REVERSIBILITY OF THE INHIBITION OF TRUE CHOLINESTERASE BY PHYSOSTIGMINE

by

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Since the discovery of cholinesterase by LOEWI[1] the pharmacological effects produced by physostigmine have been connected with the ability of this drug to inhibit cholinesterase *in vitro*. Recently a great deal of work has been done with diisopropyl fluorophosphonate (DFP). On account of results, obtained with DFP and other substances, doubt has risen whether a cholinesterase inhibition can really be held responsible for physostigmine-like effects, because often no parallelism between the two could be observed[4, 5, 6, 7, 8, 9].

Most of the discrepancies can be explained, when a proper use is made of the discovery of MENDEL *et al.*[2, 3], who demonstrated that 2 types of cholinesterase exist, namely the pseudo cholinesterase occurring *e.g.*, in human and horse serum and a true cholinesterase occurring mainly in nervous tissue. No connection seems to exist between pseudo cholinesterase inhibition and symptoms after DFP. Some authors[5, 7] also deny a relationship between the inhibition of *true* cholinesterase and clinical symptoms after DFP, but NACHMANSOHN[10] was able to establish a close relationship in a series of carefully controlled experiments. If *a priori* such relationship between true cholinesterase inhibition and *in vivo* symptoms was possible, the next question was, whether it was also causally *responsible* for the pharmacological effect of DFP. If this were the case, as suggested by NACHMANSOHN, a similar parallelism between cholinesterase inhibition and the clinical symptoms of physostigmine administration were to be expected. This drug has been exhaustively examined for its action on pseudo cholinesterase. For a good understanding of its pharmacological action obviously only its effect on true cholinesterase would be of importance.

In the following experiments this effect has been studied particularly in connection with one of its most critical aspects, namely that of the reversibility of the inhibition.

Any theory explaining the pharmacological action of physostigmine on the basis of enzymological data should be able to account for the ready reversibility observed *in vivo* of the symptoms of physostigmine poisoning. Therefore a study of the character of the inhibition *in vitro* seemed necessary. In a previous communication[11] the influence of the character of the substrate used for the test on the degree of inhibition was described. In the following experiments other factors, which may influence the degree of reversal of inhibition, were examined.

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

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filter paper 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 activity were carried out titrimetrically. For this purpose 2 ml of brain suspension were made up to a total volume of 10 ml with substrate solution, bromthymolblue and water. The standard for comparison contained the same amount of brain suspension and indicator, made up to a total volume of 10 ml with phosphate buffer M/15 at p_H 7.3.

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 p_H as the comparison vessel. The reaction took place at 24° C in a waterbath.

The acetylcholine used was acetylcholinchloride (ROCHE). The acetyl-β-methylcholine was given in the form of amechol (SAVORY AND MOORE). The physostigmine used was the pure base, dissolved in water.

EXPERIMENTAL RESULTS

I. INFLUENCE OF THE SUBSTRATE CONCENTRATION ON THE ACTIVITY OF CHOLINESTERASE

Before investigations into the inhibition of true cholinesterase by physostigmine could be initiated, it was necessary first to examine the mechanism of action of the enzyme itself. It could not be assumed without specific evidence that the pattern of action would be the same as that of many other hydrolytic enzymes (*e.g.*, pseudo cholinesterase), *viz.*, a first order reaction at low concentrations of substrate and a zero order reaction at higher substrate levels, the rate of the reaction at these high levels being proportional to the amount of enzyme present. Only if this state of affairs should prevail, would the usual test procedure, in which the observed activity is taken to be proportional to enzymic activity, at high substrate levels be justified.

For this purpose 2 ml of a brain suspension was mixed with amechol in a final concentration of 0.002, 0.005, 0.01, 0.02, 0.04 and 0.08 M in the usual test procedure (total volume 10 ml). It was found that a maximum of activity was reached at a substrate level of 0.01 M. Further increase of the substrate concentration did not lead to higher activity. At these concentrations straight lines were always observed, when activity was plotted against time (Fig. 1a), indicating zero order reaction. From these curves the velocity in microgrammol/min could be calculated (Table I).

