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ESTER-SPLITTING ACTIVITY OF THE ELECTROPLAX

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SUMMARY

1. The hydrolysis of acetylcholine, DL-acetyl-β-methylcholine, ethyl chloroacetate, triacetin and butyrylcholine by intact and homogenized isolated single cells and by the connective tissue between electroplax has been tested. Some connective tissue remains attached to the electroplax; a correction for this extracellular esterase was therefore applied to evaluate esterase activity of the electroplax proper.

2. The electroplax enzyme displays the characteristics of acetylcholinesterase. The connective tissue enzyme has neither the characteristics of acetylcholinesterase, human-serum cholinesterase, aliesterase nor arylesterase. In some respects, however, it resembles the cholinesterase of certain piscine plasmas.

3. Hydrolysis of low concentrations of acetylcholine by intact cells is mainly by acetylcholinesterase of the electroplax, whereas high concentrations of acetylcholine are mainly hydrolyzed by the connective tissue esterase. Both enzymes are inhibited by physostigmine. Ethyl chloroacetate is hydrolyzed by at least 2 enzymes in the electroplax, only one of which is inhibited by physostigmine.

4. There is a strong permeability barrier to the penetration of acetylcholine to the interior enzyme of the intact electroplax. This barrier is less strong for DL-acetyl-β-methylcholine, ethyl chloroacetate, triacetin and butyrylcholine.

5. Concentrations of physostigmine which block electrical activity markedly depress acetylcholine hydrolysis. Tetracaine even in much higher concentrations than required to block electrical activity only slightly inhibits acetylcholine hydrolysis. Inhibition by neostigmine is intermediate between that of tetracaine and physostigmine.

INTRODUCTION

The use of electric organs since 1937 by NACHMANSOHN and his associates has been of great value for the study of the role of the ACh system in bioelectrogenesis[1,2]. This tissue was particularly suitable for the isolation and purification of AChE which is thought to have a vital function in nerve activity. However, many questions remain open; one of special interest is the level of AChE activity when electrical activity of the electroplax is blocked by neurotropic agents acting on the ACh system.

Abbreviations: ACh, acetylcholine; EC, ethyl chloroacetate; TA, triacetin; MeCh, DL-acetyl-β-methylcholine chloride; BuCh, butyrylcholine iodide; AChE, acetylcholinesterase.

Early attempts to do this using rows of electroplax[3,4] were unsatisfactory because of permeability problems and because the esterase activity of the electroplax could not be separated from that of the connective tissue. The monocellular electroplax preparation developed by SCHOFFENIELS[5,6], has been used to determine the potency of compounds expected to interact with the ACh system[7–9]. It offered the possibility of an improved analysis of esterase activities of the intact cell.

The difficulties of correlating electrical and enzyme activity have been previously discussed[1]. The necessity of using intact single cells for determining unequivocally the minimum enzyme level compatible with unimpaired conduction has become increasingly apparent, as previously pointed out[10]. It is necessary, however, to first characterize the esterases of the electroplax by using various substrates, and to compare the rates of their hydrolysis by intact and homogenized tissue. The results of these kinetic studies were interesting in several respects, and are reported in this paper.

METHODS

Single cells were isolated from the organ of Sachs of *Electrophorus electricus*[5,6]. The isolated cell used in these investigations consisted of the electroplax (1.5–12 mm^3 volume), a layer of ground substance (less than 100 μ thick) and nerve terminals in front of the innervated membrane, a variable amount of ground substance in back of the non-innervated membrane, and finally the dense structural tissue around the edge of the cell. Only unimpaired cells were used for testing the activity of intact cells; slightly damaged cells were still satisfactory for use as homogenized cells. The mean fresh weight of about 500 cells was 45 mg with a range from 15 to 90 mg. The connective tissue used was the gelatinous ground substance between electroplax. All cells and connective tissue were dissected in oxygenated special Ringer's solution used for electroplax studies[7]. The material was blotted, weighed and added either directly to or homogenized in the incubation mixture used for enzymic measurements. The connective tissue was homogenized even though it was found that intact and homogenized connective tissue had about the same esterase activity using either ACh or EC as substrate. All homogenizations were performed by hand with Tenbroeck tissue grinders. The same Ringer's solution used for dissection was employed for enzymic determinations except that instead of the phosphate buffer 0.1 M Tris was used (pH 7.4–7.8). To this solution was added the substrate, either ACh bromide, EC, TA, MeCh or BuCh. Physostigmine salicylate, neostigmine bromide or tetracaine hydrochloride were added to the incubation mixture as indicated in the experiments.

