THE CHARACTERIZATION AND LOCALIZATION OF FROG SKIN CHOLINESTERASE*

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(Received for publication, February 18, 1958)

ABSTRACT

Frog skin cholinesterase is largely of the serum (pseudocholinesterase) type. For whole skin, the activity at 10^{-1} M AcChCl is 4.9 μl./mg. N/hr.

Tela subcutanea isolated by dissection exhibits an activity of 65 μl./mg. N/hr. at 10^{-1} M AcChCl. Since about one-tenth of the nitrogen of the skin is located in the tela subcutanea, it is estimated that more than 90 per cent of the enzyme is associated with this tissue layer.

Kirschner (8) has reported that inhibitors of cholinesterase inhibit active sodium transport in frog skin. These inhibitors are more effective when applied to the inside of the skin than when applied to the outside. The action of each inhibitor on sodium flux has the same reversibility properties as its action on the enzyme. Thus, eserine, a reversible inhibitor of cholinesterase, reversibly inhibits active sodium transport, while tetraethylpyrophosphate, an irreversible inhibitor of the enzyme, exhibits irreversible inhibition of transport.

Van der Kloot (10) has presented evidence that the sodium pump in frog muscle requires cholinesterase activity. He measured the equilibrium intracellular sodium concentration and the residual cholinesterase activity of frog sartorii which had been bathed in graded concentrations of physostigmine eserine, a cholinesterase inhibitor capable of penetrating into the muscle fibre. It was found that the equilibrium intracellular sodium concentration paralleled the degree of cholinesterase inhibition, the 50 per cent values both falling at about 10^{-4} M physostigmine.

Despite the occurrence of high cholinesterase activities in tissues in which active sodium transport is observed to occur, and the striking effects of cho-

* Supported by grants from the National Science Foundation and the United States Public Health Service, administered by B. T. Scheer. These results form a part of a thesis submitted to the Graduate School, University of Oregon, in partial fulfillment of the requirements for the Ph.D. degree.

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linesterase inhibitors referred to above, a literature search revealed only two references to cholinesterase in frog skin. Duveau and Gerebtzoff (3) report that they were unable to demonstrate cholinesterase in this tissue using a histochemical technique, while Ussing (9) states that it is "well known" that frog skin contains large amounts of specific acetylcholinesterase. No reference is made to experimental work.

The *tela subcutanea* is a cellular layer which constitutes the internal surface of the dermis (5). Fleming (4) has found that skins from which this layer has been removed by dissection no longer actively transport sodium ions. The permeability of such skins to both Na^+ and K^+ is markedly increased.

METHODS

Two methods were used to assay cholinesterase. The Warburg method of Ammon was used on pooled tissues in those experiments in which substrate specificity and substrate concentration dependence of activity were studied. Two modifications were made in this method. As is well known, choline esters are very deliquescent. Even in the crystalline state, brief exposure to air results in the uptake of considerable amounts of water and the subsequent hydrolysis of the ester. In solution spontaneous hydrolysis continuously produces free acid and free choline. The substrate preparations used in assay, therefore, almost always are more acid than the bicarbonate-carbonic acid buffer, and since the method depends on shifts in this buffer, tipping in of the substrate usually gives some apparent hydrolysis. In the Ammon method this error is minimized by adding a small volume of enzyme solution from the side arm to a larger volume of substrate in the main chamber. Working with whole tissue, however, this is not possible. Accordingly, after gassing and equilibration, usually 30 minutes in these experiments, stop-cocks were opened, substrate tipped in, manometers adjusted to the zero setting (150 mm.), and stop-cocks closed. The maximum time elapsed between addition of substrate and closing of stop-cocks was 2 minutes. Half molar substrate solutions were prepared fresh every 2 or 3 weeks, and concentrations used in assay were prepared by serial dilution of this solution for each run. Warburg assays were performed at pH 8.0 and 36.2°C.

For the assay of individual skins, the Warburg method is not suitable because of the large amounts of skin required. For this reason a modification of Hestrin's hydroxamate method (Hestrin (6), Bonting (2)) was used. This method is capable of assaying activities in as little as 15 mg. of tela subcutanea, or 125 mg. of whole skin.

Reagents:

Buffer.—1.03 gm. per cent sodium barbital, 1.06 gm. per cent Na_2CO_3, 0.95 volume per cent concentrated HCl (sp.gr. = 1.19), 1.09 gm. per cent $MgSO_4 \cdot 7H_2O$, and 0.2 gm. per cent KCl. pH is adjusted to 8.0 with concentrated HCl.

Hydroxylamine Solution.—Equal volumes of 28 per cent NaOH and 14 per cent $NH_2OH \cdot HCl$ are mixed immediately before use.

Color Reagent.—2.25 gm. per cent $FeCl_3 \cdot 6H_20$, 3.6 vol. per cent conc. HCl.

Substrate Solutions.—These were made up at 12×10^{-2} M and 8×10^{-3} M in barbital buffer. Fresh solutions were prepared every other week.

