ELECTRICAL ACTIVITY IN ELECTRIC TISSUE

II. EVALUATION OF ESTERASE ACTIVITY IN INTACT ELECTROPLAX*

by

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INTRODUCTION

Studies in this laboratory had shown that the effect of certain substances on nerve conduction can be attributed to inhibition of the enzyme acetylcholinesterase[1]. For the effect of certain other substances, which are relatively mild inhibitors of acetylcholinesterase *in vitro*, a different interpretation had emerged[2]. It was desirable to supplement other tests of these theories by simultaneous measurement of transmembrane potentials and acetylcholinesterase activity. The electroplax of *Electrophorus electricus* were a suitable material for this study.

The validity of the assay depended evidently on the extent to which the substrate could penetrate intact cells. A method of estimating this condition is (a) to determine the Michaelis constant (K_m) of the substrate using ground cells; (b) to determine the apparent K_m of the substrate using intact cells; (c) to calculate the ratio of these two quantities, which gives the fraction of external substrate concentration prevailing within the cell; (d) to determine the enzymic activity of whole, and subsequently ground, cells to determine whether the rise in activity on grinding can be accounted for solely by the increase in effective substrate concentration brought about by destruction of the cell. The expected activity of a constant quantity of enzyme as a function of substrate concentration is taken from step (a) above.

If the increase in activity on grinding is greater than expected on this account, it must be concluded that the substrate did not assay the balance of cellular enzyme. A complete assay permits an evaluation of inhibitors. However, in order to obtain the true effect of added inhibitors in terms of the residual ability of acetylcholinesterase to hydrolyze physiological quantities of acetylcholine, a correction for the presence of added substrate during the assay may be necessary when the inhibition is competitive. The extent to which inhibitors penetrate intact cells can also be determined in this type of experiment when the enzyme-inhibitor dissociation constant (K_I) is known.

By using the criteria just outlined, it had been found that neither acetylcholine nor its tertiary amine analogue, dimethylaminoethyl acetate, can penetrate to more than 15% of the acetylcholinesterase of intact electroplax in sufficient concentration to permit

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assay. Ethyl monochloroacetate had been shown to be hydrolyzed relatively well by purified acetylcholinesterase[3] from the electric organ of *Electrophorus electricus*, presumably in consequence of a HAMMETT[4] type substitution effect of the chloro group. The correlation study of electrical and acetylcholinesterase activity was therefore undertaken with this substrate[5]. The results of the assay in terms of the method stated are described in detail in the present paper.

METHODS

The methods used were previously described[5]. They had been designed to minimize biological variation. It was found that the absolute enzymic activity of tissue specimens could vary considerably; however, the six segments of six electroplax each constituting a single experiment were quite similar in that respect and could be compared reproducibly for effect of inhibitors, variation in substrate concentration, etc.

Tissue or ground material were exposed to inhibitors for 20 to 30 minutes prior to assay. The substrate was added to the sidearm of Warburg vessels in 0.1 ml ethanol near the end of the gassing period to avoid evaporation. The usual manometric technique was used at 25° C and pH 7.6. During the 30 minute period of observation, the reaction reached 10% of completion, and the rate of hydrolysis remained constant, indicating that a steady state existed with respect to penetration of substrate and of reversible, competitive inhibitors.

The data presented are corrected for spontaneous hydrolysis of substrate. Average deviations in experiments involving intact cells usually did not exceed 10%, those in experiments using pooled enzyme material were less than 5%. Probable errors are indicated except for the calculated data in Table IV, in which case they were not individually determined.