1 to 4 cells were added to or homogenized in 1–80 ml of incubation mixture depending on enzyme activity. 30–80 mg of connective tissue were homogenized in 3–6 ml of incubation mixture. Measurements of ester concentration were made on aliquots of incubation mixture removed immediately before addition of cells or connective tissue, and on samples removed at intervals thereafter. In most experiments the addition of cells or tissues diluted the substrate concentration less than 3 %, so no precautions were necessary. In the experiments where a relatively large amount of tissue was incubated in a small volume, the control samples were removed 30 min after their addition rather than immediately before. Depending on enzyme activity the incubation proceeded for 2–8 h during which time the tissues or cells were incubated with shaking at room temperature. Hydrolysis of substrate was allowed to

proceed to about 25% of completion. The actual concentrations of MeCh are given although only one of the two isomers is hydrolyzed[11,12]. Hydrolytic activities were determined with the colorimetric procedure of HESTRIN[13]. All results with EC were corrected for the relatively large spontaneous hydrolysis. The pH of the incubation mixture during the course of the experiments did not decrease more than 0.2 pH unit except for the experiments with high concentrations of EC in which the pH dropped to as low as 6.8 in 3 h; this time period was therefore the longest period of incubation which could be used. At pH 6.8 the hydrolysis of EC by cells is about 85 % that at pH 7.8. Control experiments indicated that when ACh or MeCh were substrates the amount of choline formed produced only about 10% inhibition, which, being relatively constant in all tubes, was not corrected.

Calculation of results

In agreement with earlier results[3] it was found best to relate the esterase activities of intact and homogenized cells to the number of cells used rather than to their weight. The variations in activity were smaller under these conditions indicating that the enzyme activities of cells are similar regardless of weight.

It is possible to obtain pure connective tissue but not electroplax uncontaminated with connective tissue. An estimate was made of the percentage of cell weight which actually was connective tissue. 10 cells were dissected in the usual manner and weighed; then the connective tissue was trimmed off and weighed. This careful removal of most of the connective tissue could not be employed in the actual experiments since in the process the cells were injured. The connective tissue constituted about 50 % of the weight of the electroplax plus connective tissue, *i.e.*, what is referred to as cell. Probably it forms about 60–70 % of the weight since it was not possible to remove it all in the above experiments. The mean esterase activities with intact and homogenized cells were calculated both uncorrected and corrected for connective tissue, assuming that 50% of the weight of each cell was connective tissue. The esterase activity of this weight of connective tissue was subtracted from the total activity. The mean values $\pm$ 1 standard deviation of the mean (standard error) are shown in the figures for the uncorrected values. Since the corrected values are only estimated, and not based entirely on experimental determination it was not thought justified to attach a standard deviation to this calculated value. Although not recorded, standard deviations were calculated and were found to be about the same as for the uncorrected values.

Approximate K_m values and maximum velocities (V_{max}) were calculated from double reciprocal plots of velocity *versus* substrate concentration. To obtain an estimate of the variation in the K_m plot, not only the reciprocal of the mean velocity was plotted but also the reciprocal of the mean $\pm$ 1 standard error.

RESULTS

In Fig. 1 are shown the hydrolytic activities of connective tissue with the various substrates. Each of the points with ACh is based on 4 or 6 determinations, except 2 points which are based on single determinations. With the other substrates means in the figure are based on 2 or 3 experiments. MeCh was tested in concentrations of 10^{-3}–10^{-1} M (14 experiments). In half of the experiments no hydrolysis was observed

and in the other half the activities found were very low (< 0.5 μmole hydrolyzed per 50 mg/h). The irregularity of the results indicates that the low activities sometimes recorded may be due to contamination with cellular material, since it is sometimes difficult to be sure that included with the connective tissue there are none of the tips of the convoluted non-innervated membrane of the electroplax. The amount of contamination would be irregular, but probably not greater than 5% of the connective tissue weight. It thus appears likely that MeCh is not hydrolyzed by connective tissue.

The hydrolytic activity of homogenized cells, uncorrected and corrected is shown in Fig. 2. Each of the uncorrected means is based on 3 determinations except for the ACh values which are based on 3–5. Since MeCh is apparently not hydrolyzed by connective tissue no correction was necessary. The correction is significant only for the three highest concentrations of ACh, and possibly for the highest concentration of EC. As shown in Fig. 1 the connective tissue esterase activity is relatively low in the other cases.

