THE CHARACTERIZATION OF TISSUE CHOLINESTERASES

by

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Studies on true and pseudo cholinesterase[1,2] have shown that both enzymes contain an esteratic site of similar properties, but differ in the number of anionic groups; the true enzyme containing two negatively charged groups, the pseudo enzyme only one. It can thus be concluded that both cholinesterases represent modifications of the same enzyme principle and that they probably are genetically related to each other. It has been claimed in the past[3] that pseudo cholinesterase is "unspecific", *i.e.* that it does not function in the conductive process. However, since both enzymes catalyze essentially the same reactions, but at different speeds, a common physiological role becomes probable. In order to evaluate the functioning of cholinesterases in different organs, it is necessary to study the natural mixture of enzymes against their physiological substrate(s). The observation of BANISTER, WHITTAKER AND WIJESUNDRA[4], according to which propionylcholine is—at least in spleen—present in addition to acetylcholine, leads to the assumption that in different tissues different substrate mixtures may be present. However, information on this point is lacking, and at present the best approximation to natural conditions appears to be the determination of organ activity against a given substrate under varying conditions of concentration and pH.

The distribution of cholinesterases in tissues has been studied by many investigators applying either histochemical[5,6] or manometric methods. With the latter, true and pseudo-cholinesterase is distinguished by the use of specific substrates, for instance acetyl *beta*-methyl choline and benzoyl-choline or butyryl-choline[7,8]. In the present investigation an attempt is made to characterize the various types or mixtures of cholinesterases in tissues, (a) by their complete pS-activity curves and (b) by the inhibitory effect of methonium compounds.

We have shown in a recent paper[2] that for a given substrate concentration hexamethonium (= *n*-hexane-1,6-bis[trimethylammonium] salt = C6) is about 20 times more effective against pseudo- than against true cholinesterase, whereas decamethonium (= C10) is about twice as effective against the latter enzyme. On the other hand, if a single type of enzyme is present, the ratio C6:C10 remains approximately constant with changing substrate concentration. This ratio, therefore, serves as a suitable measure for a specific type of cholinesterase and is important for the understanding of the localization of the effects of certain drugs, such as alkaloids or methonium compounds.

MATERIALS AND METHODS

A complete study was carried out on the organs of several dogs, which were bled as far as possible under barbiturate anesthesia and then transfused with warm Locke's solution introduced into

References p. 519.

a femoral vein, until the perfusate collected from a carotis was practically colourless. Towards the end of the perfusion, when the heart stopped beating, circulation of the salt solution was maintained by artificial respiration with the aid of a pump. This procedure is very effective for washing out the blood from the internal organs, but less satisfactory for the mucles of the limbs, since the failing circulation impairs first the blood supply to these parts of the body. The analysis of skeletal muscles, especially of leg muscles is, therefore, much less reliable than that of inner organs.

All tissues were homogenized with Warburg buffer in a Waring blendor,—if necessary with the addition of quartz sand. Enzymic activity was calculated per gram of original tissue.

Since tissue cholinesterases are part of the fixed cell proteins, it is necessary to dissolve the latter completely in order to obtain a solution representative of the enzyme composition present in a given organ. ORD AND THOMPSON[9] tried for this purpose digestion with trypsin and reported that the cholinesterases of rats' heart or diaphragm became solubilized and that their activity remained unimpaired. We have applied this method to several organs of the dog with the result given in Table I. It is seen that highly purified preparations, such as the enzyme from the electric organ of *Torpedo*, are rapidly destroyed by trypsin. In no case did we find increased activity in the supernatant after trypsin digestion for one hour. After two hours the cholinesteratic activity was always declining. We therefore concluded that the supernatant obtained after trypsin digestion is not more representative of the tissue enzymes than the supernatant separated directly from the homogenate.

TABLE I

EFFECT OF TRYPSIN DIGESTION ON THE ACTIVITY OF SOLUBLE CHOLINESTERASES AND ON THE EXTRACTABILITY OF CHOLINESTERASES FROM TISSUE

50 mg of trypsin (Nutritional Biochemicals Corporation) were used per 100 ml of homogenate. The tissues were homogenized with twice the volume of Warburg buffer. This mixture was first incubated at 37° with or without trypsin 1 hour before centrifugation. All results are expressed as relative activities, taking the value in the absence of trypsin as 1.

