THE TRUE CHOLINESTERASE ACTIVITY OF THE BRAINS OF PHYSOSTIGMINE POISONED RATS

by

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INTRODUCTION

After the discovery of the enzyme cholinesterase (chE) by LOEWI[1] great progress was achieved by the work of MENDEL AND RUDNEY[2], who differentiated between a pseudo and a true chE. HAWKINS AND GUNTER[3] showed that pseudo-chE does not take an essential part in the neuro-humoral transmission of stimuli; they were able to inhibit pseudo-chE completely with the Hoffman-la Roche compound No. 683 without producing any clinical symptoms. When, in addition, true-chE was inhibited by the use of a higher dose of this material the characteristic symptoms of an acetylcholine (AC) poisoning became manifest. True-chE is therefore considered to be essential for the hydrolysis of AC *in vivo*. The function of pseudo-chE, which hydrolyses the esters of choline as well as other esters, is still unknown. True-chE occurs in brains and in erythrocytes, pseudo-chE in serum.

Physostigmine is known to block chE[4]. However, after the work of MENDEL AND RUDNEY[2] it became obvious that most investigations into the inhibition of chE by physostigmine *in vivo* and *in vitro* had been carried out with pseudo-chE[5, 6, 7, 8, 9]. For a proper understanding of the pharmacological action of physostigmine, it is essential to know the action of this drug on true-chE, as has been pointed out by GOODMAN AND GILMAN[10]. In our laboratory some aspects of this problem have been studied[11, 12] and in the present paper further results are reported. A second reason for these investigations is the controversy between the experimental results of MAZUR AND BODANSKY[13] and HEYMANS *et al.*[14, 15], who were not able to demonstrate a parallelism between the clinical symptoms and the degree of inhibition of true-chE when using the modern anti-cholinesterase D.F.P. and those of NACHMANSOHN *et al.*[16] and FREEDMAN AND HIMWICH[17], who did find such a parallelism.

The former authors observed that true-chE of the brain could be practically completely inhibited in the absence of any symptoms of poisoning. They interpret their results as evidence for the independence of the state of apparent health in the animal from the degree of activity of true-chE.

Contrarily the latter authors claim that a narrow relationship exists between the degree of inhibition of true-chE and a number of clinical symptoms imitating acetylcholine poisoning.

If this were so a similar relationship should exist in physostigmine poisoning and

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its demonstration would provide further evidence for the conception that the presence of a certain amount of active chE is essential for the maintenance of good health and for the prevention of the appearance of symptoms.

EXPERIMENTAL METHODS

Suspensions of rat brain for the determination of chE-activity were prepared according to a previously described method[11].

We met some difficulties resulting from variations in the activities of brain suspensions which were often very small or even absent. Several alterations were made, *e.g.*, in the duration of the crushing period, the kind of rats used, the number of rats used to provide material for the suspension, but none of these alterations proved effective. A suspension of the brain of one rat was more often found to be inactive than one prepared from the brains of two rats. Therefore, in each of the following experiments the combined brains of two rats were used.

In order to eliminate the day to day variation all experiments were accompanied by controls from the same day. To this end suspensions from the brains of pairs of rats treated with intraperitoneal injections of physostigmine were compared with suspensions from pairs of normal rats. The rats used for each experiment belonged to the same sex and were approximately of the same weight. Great care was taken that the two rats used for one suspension showed the same clinical symptoms at the time of the killing. To achieve this, sometimes one of the two rats had to be given an extra dose of physostigmine. A dose of physostigmine varying from 0.05 to 0.1 mg proved never, a dose of 0.15 to 0.5 mg nearly always lethal.

The course of the clinical symptoms was followed; the rats were killed at varying stages of intoxication. Completely inactive suspensions were not considered.

ChE was determined titrimetrically with AC as a substrate according to the method mentioned before[11]. The use of AC as a substrate was permissible, because brain was shown to contain only true-chE[2, 11] and under the circumstances preferable to amechol (acetyl-β-methylcholine), the "specific" non-physiological substrate for true-chE, according to previous experiments (COHEN *et al.*[11]).