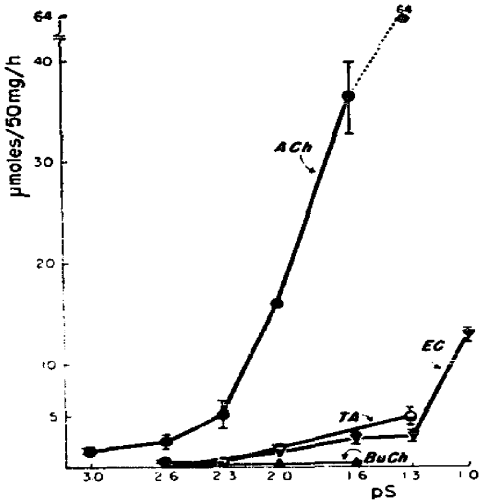

Fig. 1. Hydrolysis of ACh, EC, TA and BuCh by connective tissue esterase. No hydrolysis of MeCh was observed. pS = negative log of molar substrate concentration. The vertical bars indicate ± standard error (standard deviation of the mean).

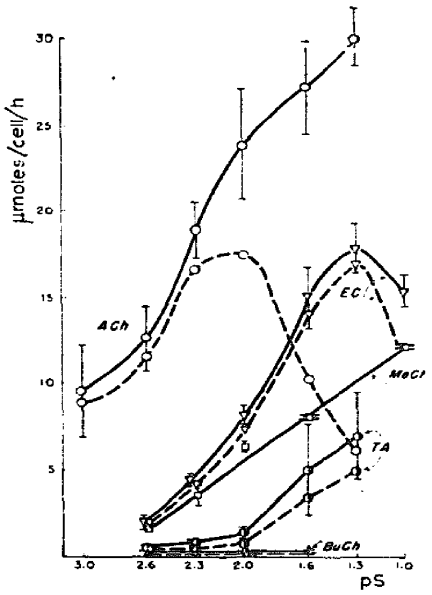

Fig. 2. Hydrolysis of ACh, EC, MeCh, TA and BuCh by homogenized isolated single electroplax. ———, uncorrected values; – – –, the values after subtraction of the estimated activity of connective tissue contamination. There is no correction for hydrolysis of MeCh.

The ability of intact cells to hydrolyze EC, TA, BuCh and MeCh are shown in Fig. 3. The corrections for connective tissue are relatively small. Each of the points (uncorrected values) is based on 3 experiments except for the EC values which are based on 4 or 5. Comparison of Figs. 2 and 3 shows that the rate of hydrolysis of EC by intact cells is 50–80% that of homogenized cells. With MeCh the intact cells have about 30% the activity of homogenized cells at the 3 lower concentrations and 80% at the highest concentration.

The results obtained with ACh and intact cells are shown in Fig. 4. For comparison we have included from Fig. 2 the results with ACh and homogenized cells. 4–6 determinations were performed at each concentration of ACh using intact cells.

The data recorded in Figs. 1–4 were used to calculate the apparent Michaelis constant (K_m) and the apparent maximum velocities (V_{max}). These are only the apparent constants since the activity is not tested with single enzymes in solution, but the hydrolysis of substrates is measured by possibly several different esterases.

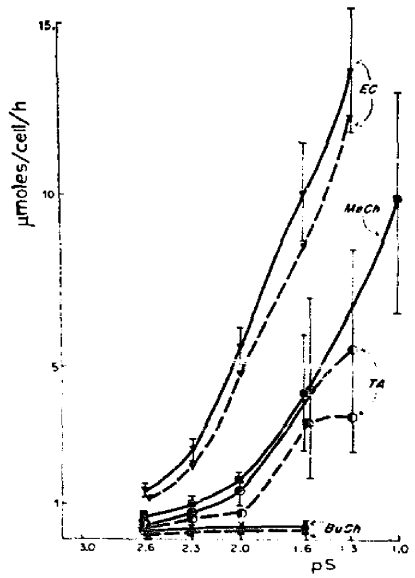

Fig. 3. Hydrolysis of EC, MeCh, TA and BuCh by intact isolated single electroplax. ———, uncorrected values; – – –, estimated activities after correction for activity of connective tissue.