At lower concentrations, however, (0.005 and 0.002 M), the curves were of the dying out type (Fig. 1b). To investigate whether these curves represented the expected first order reactions, it was examined, whether they fitted the formula for the monomolecular reaction $k_1 = \frac{1}{t} \log \frac{a}{a-x}$ * (I).

The amount hydrolysed could be calculated at any moment from the amount of NaOH that had been added at the corresponding time. Table II, computed from curve 3

* in which t = time,
a = initial substrate concentration,
x = concentration of hydrolysed substrate,
k_1 = k (= velocity constant)·2.3026.

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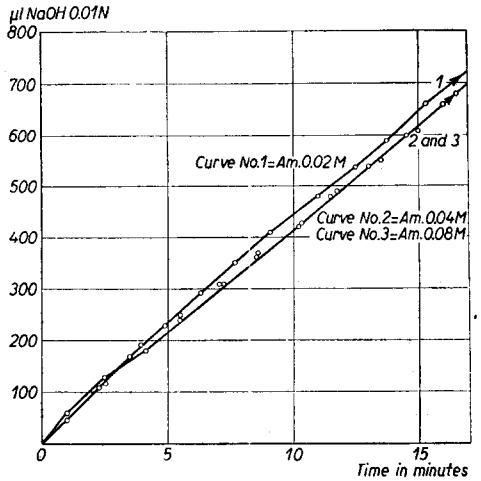

Fig. 1a

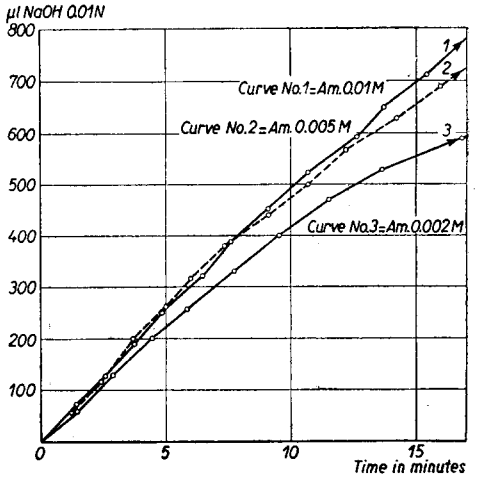

Fig. 1b

Activity of rat brain cholinesterase at various concentrations of amechol

TABLE I (see Fig. 1a)

Substrate concentration	Velocity in microgrammol/min
Amechol 0.08 M	0.40
,, 0.04 ,,	0.41
,, 0.02 ,,	0.40
,, 0.01 ,,	0.41

TABLE II (see Fig. 1b, curve 3)

Time in minutes	µl NaOH 1/100 N	$\log \frac{a}{a-x}$
5.54	260	0.0607
9.32	400	0.0969
13.40	530	0.1336
16.52	590	0.1492
20.10	650	0.1703

of Fig. 1b (amechol 0.002 M), gives various values for $\log \frac{a}{a-x}$ for corresponding times.

When $\log \frac{a}{a-x}$ is plotted against time a straight line results (Fig. 2) proving that the values obtained are related according to formula (I) and that therefore the hydrolysis of low concentrations of amechol follows a monomolecular reaction pattern. From the slope of this line k_1 can be calculated and this value divided by 2.3026 gives a velocity constant k of 0.0044. It will be seen that in the first part of the curve the slope is constant, suggesting a monomolecular reaction. At very low substrate concentrations (corresponding to time 10 min in Fig. 2) the slope changes. Apparently the reaction is then no longer a pure first order one and consequently the k has been calculated from the first part of the curve only.

Since the velocity $v = \frac{ds}{dt} = k \cdot s^*$ for first order reactions, it can now be calculated for various low substrate concentrations. Fig. 3 pictures the relation between various substrate concentrations and the velocity v. The graph clearly shows that true cholinesterase hydrolyses amechol according to the expected pattern; *i.e.*, a monomolecular reaction at low and a zero order reaction at high substrate concentrations. In fact the line representing the first order reaction at low substrate concentrations should pass

* in which s = substrate concentration in microgrammols.

