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Acetylcholine and Cholinacetylase Content of Synaptic Vesicles

Abstract. *Acetylcholine, cholinacetylase, and acetylcholinesterase were determined in three subfractions that resulted from the osmotic shock of the "mitochondrial" fraction of the rat brain. Acetylcholine and cholinacetylase were found concentrated in the subfraction that contained mainly synaptic vesicles and some membranes, whereas the larger proportion of the acetylcholinesterase was observed in the subfraction that contained torn nerve endings. These results support the idea that synaptic vesicles are the morphological units of acetylcholine within the synapse.*

Recently a technique was developed in our laboratory (*1*) that permits the separation of synaptic vesicles from isolated nerve endings. This consists in treating with distilled water a freshly prepared "mitochondrial" fraction of the rat brain, in which the presence of numerous intact nerve endings, together with free mitochondria, synaptic membranes, and myelin fragments, had previously been demonstrated (*2*). This procedure results in the swelling and breaking of the nerve endings, with liberation of most of the synaptic vesicles. By centrifugation at 11,500*g* for 20 minutes and further centrifugation of the supernatant at 100,000*g* for 30 minutes, the osmotically disrupted mitochondrial fraction M was separated into three subfractions: (i) M_1 (composed of myelin fragments, swollen mitochondria, and remnants of disrupted nerve endings), containing 31.5 percent of the protein of the total homogenate and practically all the succinodehydrogenase; (ii) M_2 (composed mainly of synaptic vesicles and some membranes), containing 6.3 percent of the protein; and (iii) M_3, a soluble supernatant containing 11.2 percent of the protein (*1*).

In such preparations we have studied the content of acetylcholine, cholinacetylase, and acetylcholinesterase. As shown in Table 1, only about one-third of the acetylcholine and cholinacetylase remains in subfraction M_1 after the osmotic shock; the rest is distributed between M_2 and M_3. Although both acetylcholine and cholinacetylase are mainly localized in M_2, as demonstrated by the relative specific activity (rsa), the acetylcholine occurs in lower concentration. This could be explained by the greater solubilization of acetylcholine into M_3.

The fact that the highest concentration of acetylcholine and cholinacetylase is found in subfraction M_2 is interpreted as a demonstration that the synaptic vesicles are indeed the carriers of this transmitter substance, as had been postulated by De Robertis and Bennett (*3*), and that they contain the enzyme directly involved in its synthesis. The concentration of acetylcholine and cholinacetylase in the synaptic vesicles is better shown by the expression (rsa M_2)/(rsa M_1), the ratio between the concentrations of succinodehydrogenase, acetylcholinesterase, acetylcholine, and cholinacetylase in the two structural compartments of the nerve endings.

After the osmotic shock, most of the synaptic vesicles appeared to have small discontinuities or pores in the membrane which allowed phosphotungstate to penetrate, and 30 percent of the acetylcholine had been liberated into subfraction M_3. In spite of these discontinuities, which probably resulted from the hyposmotic treatment, a large part of the acetylcholine remained bound to the vesicle—a finding which suggested that it might form a complex with the synthesizing enzyme or other components in the vesicular membrane. As shown by Whittaker (*4*), the total amount of acetylcholine can be released only through more drastic procedures involving denaturation of the proteins.

The higher concentration of cholinacetylase in M_2 and its lesser solubilization into M_3 indicate that most of this enzyme is firmly bound to the structure of the vesicular membrane.

Acetylcholinesterase, although preferentially localized in cholinergic nerve endings (*2*), differs in being more finely distributed within the nerve-ending complex. Most of it remains in subfraction M_1, which contains the remnants of disrupted nerve endings. Its high relative specific activity in M_2 could be explained by the low protein content and the presence of synaptic membranes in this subfraction. However, the ratio (rsa M_2)/(rsa M_1) is rather low.

The foregoing facts and some preliminary results of the subfractionation of M_2 suggest that acetylcholinesterase may be localized in the membranes of the ending at the synaptic junction. Another interesting difference in the

Table 1. Protein content and activities of enzymes and other active substances in mitochondrial subfractions M_1, M_2, and M_3 after osmotic shock. The values for the subfractions are means expressed in percentages of the total amount of homogenate recovered and relative specific activity.* Protein was determined with the biuret method of Palladin (*7*); succinodehydrogenase (SDH), with the technique of Slater and Bonner (*8*), involving a change in optical density of a solution of ferricyanide; acetylcholine (ACh), with a modification of the rat-fundus method of Vane (*9*); and cholinacetylase (ChAc), with the method of Hebb and Smallman (*10*), in which acetylating enzyme, choline, acetate, and adenosine triphosphate are used.

Ultrastructure	Determinations (N)	M Absolute values (per gram of fresh tissue)	M_1 (Myelin; mitochondria; ruptured nerve endings) Percentage	M_1 Relative specific activity	M_2 (Synaptic vesicles; membranes) Percentage	M_2 Relative specific activity	M_3 (Soluble fraction) Percentage	M_3 Relative specific activity	(rsa M_2)/(rsa M_1)
Protein	8	33.0 mg	31.5		6.3		11.2		
SDH	5	615.5 U	85.0	2.7	2.3	0.3	0	0	0.1
AchE	3	253.1 U	37.6	1.2	14.6	2.3	0	0	1.9
ACh	4	0.75 μg	21.5	0.7	22.7	3.6	17.0	1.5	5.8
ChAc	4	140.0 U	22.7	.7	35.5	5.6	9.8	0.9	7.8

*For example, the relative specific activity for succinodehydrogenase = (percentage of recovered SDH)/(percentage of recovered protein).

distribution of acetylcholinesterase and cholinacetylase is the fact that the former is not present in the supernatant or soluble subfraction M_3.