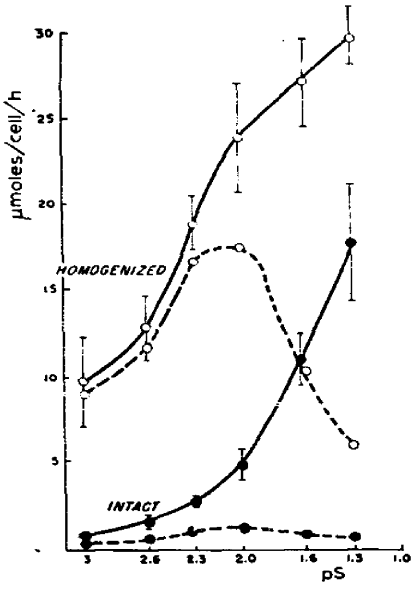

Fig. 4. Hydrolysis of ACh by intact and homogenized cells. ———, uncorrected data; – – –, estimated activities after correction for activity of connective tissue.

With intact cells we are only measuring the constants for the enzyme which can be reached by the substrate. There may even be some permeability barriers with homogenized tissues, although in brain homogenates no evidence for such an assumption was found[14]. For these various reasons and because of the relatively large standard errors noted in Figs. 1–7 the values of K_m and V_{max} can only be considered as an approximation.

Figs. 5–7 show LINEWEAVER-BURK plots for ACh, EC and MeCh with homogenized and intact cells. The V_{max} and K_m values were determined from these plots in the usual manner, they are listed in Table I. The corrected constants for TA (intact and homogenized cells) and ACh (intact cells) could not be calculated because the estimated corrected values were very irregular when plotted in the double reciprocal manner. Since connective tissue does not hydrolyze MeCh, no correction was necessary.

The effects of physostigmine on hydrolysis of EC and ACh are shown in Figs. 8 and 9. The partially purified preparation of AChE from the electric eel had an initial activity of 960 mg ACh hydrolyzed/mg protein/h. It was not possible to completely inhibit the hydrolysis of EC in intact or homogenized cells (Fig. 8). Each of

the recorded points are the uncorrected values, and are based on 2 experiments with purified AChE and 3–6 experiments for the others. As can be seen in Fig. 8 and as was also found in other experiments, it requires higher than 10^{-2} M concentration of physostigmine to inhibit the physostigmine resistant enzyme in homogenized tissue 50%, since the resistant enzyme has only about 30% of the total activity. In contrast about 10^{-7} M physostigmine inhibits about 50% of the sensitive enzyme which contributes about 70% of total enzyme activity.

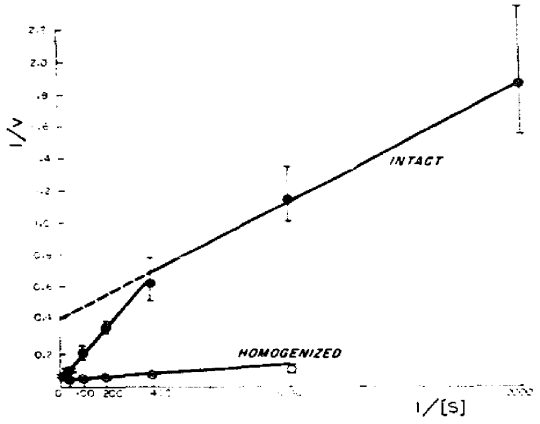

Fig. 5

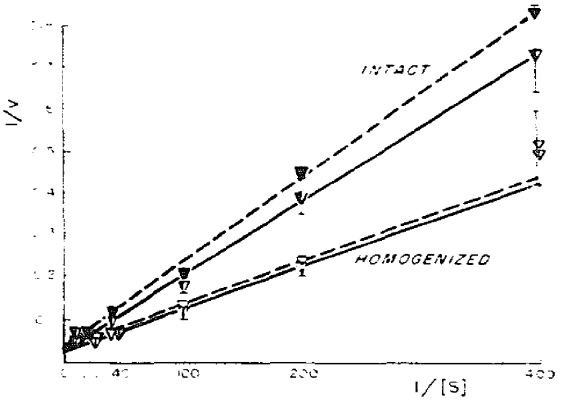

Fig. 6

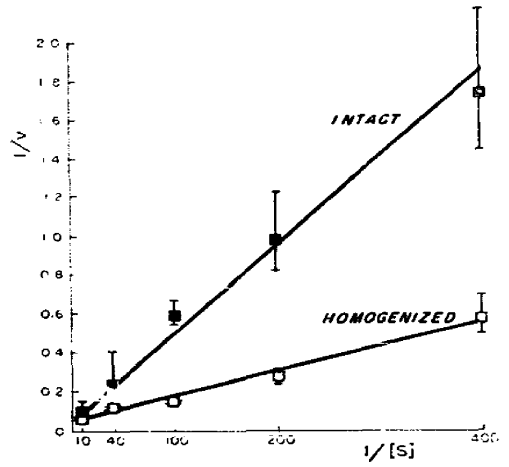

Fig. 7

Fig. 5. Reciprocal plots of velocity of hydrolysis of ACh by intact and homogenized cells as a function of substrate concentration.