The question arises whether the inhibition of chE by physostigmine, which exists at the moment the animal is killed, remains unaltered during the process of preparation and testing. During the preparation the suspension is being washed once. The following experiment proves that no reversion of the inhibition occurs after washing.

A brain suspension from 4 ♀♀ rats weighing about 240 g was prepared in the usual way, but for the last process, *viz.*, the washing. The suspension was divided into a portion A, which was incubated in the absence, and a portion B, which was incubated in the presence of physostigmine, both at room temperature.

Portion A was again divided into a portion I, which was washed after the incubation period, and a portion II, which was not washed.

Portion B, 20 ml, was incubated with 10 ml of physostigmine $4 \cdot 10^{-7}$ M for half an hour. Then portion B was divided into a portion III, which was washed, and a portion IV, which was not washed. 2 ml of portion I and II and 3 ml of portion III and IV were tested for chE-activity. The results are represented in Table I.

Table I shows that the activity of the brain suspension incubated with physostigmine after the washing was as high as before.

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TABLE I

			Activity
Portion A	I without addition,	washed	72.5
	II without addition,	not washed	83.5
Portion B	III with physostigmine,	washed	55.5
	IV with physostigmine,	not washed	54.5

The activity is expressed in μl NaOH 0.01 N. per 60 min/mg dry weight. The difference in activity between portion A. I and A. II lies within the experimental error.

NACHMANSOHN *et al.*[16] pointed out that in the case of D.F.P., free poison in the tissues, *i.e.*, poison not yet bound with the enzyme protein, may combine with the enzyme during the process of preparation. Therefore a lower chE activity is found in the final test than was originally present *in vivo*. FREEDMAN AND HIMWICH[17] could not confirm this. The following control experiment was done according to NACHMANSOHN[16].

The supernatant liquid of a brain suspension from 8 normal ♀♀ rats weighing about 130 g was tested on chE-activity. This liquid is indicated as "test solution".

Four ♀♀ rats weighing app. 125 g were given 0.2 mg of physostigmine. Consequently convulsions appeared. The rats were then decapitated just before death; the brains of each rat were halved as accurately as possible and the respective halves were thus distributed that two portions were obtained of almost the same weight and each consisting of halves of each of the four rat brains. After being crushed in a mortar portion A was suspended in water, whereas portion B was suspended in the test solution.

The activity of the test solution was 2400 μl, that of the brain suspension in water 1920 μl and that of the brain suspension in test solution 3650 μl 0.01 N NaOH/h/2 ml solution. The activity of the test solution used to suspend the brains in was therefore 3650 — 1920 = 1730 per 2 ml suspension. The activity of the test solution itself is 2400 per 2 ml. The activity of the test solution used for the suspension was measured in 2 ml of the suspension in which the proportion of brains to test solution was 1:5. Thus the actual activity of the test solution was $^6/_5 \cdot 1730 = 2080$ per 2 ml test solution. The difference between the activity of the test solution alone (2400) and of the test solution after contact with the brains (2080) amounts to 13%, which is within the experimental error (app. 20%).

This experiment was carried out using brains of rats, which had been given a lethal dose of physostigmine. Therefore the hazard of the presence of some uncombined poison was great. Nevertheless no significant additional inhibition due to this cause was observed in the experiment described. In many of the following experiments smaller non-lethal and never higher dosages were used with accordingly still slighter chances of post mortal inhibition.

Summarizing, it is clear that the method followed for the *preparation* of brain suspensions does not relevantly affect the chE-activity of brains of physostigmine treated rats.

During the *testing* of the chE-activity, *i.e.*, during the titration, 2 ml of the brain suspension is diluted to 10 ml with a substrate solution and water[11]. As this is actually a dilution process the linkage between enzyme and poison may be partly or entirely dissociated. Previous experiments[12] on the effect of dilution on the true-chE-physostig-

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mine combination, however, have shown that under the conditions of the experiment no such dissociation takes place.

On the strength of these and previous experiments we feel justified in drawing conclusions on the activity of cholinesterase *in vivo* from the results obtained with the methods used.