	Relative activity after incubation
Cholinesterase from the electric organ of *Torpedo marmorata*	0.12
Dog's serum	0.60
Dog's intestine	0.69
Dog's heart	0.98

In the case of brain the total homogenate and the two fractions, obtained by centrifugation of it, were compared, and the data are included in Table II and III. With liver and spleen it was not possible to carry out the separation into precipitate and supernatant. Therefore all experimental figures refer to the original homogenate. The homogenate of pancreas gave only a negligible amount of precipitate. Lung, heart, kidney and intestine contained such a considerable proportion of particulate matter, that the readings obtained with whole homogenate were irregular. All measurements on these organs were, therefore, carried out with the supernatant only.

Spleen was homogenized by splitting its capsule, scraping out the pulp with a spoon and treating with 2 volumes of the Warburg buffer, described below. After percolation through cotton the homogenate was used directly. Erythrocytes were washed three times with saline and counted before they were diluted with Warburg buffer. The figures obtained for the final suspension were $7.1 \cdot 10^9$ cells/ml for dog's erythrocytes and $9.0 \cdot 10^9$ cells/ml for human erythrocytes.

Enzymic activity was determined manometrically at the acetylcholine concentrations indicated in the text. The buffer used as medium for these experiments contained 0.1 *M* NaCl, 0.026 *M* $NaHCO_3$ and 0.04 *M* $MgCl_2$. The pH was adjusted to 7.3. The gas phase consisted of 95% air and 5% CO_2. In the experiments with inhibitors, the enzyme and the inhibitor were incubated 1 hour before addition of the substrate. Acetylcholine was used at $4 \cdot 10^{-3}$ and $6 \cdot 10^{-2}$ *M*. The lower concentration is near to the pS-optimum of true cholinesterases. The higher one was selected—although it does not give the maximum rate of hydrolysis with pseudo-cholinesterase—because it is the limiting concentration at which spontaneous hydrolysis of the substrate can still be neglected.

K_m-values were derived by the method of DIXON[10] and I_{50}-values were obtained graphically by plotting percent inhibition versus log inhibitor concentration.

n-Hexane-1,6-bis [trimethylammonium] bromide (= hexamethonium or C6) and decamethonium (= C10) were gifts from Messrs. Allen and Hanburys Ltd., Manchester, England.

References p. 519.

TABLE II

CHOLINESTERATIC ACTIVITY OF VARIOUS ORGANS

Activity is given in μmoles CO_2/h per gram of original tissue, but for the erythrocytes and serum the value reported refers to 1 ml. All measurements were carried out at 37°.

Enzyme source	*Activity at:* (1) ACh $4 \cdot 10^{-3}$ M	(2) $6 \cdot 10^{-2}$ M	*Ratio of activities 2/1*	K_m
Pooled human serum	20.5	36.5	1.8	$5.9 \cdot 10^{-3}$
Pooled dog's serum	13.9	26.3	1.9	$5.8 \cdot 10^{-3}$
Pooled human erythrocytes	115	41.4	0.36	$8.7 \cdot 10^{-4}$
Pooled dog's erythrocytes	10.9	4.8	0.39	$8.5 \cdot 10^{-4}$
Dog's brain, precipitate	40	14.8	0.37	$2.0 \cdot 10^{-3}$
Dog's brain, supernatant	5.8	4.15	0.72	$5.3 \cdot 10^{-4}$
Dog's brain, whole	48	23.1	0.48	$1.7 \cdot 10^{-3}$
Dog's liver	71	146	2.1	$5.8 \cdot 10^{-3}$
Dog's pancreas	21	44	2.1	$5.8 \cdot 10^{-3}$
Dog's intestine	6.3	10	1.6	$3.9 \cdot 10^{-3}$
Dog's heart	1.13	1.71	—*	—
Dog's kidney	0.8	1.9	2.3	
Dog's lung	1.2	2.2	1.8	—
Dog's spleen	8.4	4.1	0.49	$2.6 \cdot 10^{-3}$

* This ratio cannot be taken for comparison because of the presence of two maxima (see Fig. 3).

RESULTS

1. The complete pS-activity curves, which are represented in Figs. 1–3, can clearly be divided into 3 groups:

(a) Liver, pancreas and intestine behave like serum, and all of them reach their maximum activity at about 0.1 *M* acetylcholine (Fig. 1). In accordance, the K_m-values for this group of organs are practically identical (see Table II).