Fig. 6. Reciprocal plots of velocity of hydrolysis of EC by intact and homogenized cells. ———, uncorrected activities; – – –, estimated activities after correction for connective tissue.

Fig. 7. Reciprocal plots of velocity of hydrolysis of MeCh by intact and homogenized cells as a function of substrate concentration.

It was possible to almost completely inhibit the hydrolysis of ACh in intact or homogenized cells of connective tissue (Fig. 9 data uncorrected). Although not shown in Fig. 9 a similar curve for physostigmine inhibition of ACh hydrolysis in homogenized cells was obtained with $1 \cdot 10^{-3}$ M ACh as substrate as is recorded for $1 \cdot 10^{-2}$ M ACh. The data for homogenized and intact tissue are not corrected in Figs. 8–10 because it is felt that with the use of inhibitors too many assumptions would have to be made, *i.e.*, not only the percentage of connective tissue but also the percentage inhibition by physostigmine of connective tissue. The corrections would be relatively

TABLE I

APPARENT V_{max} AND K_m VALUES FOR SUBSTRATES OF ELECTRIC TISSUE ESTERASES

Substrate	Tissue	V^*_{max}		$Km\,(M)$	
		Uncorrected	Corrected	Uncorrected	Corrected
ACh	Homogenized cells	30	20	$4\cdot10^{-3}$	$2\cdot10^{-3}$
	Intact cells	(a)** 30	—	$50\cdot10^{-3}$	—
		(b) 2.5	—	$2\cdot10^{-3}$	—
	Connective tissue	100		$8\cdot10^{-2}$	
EC	Homogenized cells	40	25	$4\cdot10^{-2}$	$2.5\cdot10^{-2}$
	Intact cells	40	25	$7\cdot10^{-2}$	$5\cdot10^{-2}$
	Connective tissue	17		$12\cdot10^{-2}$	
MeCh	Homogenized cells	20	—	$3\cdot10^{-2}$	—
	Intact cells	20	—	$10\cdot10^{-2}$	—
	Connective tissue	0		—	
TA	Homogenized cells	25	—	$15\cdot10^{-2}$	—
	Intact cells	25	—	$20\cdot10^{-2}$	—
	Connective tissue	20		$20\cdot10^{-2}$	
BuCh	Homogenized cells	0.4	0.3	$2.3\cdot10^{-3}$	$2.2\cdot10^{-3}$
	Intact cells	0.4	0.3	$3.0\cdot10^{-3}$	$2.2\cdot10^{-3}$
	Connective tissue	0.4		$8.0\cdot10^{-3}$	

* Intact and homogenized cells; μmoles hydrolyzed/cell/h. Connective tissue; μmoles hydrolyzed/50 mg/h.

** a, Based on $5\cdot10^{-2}$–$2.5\cdot10^{-3}$ M ACh; b, based on $2.5\cdot10^{-2}$–$5\cdot10^{-4}$ M ACh.

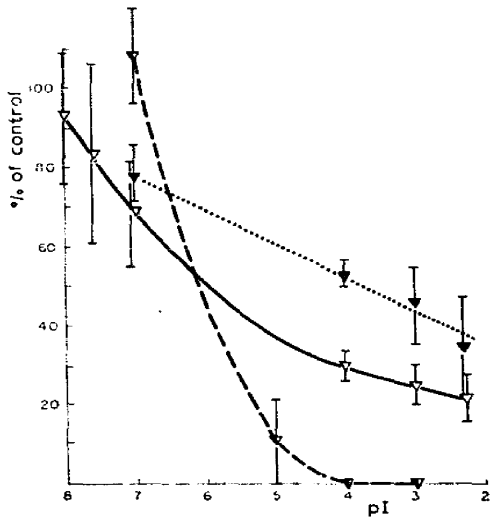

Fig. 8. Inhibition by physostigmine of the hydrolysis of $5\cdot10^{-2}$ M EC. ▼···▼, intact cells; ▽—▽, homogenized cells; ▼———▼, purified AChE. pI = negative log of molar concentration of inhibitor.