EXPERIMENTAL RESULTS

The symptoms of a physostigmine poisoning are well-known[18, 19]. They appear roughly in the following succession: chattering of teeth, shaking of the head, fibrillations of the voluntary muscles of the trunk and later also of the extremities, which become more intense until the animal shows a pumping movement of the front legs; there is salivation and lachrymation. After a non-lethal dose the symptoms gradually decrease and the animal recovers in about an hour's time, whereas after a lethal dose the animal dies from vehement convulsions accompanied by tonic contractions of the extensor muscles of the hind legs.

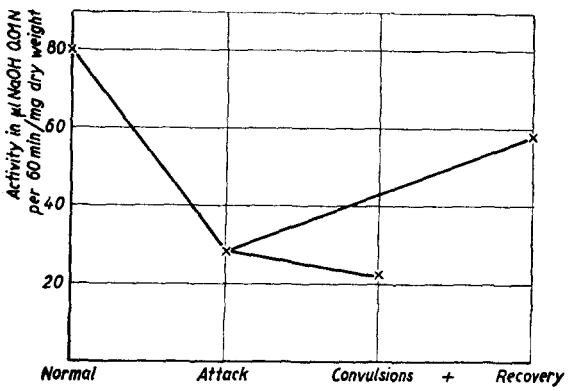

Fig. 1. True chE-activity of brains of rats treated with physostigmine

Table II and the graph of Fig. 1 give a survey of the results of the experiments described in this paper.

To facilitate the interpretation the vigour of the attack was registered using the following symbols:

\+ chattering and muscle fibrillation of the trunk but not of the extremities;

\+ + extensive muscular fibrillations of the extremities.

The experiments arranged in a horizontal column were carried out in the course of one day. *N* is the true-chE activity of brains from normal control animals; *d* means that the animal was decapitated during an attack and *a* when entirely recovered after an attack, of an intensity indicated at the head of the column. The activity is expressed in µl NaOH 0.01 N/h/mg dry weight.

Although the spread in the various groups is considerable, it was found that compared with the control values of the corresponding day the true-chE activity of the brains during a non-lethal physostigmine attack is always decreased; also, that the true-chE activity of the brains of recovered animals is higher than that of animals during an attack but lower than that of normal animals.

The average true-chE activity for the brains of normal animals from all experiments is 80.5; that for all animals which were decapitated during an attack 29.3; that for all surviving animals 58.6 and that for those animals, which were decapitated during vehement convulsions 24.4.

The difference between the chE-activities for normal animals and those killed during the attack is clearly significant: $P = < 0.01$.

The difference between the chE-activities in animals during the attack and those entirely recovered is also clearly significant: $P = < 0.01$.

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TABLE II

CHOLINESTERASE ACTIVITY OF THE BRAINS OF RATS TREATED WITH PHYSOSTIGMINE

Exp. No.	N	+		++		Convulsions	App. weight of rats	Sex
		d	a	d	a			
1	112.5	37.0			86.0		100 g	♂♂
2	138.5	57.5	101.0				100 ,,	♂♂
3	86.0				50.5		100 ,,	♀♀
4	89.0				60.5		100 ,,	♀♀
5	68.0			31.5			340 g	♂♂
6	86.5				45.5		180 ,,	♂♂
7	68.5			16.5	58.0		180 ,,	♂♂
8	85.0				61.0		150 ,,	♂♂
					47.0			
9	64.9					23.6	220 g	♀♀
						32.6		
10	42.1					36.0	220 ,,	♀♀
						30.5		
						30.5		
11	91.0						320 ,,	♀♀
12	55.6				58.8	17.1	260 ,,	♀♀
						16.6		
						22.8		
13	88.8			15 4		16.6	320 g	♂♂
14	85.7				33.0	26.4	310 ,,	♂♂
15	44.7				44.8	20.8	300 ,,	♂♂
						23.2		
						21.0		
16	88.6							
17	84.2						300 g	♂♂
18	83.3							
19	90.0							
20	88.8							
21	48.9			28.5			220 g	♀♀
				24.5			190 ,,	♀♀
				23.6			230 ,,	♀♀

For explanation see text.

