Biology. — *The significance of the substrate in the testing of true cholinesterase.* By J. A. COHEN, F. KALSBEEK and M. G. P. J. WARRINGA. (From the Medical Biological Institute of the National Defence Research Council T.N.O.) (Communicated by Prof. P. J. GAILLARD.)

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MENDEL and RUDNEY (1) proved the existence of two different cholinesterases; the true cholinesterase occurring mainly in nervous tissue and in red blood corpuscules and the pseudo-cholinesterase occurring a.o. in human and horse serum. Both enzymes are able to hydrolyse acetylcholine. Specific substrates for true and pseudo-cholinesterases are acetyl-β-methylcholine (Amechol) and benzoylcholine respectively (2). These two substrates can therefore be used for the typing of cholinesterase.

In recent years it has become evident, that only the true cholinesterase and not the pseudo-cholinesterase can be connected with the symptoms of acetylcholine accumulation.

It is possible to obtain a complete inhibition of pseudo-cholinesterase with the substance No. 683 (HOFFMANN–LA ROCHE) without a trace of clinical symptoms. Only when a dose is administered, which affects also the true cholinesterase clinical symptoms occur suggestive of acetylcholine poisoning (3). So far extensive work has been done on the kinetics of the inhibition of *pseudo-cholinesterase* by physostigmine and other similar substances (4, 5, 6). Obviously only research into the mechanism of inhibition of true cholinesterase is likely to throw some light on the in vivo effect of these substances.

The experiments to be reported form part of a series of experiments carried out with the object of elucidating the kinetics of physostigmine inhibition on true cholinesterase.

During this work we met with erratic results, when the physostigmine inhibition of true cholinesterase was determined using different substrates. The inhibition proved to be dependent to a large extent on the character of the substrate used for the test.

In this paper the significance of the character of the substrate was studied.

Experimental methods.

Suspensions containing true cholinesterase were prepared as follows: —

Rats were decapitated and the brains were pooled. After ten minutes rinsing, they were dried on filterpaper and weighed. They were then

crushed in a mortar with five volumes of water. The suspension was strained through gauze and spun and the precipitate resuspended in the original volume of water. Estimations for cholinesterase and lipase activity were carried out titrimetrically. For this purpose 2 cc. of brain suspension were made up to a total volume of 10 cc. with substrate solution, bromothymolblue and water. The standard for comparison contained the same amount of brain suspension and indicator, made up to a total volume of 10 cc. with phosphate buffer M/15 at pH 7.3 (glass electrode). During 15 minutes the rate was determined at which 0.01 N NaOH had to be added to the reaction mixture, in order to keep it at the same pH as the comparison vessel. The reaction took place at 24° C. in a waterbath.

The concentrations used were 0.02 M for acetylcholine and for amechol, 0.006 M. for benzoylcholine and 0.3 % for tributyrine.

The activity of the extracts was expressed in mm^3. NaOH 0.01 N. which had to be added per 60 minutes to neutralize one mg. of brain (dry weight).

Experimental results.

It was found that no hydrolysis of benzoylcholine took place by brain suspensions confirming MENDEL and RUDNEY's observations, that no pseudo-cholinesterase occurs in brain.

We were also able to confirm the results of NACHMANSOHN et al. (7), who found, that the hydrolysis of acetylcholine is usually much more rapid than that of amechol. We found no constant ratio in various brain suspensions between the rates of hydrolysis for amechol and acetylcholine.

Next the effect of physostigmine on the hydrolysis rate of various substrates was investigated.

It is possible to add so much physistigmine to a brain suspension that hydrolysis of amechol is completely or nearly completely stopped.

Such a system is still able to hydrolyse acetylcholine at a fair rate. This is clearly demonstrated by the experiment described in table I, which is representative for a series of experiments, giving similar results. This phenomenon cannot be explained by the presence of a pseudo-cholinesterase in brain, which hydrolyses acetylcholine but not amechol, as it has been shown that pseudo-cholinesterase does not occur in brain.

The possibility that ordinary esterase, not inhibited by the concentration of physostigmine used in our experiments, would hydrolyse acetylcholine, seems highly unlikely in view of MENDEL and RUDNEY's results, indicating, that ordinary esterases do not hydrolyse acetylcholine at all.

We were able to show that the main esterase occurring in brain, the brain-lipase, cannot be responsible for the observed effect. The experiment reported in table I shows that a concentration of physostigmine which almost completely knocks out acetylcholine hydrolysis has little or no influence on tributyrine hydrolysis by the same suspension.

TABLE I.

Substrate	Activity [1]
Amechol	100
Amechol + 1 cc. physostigmine 2×10^{-4} M	14
Acetylcholine	121.5
Acetylcholine + 1 cc. physostigmine 2×10^{-4} M	72.5
Acetylcholine + 1 cc. physostigmine 8×10^{-4} M	0
Tributyrine	143.5
Tributyrine + 1 cc. physostigmine 8×10^{-4} M	125.5

[1]) The activity is expressed in mm^3 NaOH $\frac{N}{100}$ in 60 minutes per mg dry weight. Brain suspension from rats.

In a separate experiment we were able to show, that the total inhibition of tributyrine hydrolysis by brain requires a 1000 fold stronger concentration of physostigmine than the inhibition of the hydrolysis of acetylcholine. This result strongly suggests that brain-lipase cannot be held responsible for part of the acetylcholine hydrolysis.