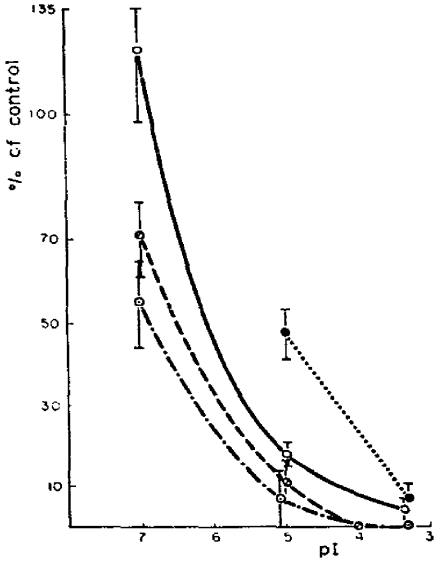

Fig. 9. Inhibition by physostigmine of the hydrolysis of $1\cdot10^{-2}$ M ACh (intact and homogenized cells and connective tissue) or $5\cdot10^{-3}$ M ACh (purified AChE). ●···●, intact cells; ○—○, homogenized cells; ⊙–·–⊙, connective tissue; ⊘———⊘, purified AChE.

minor ... Figs. 8 and 9 except with ACh as substrate in intact cells. Each of the mean values of Fig. 9 is based on 2 experiments except the values with homogenized cells which are based on 4 experiments each.

Tetracaine is a very poor inhibitor of the hydrolysis of ACh by intact cells (Fig. 10), while physostigmine is a potent inhibitor (Fig. 9). With $5 \cdot 10^{-2}$ M ACh as substrate in intact cells the great majority of the hydrolysis is due to connective tissue. As shown in Fig. 9 however, the inhibition of cellular or connective tissue esterase by physostigmine is similar. Each of the points in Fig. 10 is the mean of 3 determinations.

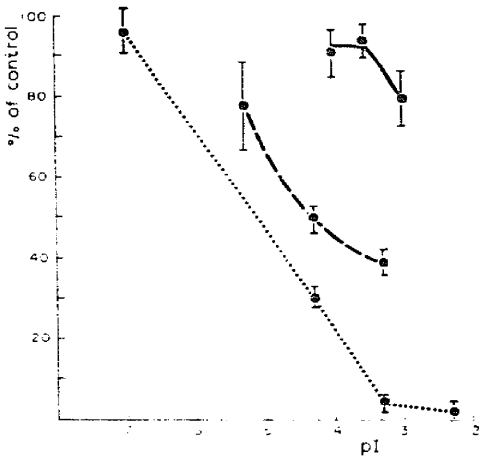

Fig. 10. Effect of physostigmine, neostigmine and tetracaine on hydrolysis of $5 \cdot 10^{-2}$ M ACh in intact cells. ●···●, physostigmine; ●--●, tetracaine; ●---●, neostigmine.

DISCUSSION

For correlating the effects of compounds on AChE with those on electrical activity, it is impossible to use homogenized electroplax. Since high concentrations of inhibitor must be applied to overcome the structural barrier, excess inhibition of the enzyme will occur during the homogenization. Intact segments of tissue are also not suitable, because the greater permeability barriers prevent sufficient concentrations of substrate reaching the esterase in the interior of the segments. This is obviously the explanation for the low rate of hydrolysis of EC found with segments[3]; the rate is about 6 times lower than that with the single electroplax reported here. Moreover, the use of segments does not permit a separation of the hydrolysis of connective tissue from that of the electroplax, whereas with single cells we can determine the activity of connective tissue and estimate the activity of the electroplax. SCHLEYER *et al.*, using segments of tissue[3,4], were able to demonstrate that certain compounds block electrical activity without affecting AChE; their data thus indicate that this action must be attributed to an effect on the receptor. However, the observations with compounds acting on cholinesterase are unsatisfactory for the reasons just outlined; they do not permit an evaluation of cholinesterase activity in relationship to electrical activity. For such studies single electroplax preparations must be used. This preparation has provided, during the last few years, a much more accurate and

reliable picture of the effects of compounds on the various electrical characteristics and has removed many difficulties and contradictions of earlier studies[7–9].

It appeared essential to first characterize the esterases of the electroplax and find a substrate which penetrates to all the available esterase in the intact preparation before attempting a correlation of effects of inhibitors on AChE and electrical activity. A pertinent result of the data reported is the evidence that the esterase activity of homogenized cells, after correction for connective tissue contamination (Fig. 2) displays a pattern of activity towards various substrates similar to that of purified AChE from electric eel[15–17]. With ACh as the substrate the typical bell-shaped curve is obtained, and MeCh is actively hydrolyzed. No substrate inhibition is found with MeCh. There appears to be substrate inhibition with EC, which would confirm a previous observation[4], although this is not quite certain because of the scattering of the data (Fig. 2).