These results (*5*) are indicative of a fine compartmentalization of enzymes and active substances within the nerve-ending complex (*6*).

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Dieldrin Susceptibility: Partial Restoration in Anopheles Selected with a Carbamate

Abstract. *Selection of dieldrin-resistant* Anopheles albimanus *Wied. and normal* Culex pipiens quinquefasciatus *Say with* m-*isopropylphenyl methylcarbamate for 21 and 30 generations, respectively, resulted only in vigor tolerance to this and other aryl methylcarbamates. However, it caused substantial restoration of dieldrin- and DDT-susceptibility in* Anopheles *by increasing the frequency of homozygous susceptible phenotypes from 10 to 83 percent.*

One of the most serious problems confronting the malaria eradication program of the World Health Organization is the selection of suitable insecticides to replace DDT and dieldrin where these have been rendered ineffective by the development of resistance in the vector mosquitoes. Malaria is transmitted exclusively by anopheline mosquitoes, and of the 85 described species of *Anopheles* at least 28 (*1*) are known to have developed resistance to one or both of these insecticides. The organophosphorus insecticide malathion has been tentatively suggested as a substitute (*2*), but resistance to this compound has already appeared in field populations of *Culex tarsalis* Coq. in California (*3*) and in Puerto Rican populations of *Aedes aegypti* L.; the latter is also resistant to DDT and dieldrin (*4*). *Aedes nigromaculis* Ludl. has shown increased tolerance to the organophosphorus insecticide parathion in California (*5*). This situation emphasizes the need for thoroughly testing potential new insecticides, not only for toxicity to resistant strains but also for the degree of resistance that they can induce.

Research on new insecticides in this laboratory has revealed that several carbamic acid esters, such as *m*-isopropylphenyl methylcarbamate, and *m-sec*-butylphenyl methylcarbamate are highly toxic to *Culex pipiens quinquefasciatus* Say and *Anopheles albimanus* Wied., even more so than DDT, dieldrin, malathion, parathion and fenthion (*6*). Selection of house flies (*Musca domestica* L.) with *m*-isopropylphenyl methylcarbamate resulted in high levels of resistance to this material and to related aryl methylcarbamates (*7*); the resistance is due primarily to ability of flies to metabolize the carbamate at a rate commensurate with its penetration into the insect (*8*).

The activity of carbamates in mosquitoes is peculiarly different from the activity in house flies, in that several compounds highly toxic to the house fly are ineffective against mosquitoes, and vice versa (*9*). Many carbamates are synergized to a remarkable degree by piperonyl butoxide in house flies (*10*) but only slightly in mosquitoes (*6*). These observations suggest the presence of distinctly different defense mechanisms against carbamates in mosquitoes, and prompted the authors to explore the potentialities of resistance development in mosquitoes through selection with *m*-isopropylphenyl methylcarbamate.

The mosquitoes used were a laboratory strain of *Culex pipiens quinquefasciatus* of normal susceptibility to insecticides, and a strain of *Anopheles albimanus* from Panama (*11*) consisting of dieldrin-susceptible, -hybrid and -resistant phenotypes in a ratio of approximately 10 : 44 : 46. This latter strain also showed approximately tenfold tolerance to DDT at the LC_{50} level. The selection technique consisted of first exposing for 24 hours all 4th-instar larvae of each generation to a solution of *m*-isopropylphenyl methylcarbamate (*12*) in tap water at concentrations which produced 80 to 95 percent mortality, and then propagating the survivors. About 3000 larvae were used in each generation; selection extended over 30 generations of *Culex* and 21 generations of *Anopheles*.

At the end of selection, larval susceptibility to *m*-isopropylphenyl methylcarbamate was 2 times as low in *Culex* and 2.7 times as low in *Anopheles* as at the beginning (Fig. 1). Susceptibility to the related methylcarbamates *o*-isopropylphenyl, *o*-isopropoxyphenyl, *m-sec*-butylphenyl, *m-tert*-butylphenyl and 4-methylthio-3,5-xylyl, remained virtually unchanged (less than two times at the LC_{50}), except for Sevin (1-naphthyl methylcarbamate) which was 5.2-fold less effective against *Culex* than initially. There was no change in the slopes of the dosage-mortality regression lines of the selected strains compared with those of the parental strains, suggesting that the small reduction in susceptibility to *m*-isopropyl-

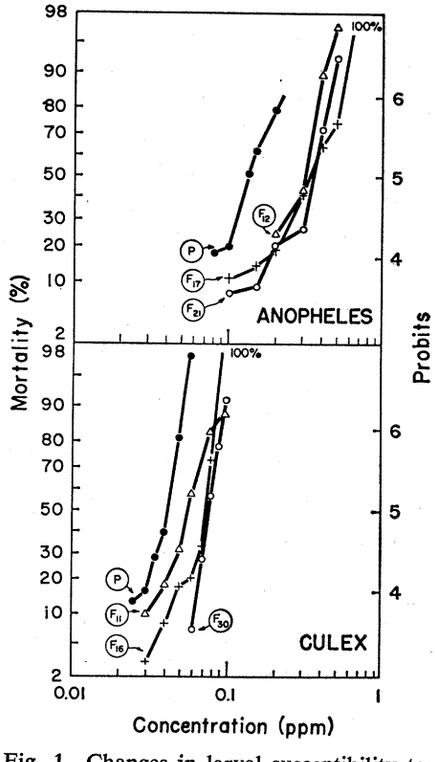

Fig. 1. Changes in larval susceptibility to *m*-isopropylphenyl methylcarbamate in *Anopheles* and *Culex* in the course of selection with this compound (*21*).