RESULTS

The range of absolute values of ethyl chloroacetate splitting by the segments of electroplax employed has been given in the preceding paper[5], together with the effect of concentrations of acetylcholinesterase inhibitors which cause block of conduction after exposure for the same length of time. Table I, column 1, shows the effect of both larger and smaller amounts of eserine and prostigmine. Column 2 of the Table shows the effect of increasing concentrations of these agents on ground electroplax. It is apparent that a plateau occurs in both instances, though not at exactly the same degree of inhibition. Therefore, failure of the inhibition to increase with external inhibition concentration cannot be due to lack of its penetration of the intact cell. The presence of a second enzyme hydrolyzing ethyl chloroacetate is indicated. The eventual decline in residual hydrolytic acitivity, again both in whole and ground cells, supports this conclusion. Additional evidence is the fact that, in the presence of 10 μg/ml eserine to inhibit the acetylcholinesterase, ground cells can still hydrolyze ethyl chloroacetate but not acetylcholine. Moreover, the ratios of initial velocities of hydrolysis, acetylcholine 0.004 M, ethyl chloroacetate 0.01 M, for ground tissue and for purified acetylcholinesterase differ. The latter preparation does not hydrolyze ethyl chloroacetate in presence of 10 micrograms per ml eserine. These data are presented in Table II. Assigning 60% of the ethyl chloroacetate splitting activity of ground cells to acetylcholinesterase when the substrate concentration is 0.01 M, one obtains, using the first two concentrations listed in Table I, column 2, a value for the K_I of prostigmine ($0.7 \cdot 10^{-7}$ moles per liter) which is similar to the value ($1.6 \cdot 10^{-7}$ M) known from determinations on purified acetylcholinesterase[6].

The further treatment of the data is therefore based on the conclusion that the tissue contains (a) acetylcholinesterase which hydrolyzes ethyl chloroacetate as well as acetylcholine and is inhibited completely by less than 10 μg/ml eserine or prostigmine; (5.5 times as much inhibitor should be required for the same degree of inhibition when acetylcholine is the substrate because of its more favorable Michaelis constant, see

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TABLE I

EFFECT OF ACETYLCHOLINESTERASE INHIBITORS ON ETHYL CHLOROACETATE HYDROLYSIS BY INTACT AND GROUND ELECTROPLAX

(Sachs Organ of *Electrophorus electricus*). 25° C. pH 7.6. Substrate concentration, 0.01 *M*.

Compound	*Concentration micrograms per ml*	*Per cent remaining activity**		
		1. Intact cells	*Prob. error*	*2. Ground cells (± 7%)*
Eserine	0.06	75	9	
	0.6	64	9	
	3.6	47	11	
	10			34
	20	45	5	
	60			32
	100	44	7	
	300			24
	500	31	7	7
	1500	18	4	14
Prostigmine	0.06	88	8	60
	0.3			49
	0.6	74	9	
	1.5			40
	3	59	6	
	7.5			40
	20			44
	30	52	7	
	75	41	11	
	100			40
	300	49	6	
	500			28
	1500			17
	5000	32	4	
	10000	17	3	
	30000	18	2	
Tertiary amine analogue of prostigmine	20			44
	500	9	2	0

* Initial velocity of hydrolysis relative to controls.

below); (b) another enzyme which also hydrolyzes ethyl chloroacetate, though not acetycholine, and requires larger concentrations of eserine or prostigmine for its inhibition. Using the last three values from Table I, column 2, for eserine (300 to 1,500 μg/ml) and the last two for prostigmine (500 and 1,500 μg/ml), where partial inhibition of the second enzyme has occurred, one obtains K_I values of $4 \cdot 10^{-4}$ and $2 \cdot 10^{-3}$, respectively, for the two inhibitors. Table I also shows the effect of the tertiary amine analogue of prostigmine on the two enzymes. This substance appears to be a stronger inhibitor of the second enzyme than either eserine or prostigmine, whereas its K_I for acetylcholinesterase is about two orders of magnitude larger than that of eserine and acetylcholinesterase (unpublished data obtained in this laboratory).

The second enzyme was further tested with respect to its ability to hydrolyze other esters. Ground electroplax, in the presence of 10 μg/ml eserine, show no measurable activity toward 0.01 molar dimethylaminoethyl acetate or ethyl acetate (see Table II). That such activity in the case of ethyl chloroacetate is indeed a hydrolysis of the ester linkage was shown by simultaneous manometric and colorimetric observation of the

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TABLE II

EVIDENCE FOR THE PRESENCE IN ELECTROPLAX OF A SECOND ENZYME HYDROLYZING ETHYL CHLOROACETATE, BUT NOT ACETYLCHOLINE, DIMETHYLAMINOETHYL ACETATE OR ETHYL ACETATE

Substrate	*Enzyme preparation*	*Effect of 10 microgram ml eserine % remaining activity**	*Relative activity of controls*
Ethyl chloroacetate 0.01 *M*	ground cells	34 ± 2	1
Acetylcholine 0.004 *M*	ground cells	3	14
Dimethylaminoethyl acetate, 0.01 *M*	ground cells	3	
Ethyl acetate 0.01 *M*	ground cells	1	
Ethyl chloroacetate 0.01 *M*	purified acetylcholinesterase	1	1
Acetylcholine 0.004 *M*	purified acetylcholinesterase	—	26

* Initial velocity of hydrolysis relative to controls.

