energy.

All these observations taken together are a strong support for the assumption of ACh cycle in the electric tissue may not be conclusive. As a working hypothesis they integrate best our present knowledge.

SUMMARY

The acetylcholine cycle in the electric organ of the fish was analyzed with methods of biochemistry and especially enzyme chemistry ; the ester was integrated into the metabolism of whole neuron and associated with its primary function, with conduction.

Acetylcholinesterase activity has been determined by both biological and chemical methods ; especially Warburg manometric technique has been reported. A simple and rapid colorimetric method for the determination of acetylcholine and related ester has been described.

The methods for the second line of this investigation has beer reported concerning assay method of coenzyme A, preparation of acetyl coenzyme and assay of the reaction mediated by cholineacetylase by nitroprusside test for the free -SH groups.

In most cases, the experiment has been carried out in Krebs' bicarbonate Ringer's solution(KRB).

The electric organ of the fish is a most favorable material correlating to

the electrical and chemical events for many reasones.

In spite of high concentration of enzyme, high water (92%) and low protein (1—2%) were contained in the electric organ of fish.

The previous literature on choline esterase has been exhaustively reviewed concerning the type, inhibitor, classification and distribution, and physiological function.

Slice and its homogenized electric tissue and cell free extract were prepared as enzyme solution.

Acetylcholinesterase activity was estimated on the electric organ of Japanese electric fish, *Narke japonica* and *Raja.*

The electric organ of *Raja pulchra* hydrolyzed mg. acetylcholine per g. dry tissue is 1400, which is more than 20 times as fast as that of some mammalian brain. Namely, organ can split in 60 min. as amount of ACh equivalent to 1—1.5 times as their own weight.

More powerful acetylcholinesterase activity has been obtained from electric organ of *Narke japonica.* The organ splits mg. ACh per mg. protein as follows : 45—80 (homogenate), 90—150 (cell free extract) and 200—300 (1st Amm. sulfate).

AChE activity was estimated in successive slice of a piece of the organ. The rhythmical change of enzyme activity has been obtained, corresponding to a change of the fine structure of the electric plate.

When enzyme activity was estimated with caudal muscle, the highest activity was found in *"lateral"* which might be transmitted to the electric organ. Optimum substrate concentration is pS, 2.3 (0.004M ACh) and optimum pH is about 8.

The higher concentration of the enzyme is, the lower its activity. The values obtained by both Hesterin's and Warburg method were compared and discussed. No effect has been obtained to AChE activity on addition of sugar as energy source. QACh values obtained by both manometric and colorimetric method were compared and discussed.

Physiological significance of acetylcholine esterase has been briefly discussed in connection with the role of the system acetylcholine-cholinesterase in the chemical mechanism of generation of bioelectricity and pharmacological effect of acetylcholine.

Second line of the investigation was pursued preliminarily under the assumption that choline should be readily acetylated by the action of coenzyme-apoenzyme system which has same role of condensing enzyme (KREBS) on that pyruvate-citrate reaction in TCA cycle. From the results obtained acetylation promotive factor in the electric organ should be identical to the coenzyme commonly such as in all the living organisms.

A method is described for assay of CoA. Electric organ extract which undergoes autolysis losses its ability recovered by addition of CoA. A unit of CoA is defined as that amount which reactivates Lipmann's sulfanilamide system

to half the maximum activity.

The isolation of CoA from the electric organ is described. CoA has been purified to certain degree by treatment with barium salts which precipitated the CoA. This preparation assayed 85 unit per mg. electric tissue.

Cholineacetylase could be extracted from electric organ cell free extract. In addition to ATP as energy source and choline and acetate as substrates, fluoride and eserine must be present in order to inhibite the action of ATPase and AChE respectively.

From one g of fresh electric tissue, enzyme solutions were obtained which form 210μg. of ACh per hour.

Choline-acetylating system of the electric organ has been purified and concentrated by fractional ammonium sulfate precipitation.

In this procedure 150—200 mg. of ACh formed per g. per hr.

Enzyme contains active sulfhydryl groups which are readily inactivated by monoiodoacetic acid in low concentration. The -SH groups are easily oxidized by air.

Presence of cysteine improves the yielde considerably and in sufficiently high concentrations may prevent any effct of oxygen.

Optimum condition of choline acetylating system has been determined. During synthesis of ACh, acetyl donor is one of key points of considerable problem on cholineacetylase.

In ACh synthesizing system of electric organ, the added acetate was found to be an essential component and the unique acetyl donor.

A pyrophosphate split of ATP, leaving AMP, as product of reaction :

$$ATP + CoA + Acetyl \rightleftharpoons AMP + PP + acetyl\text{-}CoA \quad (1)$$

In present experiment with choline as acetyl acceptor, CoA acts catalytically, being produced in the second irreversible reaction :

$$Acetyl\text{-}CoA + choline \rightleftharpoons CoA + acetylcholine \quad (2)$$

Acetylation problems developed by mainly Lipmann's brilliant investigation were considerated in detail relating to acetylcholine synthesizing system.

General discussion and conclusions of the present investigation has explained new conception concerning integration of acetylcholine in metabolic cycle of the electric organ of the fish.

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