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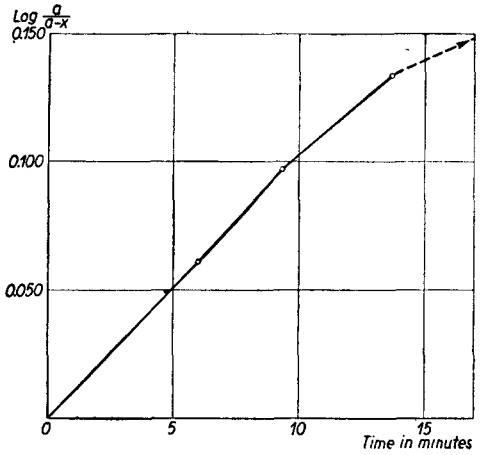

Fig. 2. Values for t and log $\frac{a}{a-x}$ from curve No. 3 (Fig. 1b).

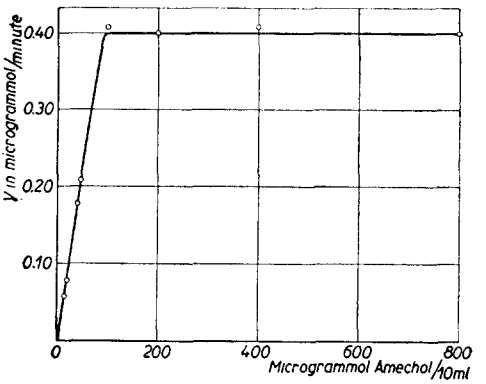

Fig. 3. Substrate velocity curve for rat brain cholinesterase hydrolysis of amechol. The data are computed from Table I (for the horizontal part of the curve) and from the calculated value for k substituted in the formula $v = k \cdot s$ (for the oblique part of the curve).

over continuously into the straight horizontal line representing velocity at excess substrate concentrations. Our line, which is obtained by connecting calculated points and which shows an abrupt bend, is therefore inexact in this respect.

In other words, at low substrate concentrations the rate of the hydrolysis depends on the concentration of the enzyme and that of the substrate, at higher substrate concentrations only on the concentration of the enzyme.

That at high substrate concentrations the reaction rate was proportional to the enzyme concentration was demonstrated in many experiments. Our enzyme activity tests were done with an excess of substrate to eliminate interference of the substrate concentration. The substrate concentration used throughout was 0.02 M and therefore well above the 0.01 M found to be the smallest concentration required in order to work in the zero order part of the substrate-velocity curve, where enzymic activity only is limiting the reaction rate. The MICHAELIS constant was $4.5 \cdot 10^{-3}$ molar.

II. THE REVERSIBILITY OF THE INHIBITION OF TRUE CHOLINESTERASE BY PHYSOSTIGMINE

a. Reversibility by substrate competition

Titrations for cholinesterase activity were carried out as usual but in presence of 1 ml of a physostigmine solution, which was allowed to act on the enzyme two minutes prior to the initiation of the reaction by addition of amechol. The period of two minutes was arbitrarily chosen; it seemed advisable, however, to have a fixed time of interaction in all experiments. Fig. 4 represents the rate of the reaction (by plotting μl NaOH, required to neutralize the acid formed, against time) for final concentrations of physostigmine of $1.2 \cdot 10^{-6}$, $2.4 \cdot 10^{-6}$, $0.4 \cdot 10^{-5}$, $0.5 \cdot 10^{-5}$, $0.6 \cdot 10^{-5}$, $0.7 \cdot 10^{-5}$, $0.8 \cdot 10^{-5}$ and $1.5 \cdot 10^{-5}$ M. The most intriguing feature is, that all curves after a certain period become bent; the latent period before the beginning of the bending is longer at smaller, shorter at larger concentrations of physostigmine. The initial protection against the physostig-

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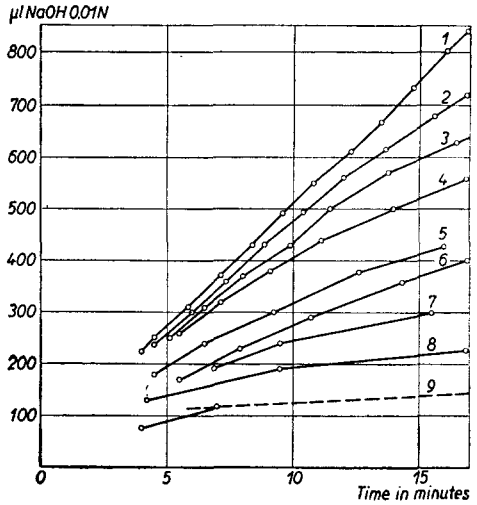

Fig. 4. Inhibition of rat brain cholinesterase by various concentrations of physostigmine. 2 ml rat brain suspension.