Using the main electric organ of electric eel, rather than the Sachs organ, which we used, it was found that freshly homogenized tissue and purified AChE from electric eel have the same pattern of activity towards various substrates[16]. In contrast we find a markedly different pattern of activity between freshly homogenized cells from the Sachs organ, uncorrected for connective tissue, and that reported for purified AChE. The difference in results is probably because in the main organ the cells are much more closely packed than in the Sachs organ with correspondingly less connective tissue between the cells. Using the main organ therefore the esterase activity of connective tissue was probably negligible relative to that of the cells, and therefore did not significantly perturb the pattern of activity towards the various substrates.

The connective tissue esterase is not the AChE type as evidenced by the lack of substrate inhibition with ACh, and the lack of hydrolysis of MeCh (Fig. 1). The results with BuCh indicate that it is not of the butyrocholinesterase (human serum esterase) type. Aliesterases (B-esterases) and arylesterases (A-esterases, aromatic esterases) have been described in vertebrate and insect tissue[18–22]. Since the above two esterases do not hydrolyze choline esters and are not inhibited by physostigmine the connective tissue esterase activity is not exclusively of either type. There may of course be a mixture of several esterases in the connective tissue. In some respects the pattern of esterase activity in connective tissue resembles that in various piscine plasmas, which hydrolyze ACh at a higher rate than BuCh or MeCh (see refs. 21, 22).

An important aim of this investigation was to determine the penetrations of the various substrates into the intact cell and their ability to react with the hydrolytic enzymes. It was hoped that a double reciprocal plot of the data shown in Figs. 1–4 would provide information as to the extent of the permeability barriers to these substrates. If there is no absolute permeability barrier the V_{max} values for intact and homogenized cells would be identical. Also if there is no absolute permeability barrier, any difference in K_m values for homogenized and intact cells indicates a relative permeability barrier. The ratio of homogenized to intact K_m gives an approximate indication of the fraction of external substrate concentration which is within the cell[4]. While the above analysis is theoretically satisfactory, several practical difficulties were encountered. Since the activity measured is not that of a single purified enzyme, the values are only approximations, although probably fairly good ones. A more serious difficulty is the variability of the data, as shown in the figures. The values of K_m and V_{max} in Table I are estimated by fitting the best possible line

through the mean values of the double reciprocal plots. It is, however, possible in most of the cases to fit different lines to the data which would pass within $\pm$ 1 standard error, and which would give different K_m and V_{max} values. Some reservation must therefore be attached to the analysis of the data based on K_m and V_{max} values.

The V_{max} values (Figs. 6, 7 and Table I) of intact and homogenized cells with each substrate appear identical indicating no absolute permeability barrier. The different apparent K_m values for homogenized and intact cells (Table I) indicates a relative permeability barrier.

The results with ACh are more difficult to interpret than with the other substrates, since there are apparently at least two enzymes hydrolyzing ACh with differing K_m values as indicated by the break in the K_m curve (Fig. 5). Two V_{max} and two K_m values for ACh and intact cells are given in Table I, one based on the steep and the other on the flat part of the curve. There was an indication of a break in the curve with homogenized cells also, although it is not so obvious (Fig. 5), probably because the ratio of electroplax esterase to connective tissue esterase is so much greater in the homogenized than in the intact cell (Fig. 4). Based on the data of Fig. 4 it appears that the apparent K_m value with intact cells (Fig. 5) based on the flat part of the curve is an average value of about an equal mixture of electroplax and connective tissue enzyme. The K_m for the steep part of the curve is mainly that of the connective tissue esterase. In agreement with this interpretation the apparent K_m for the connective tissue enzyme with ACh as substrate ($8 \cdot 10^{-2}$ M) is similar to that with high concentrations of ACh in intact cells ($5 \cdot 10^{-2}$ M). The K_m for the electroplax enzyme itself is therefore probably lower than $2 \cdot 10^{-3}$ M which is the calculated K_m for the mixture of the two enzymes at low substrate concentrations in intact cells and is also the corrected K_m for homogenized cells. The K_m of purified AChE with ACh as substrate is about $1 \cdot 10^{-4}$ M (see ref. 23).