TABLE III

HYDROLYSIS OF ETHYL CHLOROACETATE BY GROUND ELECTROPLAX AFTER INHIBITION OF ACETYLCHOLINESTERASE

(by 10 micrograms per ml eserine)

Method of analysis	*Substrate*	*Interval min*	*Extent of reaction per cent*
Manometric	ethyl chloroacetate	100	40 ± 2
Colorimetric	ethyl chloroacetate	100	42
Colorimetric	acetylcholine	40	4

reaction. The latter method[7] is a test for the ester linkage. With both methods the same extent of reaction was obtained. These data are shown in Table III.

We return now to the analysis of the completeness of the acetylcholinesterase assay of intact cells. It was necessary to determine the K_m of ethyl chloroacetate and acetylcholinesterase in the presence of the other enzyme. This was again done by appropriate experiments with and without 10 micrograms per ml eserine, all on the same preparation of pooled ground cells. The velocity of hydrolysis of ethyl chloroacetate by acetylcholinesterase as a function of substrate concentration could, of course, only be obtained from these data by difference. In the case of whole cells the task was further complicated by the variation in their enzymic activity (see the preceding paper of this series[5]) which required that only cells from a single tissue preparation (six specimens) be compared as to their absolute enzyme activity. At the same time it seemed desirable to have at least duplicates at each substrate concentration and at least three different substrate concentrations. Consequently, the attempt to determine the apparent Michaelis constant required two steps: 1. measuring the variation of total enzymic activity with substrate concentration; 2. measuring the degree of inhibition of total enzymic activity by 10 μg/ml eserine at each substrate concentration. Constructing the contribution of acetylcholinesterase to the total activity in this manner, it was found that the acetylcholinesterase activity of intact cells show a velocity maximum at 0.03 *M* ethyl chloroacetate.

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The same is true for ground cells as well as for the purified acetylcholinesterase preparation mentioned above, when the three concentrations used are 0.01, 0.03 and 0.06 *M* ethyl chloroacetate. The acetylcholinesterase activity of whole cell segments as used in these experiments becomes too small at lower substrate concentrations to determine the actual apparent K_m. However, the appearance of the velocity maximum at about the same point as with ground cells makes it clear that the penetration of ethyl chloroacetate to as much of the enzyme of intact cells as participates in its hydrolysis is substantially complete. The Michaelis constant obtained for ethyl chloroacetate and acetylcholinesterase in ground cells is 0.015 *M*. This is in satisfactory agreement with the value obtained with the purified acetylcholinesterase preparation (0.011 *M*), in which the second enzyme is absent. Some of these data are collected in Table IV.

The K_m of ethyl chloroacetate with the second enzyme of the ground cells ranged from 0.07 to 0.17 *M* in different experiments, and about the latter apparent value was obtained with intact cells. A more accurate determination was precluded by the large value of the constant, since spontaneous hydrolysis of the substrate becomes formidable relative to enzymic when the required high substrate concentrations and the manometric technique are used.

The comparison of whole and subsequently ground cells for their acetylcholinesterase activity again involved use of 10 microgram per ml eserine and subtraction of the contribution of the other enzyme from the total. The data are summarized in Table V. These experiments were done at 0.03 and 0.06 *M* ethyl chloroacetate. It will be seen that the total enzymic activity rises regularly on grinding while the activity of the second enzyme falls to a variable extent. The acetylcholinesterase activity rose on grinding in each of four separate experiments, giving an average of 63% (= 10) activity of whole cells relative to ground. This may be attributed to failure of the substrate to reach the remainder of the cellular enzyme in substantial concentration.