(b) Brain, spleen and erythrocytes exhibit very similar pS-activity curves (Fig. 2) with a maximum at 2.0–2.2. These values are somewhat lower than the optimum of acetylcholinesterase from *Torpedo* and *Electrophorus* ($pS_{opt} = 2.3$). For the calculation of K_m-values in this group only the measurements at low substrate concentrations were used, *i.e.* in a region where auto-inhibition due to the formation of ES_2-complexes can be neglected. It is remarkable that the K_m-values of both human and dog's erythrocytes are identical and lie between the figures for the soluble and insoluble portion of brain.

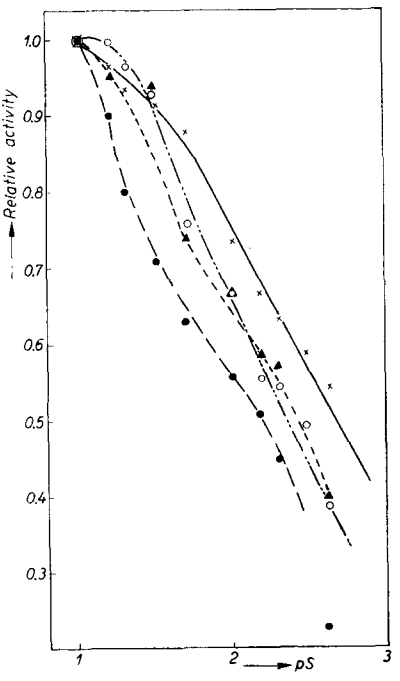

Fig. 1. Cholinesteratic activity of dog's tissues as function of pS (= the negative logarithm of the concentration of acetylcholine). (Organs showing the pseudo-type of enzyme.)

×——×——× Intestine ▲– – –▲– – –▲ Serum
○—— – – ——○ Liver ●— —●— —● Pancreas

References p. 519.

TABLE III

INHIBITORY EFFECT OF HEXA- AND DECAMETHONIUM ON CHOLINESTERASES FROM VARIOUS SOURCES

I_{50}-values were determined at 37° at two standard concentrations of acetylcholine. The ratio I_{50} (higher substrate conc.) : I_{50} (lower substrate conc.) is given after each pair of values. The last column tabulates the ratio of I_{50}-values, C6:C10, at both concentrations of substrate.

Source of enzyme	I_{50}(M) of hexamethonium at ACh		Ratio	I_{50}(M) of decamethonium at ACh		Ratio	C6:C10 for ACh	
	$4 \cdot 10^{-3}$ M	$6 \cdot 10^{-2}$ M		$4 \cdot 10^{-3}$ M	$6 \cdot 10^{-2}$ M		$4 \cdot 10^{-3}$ M	$6 \cdot 10^{-2}$ M
A. Serum cholinesterase*	$9.7 \cdot 10^{-4}$	$1.1 \cdot 10^{-2}$	11.3	$4.6 \cdot 10^{-5}$	$3.5 \cdot 10^{-4}$	7.6	21.1	31.4
Pooled human serum	$8.2 \cdot 10^{-4}$	$1.0 \cdot 10^{-2}$	12.2	$3.0 \cdot 10^{-5}$	$2.9 \cdot 10^{-4}$	9.7	27.3	34.5
Pooled dog's serum	$3.4 \cdot 10^{-3}$	$2.2 \cdot 10^{-2}$	6.5	$2.0 \cdot 10^{-4}$	$1.5 \cdot 10^{-3}$	7.5	17.0	14.7
Dog's intestine	$8.0 \cdot 10^{-3}$	$5.2 \cdot 10^{-2}$	6.5	$4.0 \cdot 10^{-4}$	$3.2 \cdot 10^{-3}$	8.0	20.0	16.3
Dog's liver	$3.1 \cdot 10^{-3}$	$2.8 \cdot 10^{-2}$	9.0	$2.7 \cdot 10^{-4}$	$1.8 \cdot 10^{-3}$	6.7	11.5	15.6
Dog's pancreas	$3.0 \cdot 10^{-3}$	$2.9 \cdot 10^{-2}$	9.7	$3.0 \cdot 10^{-4}$	$1.6 \cdot 10^{-3}$	5.3	10.0	18.1
Dog's stomach	$6.2 \cdot 10^{-3}$	$3.3 \cdot 10^{-2}$	5.3	$1.2 \cdot 10^{-4}$	$1.9 \cdot 10^{-3}$	15.8	51.7	17.4
Dog's lung	$9.0 \cdot 10^{-3}$	$3.5 \cdot 10^{-2}$	3.9	$5.0 \cdot 10^{-4}$	$4.7 \cdot 10^{-3}$	9.4	18.0	7.5
B. *Torpedo***	$3.1 \cdot 10^{-3}$	$(> 1 \cdot 10^{-1})$	—	$6.7 \cdot 10^{-6}$	$2.1 \cdot 10^{-4}$	31.3	463	—
*Electrophorus***	$1.9 \cdot 10^{-2}$	$(> 1 \cdot 10^{-1})$	—	$2.0 \cdot 10^{-5}$	$5.6 \cdot 10^{-4}$	28.0	950	—
Pooled human erythrocytes	$2.4 \cdot 10^{-2}$	—	—	$1.0 \cdot 10^{-4}$	$2.4 \cdot 10^{-3}$	24.0	240	—
Pooled dog's erythrocytes	$3.2 \cdot 10^{-2}$	—	—	$1.3 \cdot 10^{-4}$	$2.1 \cdot 10^{-3}$	16.2	246	—
Dog's brain, whole	$2.1 \cdot 10^{-2}$	—	—	$2.0 \cdot 10^{-4}$	$7.5 \cdot 10^{-3}$	37.5	105	—
Dog's brain, precipitate	$2.3 \cdot 10^{-2}$	$(> 10^{-1})$	—	$1.7 \cdot 10^{-4}$	$5.2 \cdot 10^{-3}$	30.6	135	—
Dog's brain, supernatant	$5.0 \cdot 10^{-2}$	$(> 10^{-1})$	—	$1.5 \cdot 10^{-4}$	$7.1 \cdot 10^{-3}$	47.3	357	—
Dog's spleen	$5.6 \cdot 10^{-2}$	—	—	$2.8 \cdot 10^{-4}$	—	—	200	—