The V_{max} values for total hydrolysis of ACh by intact and homogenized cells appear to be the same (Fig. 5) indicating no absolute permeability barrier, however, at very high concentrations of ACh we are mainly measuring the hydrolysis of ACh by connective tissue in which we had previously found no barrier to the penetration of ACh (see METHODS). There may actually be an absolute permeability barrier to the penetration of ACh in the intact electroplax. The data recorded in Fig. 4 indicates that the barrier may be either very great or possibly even absolute. The corrected esterase activity of intact cells at the optimal ACh concentrations is only about 7 % that of homogenized cells (corrected values, 10^{-2} M) (Fig. 4). Since it has been estimated that the synaptic regions of the electroplax occupy 3–6 % of the surface area[1], it is possible that the activity measured with the intact cell is mainly due to synaptic plus nerve terminal esterase. The barrier to ACh in the electroplax is much greater than in other preparations such as rabbit vagus[10] or crab nerve[24]. For the reasons discussed the ratio of homogenized to intact K_m for ACh would not be an accurate index of the permeability barrier.

The data in Figs. 1–7 and Table I indicate that there is some barrier to the penetration of all the substrates, being least for TA and BuCh. Since MeCh is only hydrolyzed by the electroplax, and not by the connective tissue it would be the ideal substrate for determination of AChE in the intact electroplax if it were more soluble. In the rabbit vagus nerve MeCh penetrates very readily[10], however, as seen in Figs. 2, 3 and 7, there is a substantial barrier to its penetration in the electroplax.

There appear to be in the electroplax at least two esterases hydrolyzing EC, one of which is inhibited by physostigmine, the other not (Fig. 8). The resistant enzyme (probably an aliesterase) accounts for about 30 % of the total activity with 70 % of the activity being inhibited by physostigmine. SCHLEYER had also found that physostigmine did not completely inhibit the hydrolysis of EC (see ref. 4).

Physostigmine readily inhibited ACh hydrolysis in connective tissue and homogenized cells (Fig. 9, uncorrected data). It is surprising that 10^{-5} M physostigmine does not inhibit ACh hydrolysis in intact cells as much as in connective tissue, since at a concentration of $1 \cdot 10^{-2}$ M ACh about 80 % of the hydrolysis is due to contamination with connective tissue. It is possible that the cells used in these 2 experiments had a smaller proportion of connective tissue contamination. With the homogenized cells only about 25 % of the total activity is due to connective tissue contamination (Fig. 4).

Arylesterases and aliesterases are the only esterases known that are not inhibited by physostigmine but they do not hydrolyze ACh (see ref. 22), therefore it was to be expected and was found that the hydrolysis of ACh is inhibited by physostigmine.

It was hoped to correlate inhibition of AChE with effects on electrical activity, however, as mentioned previously none of the substrates penetrate at a sufficiently high rate into the intact cell. Therefore when an inhibitor is used the status of the unassayed enzyme would remain in doubt. Experiments were, however, performed to find out what information could be obtained using a high concentration ($5 \cdot 10^{-2}$ M) of ACh as substrate, where the total activity of intact cells is about 60 % that of homogenized cells (Fig. 4, uncorrected values). Only a small amount of the total activity in intact cells is, however, due to cellular enzyme, the majority of ACh hydrolysis being due to the connective tissue. Tetracaine even at 10^{-4} M has marked effects on the electrical activity of single electroplax[6] whereas 10^{-3} M tetracaine only inhibits enzymic activity 20 % (Fig. 10). Although we are mainly measuring the effect of tetracaine on connective tissue esterase it has also been found that tetracaine is a relatively weak inhibitor of purified AChE (see ref. 25). Tetracaine effects on electrical activity are therefore probably entirely due to competition with ACh for a receptor component of the membrane. $5 \cdot 10^{-4}$ M physostigmine markedly inhibits electrical activity[6], and also inhibits the hydrolysis of ACh in intact cells at least 90 % (Figs. 9, 10). EC hydrolysis of intact cells is also markedly inhibited by this concentration of physostigmine (Fig. 8), if we subtract the 30 % of the hydrolysis which is due to a second enzyme. Inhibition of esterase is therefore a major factor in the effects of physostigmine on the electrical activity of the electroplax.

Inhibition by neostigmine is smaller than that with physostigmine (Fig. 10). The main factor is probably its inability to penetrate to that portion of the enzyme which is within the cell. In addition, neostigmine is only 1/3 as potent as physostigmine in inhibiting AChE in solution[26]. The block of synaptic transmission by neostigmine in relatively low concentration in intact electroplax is most likely not only due to inhibition of AChE, but probably involves direct interaction with the receptor in the membrane as occurs at certain neuromuscular junctions[27]. The rapid depolarizing action of the compound supports this assumption. For a more satisfactory and more quantitative relationship between electrical and AChE activity it would be necessary to use a better penetrating substrate than we have as yet been able to find.

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