The apparent lability of the second enzyme was not investigated further. It may be due either to the grinding itself or to its use at room temperature. It did not occur consistently. For instance, in other experiments the second enzyme contributed 28 to

TABLE IV

REPRESENTATIVE DATA ON THE CONTRIBUTION OF ACETYLCHOLINESTERASE TO ETHYL CHLOROACETATE HYDROLYSIS AT DIFFERENT SUBSTRATE CONCENTRATIONS. INTACT AND GROUND ELECTROPLAX, AND PURIFIED ACETYLCHOLINESTERASE

Enzyme	*Initial velocity of hydrolysis micromoles per hour Ethyl chloroacetate, M*			*Apparent* K_m *M*	K_m *M*
	0.01	0.03	0.06		
Intact cells plus 10 micrograms/ml eserine	1.0	5.6	8.1	approx. 0.17	
Acetylcholinesterase of intact cells (calculated, see text)	3.5	8.4	7.7		
Ground cells plus 10 micrograms/ml eserine	2.0	4.5	7.2		approx. 0.07 (up to 0.17 in other expts.)
Acetylcholinesterase of ground cells (calculated)	5.1	8.5	7.3		0.015
Purified acetylcholinesterase	6.7	10.9	9.3		0.011

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TABLE V

HYDROLYTIC ACTIVITY OF INTACT RELATIVE TO SUBSEQUENTLY GROUND ELECTROPLAX
(Activity of ground cells = 100)

	Ethyl chloroacetate	
	0.03 M	*0.06 M*
Total activity	71, 78	81, 69
In presence of 10 micrograms/ml eserine	114, 150	106, 106
By acetylcholinesterase (calculated)	55, 69	71, 55
Fraction cellular acetylcholinesterase assayed	63% ± 10	

34% to the total enzymic activity of ground cells at 0.01 *M* ethyl chloroacetate, 35 to 30% at 0.03 *M*, and 50% at 0.06 *M*. This enzyme contributes only 26% to the total activity of ground cells in the experiments summarized in Table V, at 0.03 and 0.06 *M* substrate concentration. It should be mentioned in this connection that the experiments with prostigmine listed in Table I, column 2 (except the last at 1500 micrograms per ml), were performed on the same ground cell preparation. Such preparations proved stable during several days' storage in the refrigerator but probably differed somewhat in their ratio of enzymic components.

From the amounts of inhibitor required to produce a given effect in intact relative to ground cells (Table I), it may be estimated that eserine penetrates to the assayable enzyme of the cell quite freely, whereas an approximately tenfold concentration gradient is indicated for prostigmine.

DISCUSSION

In order to evaluate the completeness of the assay by the method presented, a simplified assumption was necessary. An electroplaque had to be considered to possess a uniform region where the acetylcholinesterase activity of intact cells is located. This is implicit in determining an apparent K_m. For that region it was then found that the substrate concentration is essentially the same as in the external medium. It followed that the cell possessed another region containing additional acetylcholinesterase (about 40%) but not accessible to sufficient substrate to contribute to the assay. Consequently, these numerical values must be considered as approximations.

Since, as has been shown, inhibition of acetylcholinesterase in the assayable portion of the cell is achieved with a smaller concentration of its inhibitors than is conduction block, and since enzymes are known to occur in excess of functional requirements, it may be assumed that the easily penetrated portion of the cell does not contain crucial elements for function in conduction. Yet ethyl chloroacetate as well as dimethylaminoethyl acetate and acetylcholine, two poor substrates for assay of intact cells, necessarily do penetrate to the functional elements, if only to small extents, since all three of these substrates of acetylcholinesterase also cause membrane depolarization and block of propagation([5] and unpublished results of this laboratory). This does not imply that the cell portion where the functional elements are located must therefore be assayable, for a substrate concentration of less than 0.001 *M* in this region (tenfold gradient versus external medium) would no longer contribute measurably to the assay. On the other

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hand, depolarization can be achieved with 10^{-4} M or less of these substances in the bathing fluid.

A smaller contribution of acetylcholinesterase to the total enzymic activity of intact cells than to that of ground cells is reflected in the different degree of inhibition constituting the plateau in each case (Table I, column 1 as against column 2). Insofar as the difference is significant, it may be attributed to 1. the inaccessibility of part of the cellular acetylcholinesterase and 2. to the apparent occasional lability of the second enzyme on grinding. However, the decline on grinding in the activity assigned to the latter may suggest a different interpretation of the data, based on failure of eserine to penetrate all of the assayable portion of the cell. If such were the case, a larger difference in the level of the plateau for intact and ground cells in the presence of eserine should occur, and possibly also a difference according to whether eserine or prostigmine is used with whole cells. When a correction is made on this basis (*i.e.* taking the activity of the second enzyme in the subsequently ground cells as its true activity in intact cells and assigning the balance to acetylcholinesterase), the calculated contribution of acetylcholinesterase to the enzymic activity of intact cells is still such that a velocity maximum occurs at 0.03 M ethyl chloroacetate. This implies essentially complete penetration of the substrate to the assayable enzyme. Because of the rise in total enzyme activity on grinding, *i.e.* in the absence of eserine, it would then still not be safe to assume that the cell assay was complete. A combination of enzyme lability, complex partial penetration barriers to substrate and inhibitors, variations among cells and other factors may actually prevail. In any event, it must be concluded that the assay of intact electroplax using ethyl chloroacetate as substrate permits as yet only a partial correlation of the effect of inhibitors on conduction and on acetylcholinesterase activity in these cells. The penetration of substrates and inhibitors appears to increase with the non-polar character of the molecule.