* Fraction IV – 6 – 3 of human plasma, kindly supplied by Dr. RUTH M. FLYNN, Dept. of Biochemistry, Harvard Medical School.

** The electric organs of *Torpedo marmorata* and *Electrophorus electricus* were fractionated according to the procedure of ROTHENBERG AND NACHMANSOHN[13].

However, even the pS-activity curves of erythrocytes are not symmetrical and the slow decline for high substrate concentrations—unlike the characteristic shape of the curve of esterases from electric organs—suggests that these cells may contain a mixture of true and pseudo cholinesterase, with the former prevailing. This result is in contrast to the general belief that erythrocytes contain only one type of cholinesterase.

(c) The third group (Fig. 3) is represented by pS-activity curves with maxima at low pS-values, for example lung and kidney, for which the peak lies at 1.3. Heart muscle occupies a peculiar position: It shows a maximum at pS = 2, similar to brain, and a second rise at lower pS-values. This result clearly indicates the presence of two enzymes hydrolyzing acetylcholine.

The figures in Table II give a good picture of the relative hydrolytic activity of various tissues against acetylcholine. The ratio of the activities at the high and low substrate concentration is smaller than 1 for those organs, in which the "true" enzyme predominates. The very small total activity of heart muscle is probably an expression of the difficulty to homogenize this tissue and to extract its cholinesterases. Still greater

References p. 519.

difficulties were encountered with flat or spindle-like skeletal muscles, as represented by the diaphragm and gastrocnemius of the dog.