About 50 mg. tela subcutanea or 200 mg. residue (tissue remaining after removal of tela subcutanea) was incubated in 0.5 ml. buffer in a Dubnoff shaking metabolic incubator at 37°C. for about 15 minutes to insure temperature equilibration. To each tube was added 0.5 ml. of substrate solution or buffer (controls). Substrate controls were run at both substrate concentrations as well. The tubes were stoppered with waxed cork stoppers. After 1 hour, 0.5 ml. aliquots were taken from those tubes containing 6×10^{-2} M substrate concentration, while 0.05 ml. was removed from those containing 4×10^{-3} M. The latter transfer was performed with a 50 μl. micropipette, and these aliquots were added to tubes containing 0.45 ml. buffer. Immediately, 0.5 ml. of the alkaline hydroxylamine solution was added to each tube and the rack vigorously shaken. Color was then developed by the addition of 4 ml. of the ferric ion solution. The contents were mixed by inversion and transferred to colorimeter tubes. The color density was read in a Klett colorimeter with a No. 50 filter within 1 hour,

TABLE I

Concentration Dependence of Cholinesterase Activity

Substrate concentration (acetylcholine chloride)	Activity (μl. CO_2/mg. N/hr.)
10^{-1} M	4.9
2×10^{-2} M	1.4
4×10^{-3} M	2.3
8×10^{-4} M	0

Each value the average of 3 determinations. Ventral skin from 6 frogs used in each run (5 substrate concentrations and a control).

during which time the color was stable. Standards were run in triplicate with each series of determinations. The tissue controls never gave a readable color. Hydrolysis rates were calculated from the difference between substrate control and experimental values.

Tela subcutanea was isolated by dissection by the method of Fleming (4).

RESULTS

For whole skin assays it was necessary to use six skins for each run, usually consisting of four substrate concentrations and a control. Frogs were pithed, and the unpigmented ventral skin removed and transferred to the buffer used for assay. Each skin was then spread out on a piece of filter paper soaked in buffer and overlaid with millimeter graph paper. It was then cut up into centimeter strips of varying length. These were divided up among the Warburg flasks so that approximately the same area of skin from each frog was present in each flask. A similar procedure was used when tela subcutanea was assayed, though here graph paper could not be used and randomization was not as well insured.

The results of assay on whole skin are shown in Table I. The highest activity occurs at 10^{-1} M AcChCl; however, a small peak is observed at 4×10^{-3} M,

TABLE II

Cholinesterase Activity in Isolated Tela Subcutanea

Substrate	Substrate concentration	Activity (μl. CO_2/mg. N/hr.)	
Acetylcholine chloride	10^{-1} M	65	
	2×10^{-2} M	20	Average of two determinations
	4×10^{-3} M	28	Four and six skins, respectively
	8×10^{-4} M	8	
Butyrylcholine chloride	10^{-1} M	115	
	2×10^{-2} M	50	One determination; four skins
	4×10^{-3} M	0	
Benzoylcholine chloride	10^{-1} M	52	
	2×10^{-2} M	22	One determination; four skins
	4×10^{-3} M	5	

TABLE III

Cholinesterase Activity of Individual Skins

Skin No.	Activity (μmoles AcChCl hydrolyzed/gm. wet weight/hr.)			
	Tela subcutanea		Residue	
	6×10^{-2} M	4×10^{-3} M	6×10^{-2} M	4×10^{-3} M
124	27.2	6.7	—	—
122	—	18.6	—	—
211	46.2	—	—	—
212	44.4	11.5	—	—
251	0	29.1	—	—
28	37.1	6.5	14.2	1.1
291	25.6	9.2	−5.1	0
292	12.2	6.7	20.8	−1.2
2131	67.6	9.0	0	−2.6
2132	85.8	7.7	6.0	−3.0
Average ± S. D.	43.3 ± 22.3	13.7 ± 5.7	7.2 ± 8.7	−1.1 ± 1.5

the optimum substrate concentration of the specific acetylcholinesterase. Acetyl β-methylcholine, an ester which is hydrolyzed only by the specific enzyme, is not hydrolyzed by these preparations.

The results of assay on isolated tela subcutanea are shown in Table II. Assays performed using AcChCl as substrate showed that this tissue con-

tains more than ten times as much activity as does whole skin. Since only about one-tenth of the nitrogen of the skin is found in the tela subcutanea, more than 90 per cent of the enzyme is associated with this tissue. The assays performed with BuChCl and BzChCl confirm that the enzyme is of the non-specific type.

Table III contains the results of assay on individual skins. In this group of experiments assays were performed at two substrate concentrations; 6×10^{-2} M and 4×10^{-3} M. Bergmann (1) has used the ratio of activities at these two concentrations as a measure of the relative amounts of non-specific and specific enzyme present in various tissues. With one exception (No. 251) assays on tela subcutanea reveal the preponderance of the non-specific enzyme. The activity of residue (tissue remaining after the removal of tela subcutanea) is low and variable at both concentrations; in some cases a negative value is found. This is interpreted as representing esterification of free choline, which is generally present in these substrate preparations. In only one case is greater activity observed in the residue than in the tela subcutanea at the same concentration (No. 292).

DISCUSSION AND CONCLUSIONS

It is apparent from these results that frog skin cholinesterase is largely of the non-specific type and that it is to a great extent associated with the tela subcutanea. This second consideration is of some importance. Ussing and coworkers (9) believe that the active transport mechanism in frog skin is located in the stratum germinativum, while Fleming's results (4) indicate that the tela subcutanea is the site of some essential process in the mechanism. The action of inhibitors on the transport mechanism in this (Kirschner (8)) and other (Van der Kloot (10), Holland and Klein (7)) tissue suggests that cholinesterase plays some central role. The experimental observations presented in this paper further implicate the tela subcutanea as the site of some essential process in the transport mechanism.

The author takes great pleasure in acknowledging the debt owed to Professor B. T. Scheer, under whose direction this work was done.

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