Curve No. 1: Control. Solution containing 1 ml Am. 0.2 M.
" " 2: Solution containing physostigmine, final concentration $1.2 \cdot 10^{-6}$ M.
" " 3: Solution containing physostigmine, final concentration $2.4 \cdot 10^{-6}$ M.
" " 4: Solution containing physostigmine, final concentration $0.4 \cdot 10^{-5}$ M.
" " 5: Solution containing physostigmine, final concentration $0.5 \cdot 10^{-5}$ M.
" " 6: Solution containing physostigmine, final concentration $0.6 \cdot 10^{-5}$ M.
" " 7: Solution containing physostigmine, final concentration $0.7 \cdot 10^{-5}$ M.
" " 8: Solution containing physostigmine, final concentration $0.8 \cdot 10^{-5}$ M.
" " 9: Solution containing physostigmine, final concentration $1.5 \cdot 10^{-5}$ M.
Substrate amechol 0.02 M.

mine inhibition offered by the substrate in the straight part of the curve subsides gradually during the period of testing.

This mechanism suggests that a progressive inhibition takes place, which is probably slowed down but not prevented by the presence of excess substrate. This may be due either to a slow proceeding towards an equilibrium state depending on the concentrations of amechol and physostigmine, both competing for the active groups of the enzyme (competitive inhibition) or to a progressive inhibition by physostigmine not reversible by amechol. The second explanation seems the most likely in view of the fact that even with the lowest concentrations of inhibitor nothing like a reversal of inhibition during the test period was observed, in spite of large concentrations of substrate.

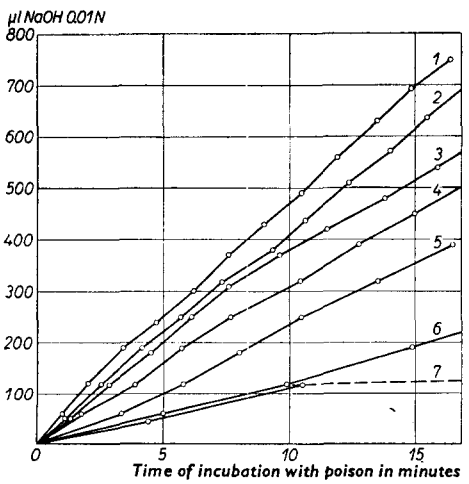

Fig. 5. Activity of rat brain cholinesterase after incubation during varying periods with physostigmine. 2 ml rat brain suspension used in all experiments.

Curve No. 1: Control. Without poison.
" " 2: Mixture containing 1 ml physostigmine (concentration $4 \cdot 10^{-8}$ M). 1 min incubation.
" " 3: Ditto, 3 min incubation.
" " 4: " " 5 " "
" " 5: " " 7 " "
" " 6: " " 10 " "
" " 7: " " 20 " "
Substrate amechol 0.02 M.

To elucidate this question a series of experiments was initiated, in which 2 ml of rat brain suspension was incubated with the same dose of physostigmine for varying

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periods at room temperature before the addition of the substrate and the other ingredients to make up the usual test solution. The results are represented in Fig. 5. Straight lines are now obtained showing that under the conditions of this experiment (low physostigmine concentration) the substrate is able to protect against further inhibition during the test period. However, as in the previous experiments no reversal of the inhibition is ever effected by the addition of substrate. Moreover the curves clearly show that during the incubation period prior to the test, *i.e.*, in the absence of substrate, a progressive inhibition is taking place as the slope of the curves becomes flatter with increasing incubation periods.