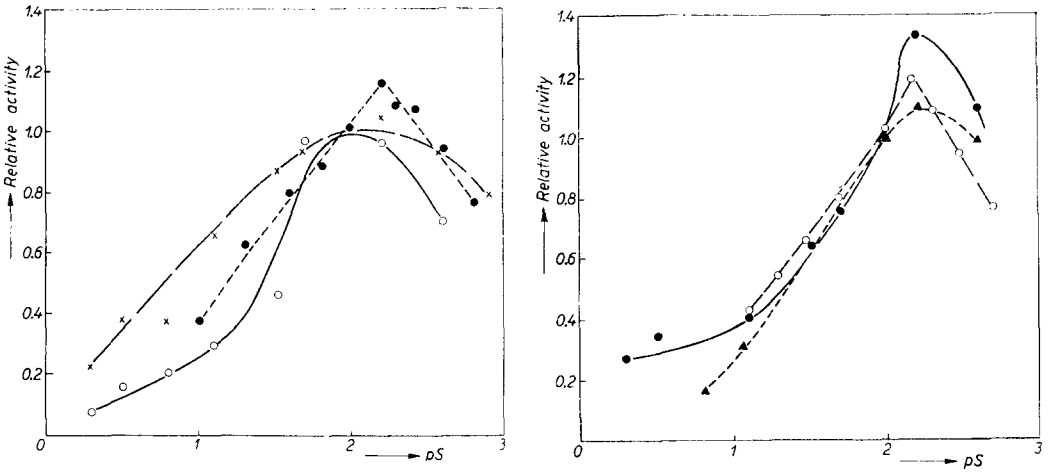

Fig. 2. Cholinesteratic activity of dog's tissues as function of pS. (Organs showing preferentially the "true" type of enzyme.)

a. ● – – – ● – – – ● Brain, whole
○ —— ○ —— ○ Brain, precipitate
× — — × — — × Brain, supernatant

b. ○ — — ○ — — ○ Spleen
▲ – – – ▲ – – – ▲ Erythrocytes
● —— ● —— ● Human erythrocytes

(Note that in this graph the activity at pS 2 has been arbitrarily chosen as 1.0, whereas in Figs. 1 and 3 the reference point is pS = 1.)

2. As a second criterion for the type of cholinesterase(s), present in various organs, the inhibitory effect of hexa- and deca-methonium was determined at two standard concentrations of acetylcholine, *viz.* $4 \cdot 10^{-3}$ and $6 \cdot 10^{-2}$ *M*. As shown in Table III, the inhibition of purified serum cholinesterase by either methonium derivative is nearly ten times stronger at the lower than at the higher substrate concentration. For the two specimens of true cholinesterase from *Torpedo* and *Electrophorus* the corresponding ratio for decamethonium is about 30. However, the affinity of hexamethonium for true cholinesterase is too small to permit the measurement of I_{50} at a substrate concentration of $6 \cdot 10^{-2}$ *M*. Still more marked differences are represented by the ratios of C6:C10, shown in the last column of Table II. We are thus in a position to classify the cholinesterases of

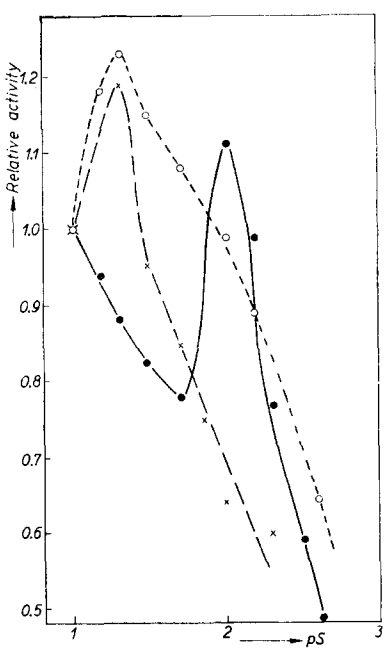

Fig. 3. Cholinesteratic activity of dog's tissues as function of pS. (Organs with intermediate or mixed types of enzymes.)

○ – – – ○ – – – ○ – – – ○ Lung
× —— × —— × —— × Kidney
● —— ● —— ● —— ● Heart

References p. 519.

various tissues on the basis of the inhibitory ratio of representative methonium compounds. Pseudocholinesterase gives for the ratio C6:C10 values between 10 and 50, while for organs with prevalent true enzyme the range is 100–1000. It thus appears that true cholinesterases of mammalian tissues are much less "specific" than the highly purified enzymes from electric organs, a fact which may be considered an indication that a greater variation exists among "true" than among pseudo types of tissue cholinesterases.

DISCUSSION

The results presented in this paper are generally in agreement with the distribution of various cholinesterases, as determined by previous authors[11,12]; however, certain discrepancies appear in a few cases. Thus, ORD AND THOMPSON[11] classified liver cholinesterase as a mixture of true and pseudo enzyme, whereas our results indicate the presence of the latter type only. Different techniques and species characteristics may be responsible for such divergencies, which, however, cannot prevent us from arriving at the important conclusion that there appears to exist in dog's tissues only one type of pseudo cholinesterase, but possibly more than one kind of true cholinesterasee. We ascribe this to the fact that in pseudocholinesterase the esteratic site is combined with only one negatively charged group, which leads to an unequivocal peptide sequence, whereas the combination with two anionic groups in the true enzyme allows for several different arrangements[13], each of which may possess a different substrate affinity. It is therefore conceivable that cholinesterases of an "intermediary" specificity exist, though our results do not clearly distinguish between such types on one hand and mixtures of pure enzymes on the other. Only in the case of heart muscle did we obtain evidence of the presence of a mixture.