The most plausible hypothesis appears to be, that the substrate itself (acetylcholine or amechol) is operative in determining the extent of physostigmine inhibition of its hydrolysis by true cholinesterase. This difference in physostigmine inhibition, when the two substrates are used cannot be directly related to the difference in hydrolysis rates, which occurs in the absence of inhibition. The experiment of table I shows that even when the latter difference is negligible, the former is still very marked. (86 % inhibition of amechol hydrolysis against 41 % inhibition of acetylcholine hydrolysis).

To elucidate this problem a new series of experiments was initiated. 2 cc. of brain suspension was incubated during periods varying from 0 to 30 minutes with a suitable concentration of physostigmine. After incubation the usual ingredients to make up the reaction mixture were added and the activity tests carried out in the ordinary way.

The results of some representative experiments are given in table II, III and IV. When amechol is used as a substrate (tables II and IV) it appears that the inhibition by physostigmine is progressive during the period of 20 minutes preceding the activity test.

The separate experiments described in tables II and III were done with different brain suspensions. The experiment of table IV was carried out with the same brain suspension which was exposed to a certain concentration of physostigmine and then tested with both substrates.

When however acetylcholine is used as a substrate, this progressive inactivation during the 30 minutes of incubation prior to the activity test is by no means reflected in the results of the activity test (table III and IV). After between 5 and 10 minutes incubation, the inhibition has reached a certain value and longer incubation has no further influence on this value.

TABLE II.

	Time of incubation of enzyme with physostigmine preceding test in minutes	Inhibition of original cholinesterase activity in %
Experiment 1	5	38
	10	54
	20	74
Experiment 2	1	15
	3	38
	5	38
	7	46
	10	69
	20	100

2 cc rat brain suspension incubated with 1 cc physostigmine M 4×10^{-8} (expt. 1) and M 4×10^{-7} (expt. 2) were used for every test. Substrate: amechol.

TABLE III.

	Time of incubation of enzyme and physostigmine preceding test in minutes	Inhibition of original cholinesterase activity in %
Experiment 1	5	0
	14	37
	30	38
Experiment 2	0	0
	5	58
	10	56
	30	57
Experiment 3	10	59
	30	59

Exp. 1. 2 cc rat brain suspension incubated with 1 cc physostigmine 0.8×10^{-6} M used per test.

Exp. 2 and 3. 0.25 cc ox nucleus caudatus suspension incubated with 1 cc physostigmine M 0.8×10^{-6} (exp. 2) and M 0.4×10^{-6} (exp. 3) used per test. Substrate: acetylcholine.

TABLE IV.

Time of incubation of enzyme with physostigmine preceding the test in minutes	Inhibition towards amechol in %	Inhibition towards acetylcholine in %
5	38	36
10	54	—
20	74	46

2 cc rat brain suspension incubated in presence of 1 cc physostigmine 4×10^{-8} M were used for every test.

Discussion.

The difference in response to the same process (incubation of the enzyme with physostigmine) for both substrates shows that after about 5 minutes of incubation a certain number of enzyme groups, active towards acetylcholine, are eliminated. Longer incubation does not increase the number of eliminated groups.

At the same time elimination occurs of enzyme groups, which are active towards amechol. This process lasts for 20—30 minutes and is therefore obviously not identical with the inactivation of acetylcholine hydrolysing groups. The same conclusion may be drawn from the experiments, in which was shown, that the percentage inhibition by a certain concentration of physostigmine is often larger towards amechol than towards acetylcholine.

It may be that the same active group is eliminated in two qualitatively different ways: — a complete inactivation (towards acetylcholine and amechol) and a partial inactivation towards amechol only. Alternatively two different groups may be involved, one active towards acetylcholine, the other towards amechol, which are affected to a different extent by the poison.

A third possibility would be the following: — when amechol is added to enzyme previously incubated with physostigmine no displacement takes place and only the intact enzyme groups are able to react. When however acetylcholine is used as a substrate some of the enzyme-inhibitor linkages are dissociated and a greater number of active groups takes part in the hydrolysis of the substrate. In that case a maximal inhibition is reached with acetylcholine after about 5 minutes incubation bringing about some sort of equilibrium. Longer incubation does not show an additional effect in subsequent activity tests possibly because linkages formed during incubation after the first 5 minutes (demonstrable with amechol) are readily dissociated when the substrate acetylcholine is added, re-establishing the equilibrium state. In this stage no preference for any one of these possibilities can be given.

The results described in this paper demonstrate, that it is not permissable to draw conclusions regarding the extent of inhibition of true cholinesterase in vivo exclusively on account of tests carried out in vitro with amechol as a substrate. It is very likely that inhibitions of cholinesterase in vivo, that is in the presence of intrinsic acetylcholine, are considerably smaller than would be concluded from in vitro tests carried out with the so called specific substrate amechol. This warning may very well apply to a great number of tests with other specific substrates, which are not identical with the naturally occurring ones.

Summary.

1. Rat brain contains true cholinesterase in addition to lipase. No pseudocholinesterase is present.

2. The percentage inhibition of enzymic activity by the same concentration of physostigmine is usually considerably higher towards amechol than it is towards acetylcholine. (A concentration of physostigmine, which entirely knocks out enzymic activity towards amechol still allows a fair amount of acetylcholine hydrolysis).
3. Lipase nor pseudo-cholinesterase can be held responsible for this difference.
4. When brain cholinesterase is incubated in the presence of physostigmine at room temperature, the inhibition proves to be progressive during 30 minutes, when activity tests are carried out in presence of amechol.

   When however acetylcholine is used as a substrate, no progressive inhibition is observed after the first 5 minutes of incubation.
5. The experiments described form a warning against conclusions concerning in vivo inhibitions on the strength of results obtained in vitro with non physiological substrates.
6. Possible explanations for the effect of the substrate on inhibition are summarised.

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