The occurrence of substrate inhibition with increasing ethyl chloroacetate concentration is of interest. This bellshaped curve effect, traditionally interpreted as enzyme-substrate supercomplex formation (MURRAY-HALDANE curve), occurs at low concentrations of bi-functional substrates of acetylcholinesterase, *e.g.* of choline esters, and has been attributed to the presence of two reactive sites on the enzyme surface[8]. It has not, until recently, been observed at comparable concentrations of substrates, such as ethyl chloroacetate, capable of forming only one point of attachment to the enzyme regardless of concentration. BERGMANN AND SHIMONI[10] have shown it to be a general property of alkyl haloacetates, whether hydrolyzed by acetylcholinesterase or liver esterase. The data of Table IV agree with their observations.

The other enzyme of the electric organ, which also splits the ester linkage in ethyl chloroacetate, showed no activity toward ethyl acetate. This is analogous to the superiority of ethyl chloroacetate over ethyl acetate as a substrate of acetylcholinesterase[3]. It may be attributed to the effect of substitution on the electronegativity of the carboxyl group. This enzyme evidently lacks an anionic site, at least at the proper distance, since it does not hydrolyze acetylcholine or dimethylaminoethyl acetate. The large value of the K_m of the enzyme and ethyl chloroacetate should be noted, however; it is possible that at higher substrate concentrations than those tested some activity toward the other substrates mentioned would have occurred.

The abundance of this enzyme in the electric organ seems remarkable. Its physiological substrate has not been identified.

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SUMMARY

1. A method of testing for the completeness of an enzyme assay in intact cells is described.
2. It was applied to the measurement of enzymatic activity in electroplax from the electric eel.
3. Ethyl chloroacetate was found to assay about 60% of the total acetylcholinesterase activity.
4. This substrate was also hydrolyzed by another enzyme abundant in the tissue. Ethyl acetate, dimethylaminoethyl acetate and acetylcholine were not split at the same concentration.
5. The experiments permit an estimate of the penetration of eserine and prostigmine into the cell and of their affinity to the second enzyme.

RÉSUMÉ

1. Une méthode de contrôle du dosage d'un enzyme dans des cellules intactes est décrite.
2. Cette méthode a été appliquée à la mesure de l'activité enzymatique dans l'électroplaque d'une cellule électrique.
3. Le chloroacétate d'éthyle permet de doser environ 60% de l'activité acétylcholinestérasique totale.
4. Ce substrat est également hydrolysé par un autre enzyme présent en abondance dans le tissu. L'acétate d'éthyle, l'acétate de diméthylaminoéthyle et l'acétylcholine, à la même concentration, ne sont pas hydrolysés.
5. Les expériences ont permis d'estimer la pénétration de l'ésérine et de la prostigmine dans la cellule et leur affinité pour le second enzyme.

ZUSAMMENFASSUNG

1. Es wird eine Methode zur Prüfung der Vollständigkeit einer Enzymbestimmung in intakten Zellen beschrieben.
2. Sie wird angewandt auf die Messung der Enzymaktivität in den elektrischen Zellen des elektrischen Aals.
3. Es wird gefunden, dass Äthylchloracetat etwa 60% der gesamten Azetylcholinesteraseaktivität anzeigt.
4. Dieses Substrat wurde auch durch ein anderes im Gewebe verbreitetes Enzym hydrolysiert. Äthylacetat, Dimethylaminoäthylacetat und Azetylcholin werden in den gleichen Konzentrationen von diesem anderen Enzym nicht gespalten.
5. Die Experimente erlauben das Eindringen von Eserin und Prostigmin in die Zelle und ihre Affinität zu dem zweiten Enzym abzuschätzen.

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