Like K_m-values and pS-optima, the ratio of inhibitory activities of C6:C10 too is a useful indicator of the type of cholinesterase prevalent in various tissues, aiding at the same time in the understanding of the localization of certain drug effects, as has been pointed out elsewhere.[2]

It is significant that the pS-activity curve of erythrocytes indicates the presence of pseudo cholinesterase in these cells. This observation forms a counterpart to the recent demonstration[14,15] that many sera contain also the true enzyme. Both dog's and human erythrocytes show the same behaviour. However, when per cell activity is calculated, the latter are about 10 times more active than the former. It becomes of interest to know whether this is due to the presence of 10 times as many active sites in human erythrocytes.

Although brain and skeletal muscles contain mainly true cholinesterase, the general distribution of cholinesterases bears no relationship to conductive function. It does not appear conceivable that in liver or spleen a sudden release of cholinesters should take place, or that such esters should exert in these organs a "specific" function, similar to the one they are assumed to have in conductive tissues[16]. A mechanism of a more general nature must, therefore, be sought to explain the physiological role of cholinesterases in the organism. The hypothesis of an enzymic buffer action of the system cholinesterase–substrate, which has previously been formulated for the conductive process[17], may perhaps be applicable to all living tissues. In fact, all esterases (and many other hydrolytic enzymes) contribute to such an effect, but the cholinesterases excel by their high reaction rates.

References p. 519.

SUMMARY

1. Trypsin digestion does not improve the yield of cholinesterases extractable from animal tissues.

2. Tissue cholinesterases can be characterized (a) by their pS-maxima and their Michaelis-Menten constants, and (b) by the ratio of the inhibitory effect of hexa- and deca-methonium at a given substrate concentration.

3. It is observed that the pseudocholinesterases present in dog's serum and tissues are identical in all respects, whereas the true cholinesterases appear to differ in specificity from one organ to the other.

4. It appears possible that like the brain, erythrocytes of dog and man also contain a mixture of true and pseudo cholinesterase.

5. It is suggested that all cholinesterases act as enzymic buffer systems, both in conductive and non-conductive tissues.

RÉSUMÉ

1. L'hydrolyse trypsique n'améliore pas le rendement en cholinestérases extractibles à partir des tissus animaux.

2. Les cholinestérases tissulaires peuvent être caractérisées (a) par leur pS-maximum et leurs constantes de Michaelis-Menten et (b) par le rapport entre les effets inhibiteurs de l'hexa- et du décaméthonium pour une concentration donnée en substrat.

3. On observe que les pseudocholinestérases présentes dans le sérum et les tissus du chien sont identiques à tous égards, tandis que la spécificité de la cholinestérase vraie varie d'un organe à l'autre.

4. Il est possible que, de même que le cerveau, les érythrocytes de l'homme et du chien contiennent un mélange de cholinestérase vraie et de pseudo-cholinestérase.

5. Les auteurs suggèrent que toutes les cholinestérases fonctionnent comme systèmes enzymatiques tampons, à la fois dans les tissus conducteurs et dans les tissus non-conducteurs.

ZUSAMMENFASSUNG

1. Trypsinvorbehandlung fördert die Ausbeute von Cholinesterase aus tierischen Geweben nicht.

2. Gewebscholinesterasen sind voneinander unterscheidbar durch (a) ihr pS-Maximum und ihre Michaelis-Mentenkonstante und (b) das Verhältnis zwischen den bei einer gegebenen Substratkonzentration durch Hexa- und Dekamethonium ausgeübten Hemmwirkungen.

3. Es wurde beobachtet, dass die in Hundeserum und -geweben vorhandenen Pseudocholinesterasen in jeder Hinsicht untereinander identisch sind, dass aber die wahren Cholinesterasen aus verschiedenen Organen in ihrer Spezifizität voneinander abweichen.

4. Möglicherweise enthalten menschliche und Hundeerythrocyten eine Mischung von wahrer und Pseudocholinesterase, wie es auch im Gehirn der Fall ist.

5. Es wird angenommen, dass sämtliche Cholinesterasen, sowohl in leitfähigen als auch in nicht leitfähigen Geweben, als enzymatische Puffersysteme wirken.

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