The difference between the chE-activities for animals during an attack and for those with fatal convulsions is not significant: $P = 0.35$.

The difference between the chE-activities of recovered animals and normal ones is significant: $P = 0.01$.

DISCUSSION

The results of our experiments with physostigmine agree with the data of NACHMANSOHN[16] and FREEDMAN AND HIMWICH[17] attained with D.F.P. Coinciding with the

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symptoms of intoxication a decrease was found of the activity of true-chE. However, whereas these authors using D.F.P. found a distinct difference in the degree of inhibition between the chE activities of brains of animals that died and those surviving such a difference could not be demonstrated by us using physostigmine. We found that the true-chE activity in the brains of clinically recovered animals is significantly higher than during the symptoms, but yet significantly lower than normal.

During the symptoms of intoxication the true-chE activity falls down to 36% of the normal value, a degree of inhibition comparable to that found by NACHMANSOHN[16] for D.F.P. in rabbits (25%). The average chE-activity of the brains of clinically recovered rats is 72% of the normal value from which it seems likely that about 30% may be inhibited without the appearance of clinical symptoms. In that case the normal enzyme activity would be app. 50% higher than that necessary for the normal functioning of the organism.

NACHMANSOHN[16] found that the chE-activity was zero or practically zero after a lethal dose of D.F.P. After a lethal dose of physostigmine we found an inhibition of the chE-activity averaging 70%. Perhaps the quantitative differences between D.F.P. and physostigmine can be accounted for by the assumption that physostigmine reaches more readily than D.F.P. a fatal concentration in certain centres essential for the maintenance of life. The enzyme may still be relatively abundant in other parts of the brain, resulting in a comparatively high overall cholinesterase value for the whole of the brains (*vid.* NACHMANSOHN[16]).

Reversibility of enzyme inhibition during the preparation could be excluded as a possible source of error.

ACKNOWLEDGEMENT

We are indebted to Prof. Dr. S. E. DE JONGH for providing us room in his laboratory and for much help and advice, and to MISS B. R. BOVENS for technical assistance.

SUMMARY

A rough parallelism exists in physostigmine poisoned rats between the clinical symptoms and the activity of the true cholinesterase of the brains.

This is demonstrated by the following experimental results:

1. The true-chE activity of the brains of physostigmine poisoned rats is significantly decreased compared with that of normal rats.

2. The true-chE activity in the brains of rats clinically recovered from a physostigmine poisoning is significantly higher than that of rats still showing symptoms, but yet significantly lower than that of normal animals.

RÉSUMÉ

Un certain parallélisme existe chez les rats empoisonnés par la physostigmine entre les symptômes cliniques et l'activité de la cholinestérase vraie du cerveau. Ceci a été démontré par les résultats expérimentaux suivants:

1. L'activité de la cholinestérase vraie du cerveau chez des rats empoisonnés par la physostigmine, est nettement diminuée comparativement à celle de rats normaux.

2. L'activité de la cholinestérase vraie du cerveau chez des rats cliniquement guéris d'un empoisonnement par la physostigmine, est nettement supérieure à celle présentée par les rats qui montrent encore des symptômes d'empoisonnement, mais reste cependant inférieure à celle présentée par des animaux normaux.

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ZUSAMMENFASSUNG

Bei mit Physostigmin vergifteten Ratten besteht ein Parallelismus zwischen den klinischen Symptomen und der Aktivität der wahren Gehirncholinesterase. Dies wurde durch die folgenden Versuchsergebnisse bewiesen:

1. Die Aktivität der wahren Cholinesterase im Gehirn von mit Physostigmin vergifteten Ratten ist gegenüber derjenigen bei normalen Ratten stark vermindert.

2. Die Aktivität der wahren Cholinesterase im Gehirn von Ratten, die eine Physostigminvergiftung überstanden haben, ist bedeutend höher als bei Ratten, die noch Symptome zeigen aber niedriger als bei normalen Ratten.

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Received February 21st, 1949