The reason for the fact *no* progressive inhibition takes place during the test period is very probably the very low physostigmine concentration (a final molarity of only $4 \cdot 10^{-9}$) in this experiment. Obviously the excess of amechol is able to protect against progressive inhibition at *low* concentrations of physostigmine, although even in this type of experiment amechol has never been able to reverse any degree of existing inhibition. This experiment therefore strongly confirms the previous one in suggesting that physostigmine produces a progressive inhibition, which is not reversible by competition by amechol, at least for the duration of these experiments.

From our previous communication[11] it follows that this conclusion on the non-reversibility of physostigmine inhibition cannot be applied blindly to substrates other than amechol.

b. Reversibility by dialysis

Suspensions of rat brain or of ox nucleus caudatus* were prepared as usual. They were then incubated during a period of 30–40 minutes with a suitable concentration of physostigmine (a relatively long incubation period was chosen to be sure that a stable condition was secured). The activity of the cholinesterase was then determined and the inhibition calculated by comparison with control suspensions, which had not been treated with physostigmine. The activity tests were carried out towards acetylcholine (final concentration in the test mixture 0.02 M).

Both inhibited suspensions and controls were then dialysed in dialysing bags against tap water at room temperature for periods up to 8 hours. Samples taken out at different times during the dialysis were tested for cholinesterase activity. The activity of inhibited enzyme preparations was compared with that of control suspensions, which had been dialysing during equal periods. The activity of the dialysed controls was found not to alter materially during dialysis and these controls were therefore omitted in later experiments in which all activities of dialysed physostigmine treated enzyme suspensions were compared with that of the undialysed control.

The results of 2 experiments are recorded in Tables III and IV.

Although reversal has not yet started 2 hours after the beginning of the dialysis, after 4 hours the original activity has practically completely returned.

It may be concluded from these experiments that the enzyme-inhibitor linkage is not materially affected by dialysis during the first 2 hours, but that 4 hours of dialysis suffice completely to reverse the inhibition.

In a similar experiment, in which pseudo cholinesterase was used for comparison, the reversal by dialysis was found to proceed much more slowly. Even after as much

* We are indebted to the Public Slaughterhouse at Leyden for providing us with ox brain. From this source also serum was obtained for the preparation of pseudo-cholinesterase.

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as 9 hours of dialysis no complete reversal was accomplished. The results of an experiment are recorded in Table V.

TABLE III

REVERSAL OF TRUE CHLOLINESTERASE INHIBITION BY DIALYSIS

Enzyme preparation: rat brain cholinesterase.
Incubated during 40 minutes prior to dialysis with an equal volume of $4 \cdot 10^{-7}$ M physostigmine at room temperature.
For every test 4 ml of the mixture were used. Concentration of acetylcholine during test: 0.02 M.

Time of dialysis in h	% inhibition compared with control enzyme suspensions
0	37
2½	37
4	0
6	0
8	0

TABLE IV

REVERSAL OF INHIBITION OF TRUE CHOLINESTERASE BY DIALYSIS

Enzyme preparation: Ox nucleus caudatus.
Incubated during 30 minutes with 4 volumes physostigmine $0.8 \cdot 10^{-6}$ M at room temperature. For every test 1.25 ml of mixture were used.
Concentration of acetylcholine 0.02 M.

Time of dialysis in h	% inhibition compared with control enzyme suspensions
0	66
2	66
3	60
3½	35
4	18
4½	5

TABLE V

REVERSAL OF INHIBITION OF PSEUDO CHOLINESTERASE BY DIALYSIS

Enzyme preparation: horse serum cholinesterase purified by ammoniumsulphate precipitation according to STEDMAN[12] diluted 1/8.
Incubated during 45 minutes prior to dialysis with 2 volumes physiostigmine $4 \cdot 10^{-7}$ M.

Time of dialysis in h	% inhibition
0	79
2½	58
4	54
6	58
9	31

DILUTION EXPERIMENTS

In the previous section it has been demonstrated that on dialysis return of activity only occurs after a latent period of several hours. In the following experiments the

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attempt was made to evoke a more ready reversal of inhibition by dilution of the enzyme inhibitor complex after physostigmine had been in contact with the enzyme at room temperature for a short period. If the inhibiting effect would be a reversible one under the circumstances of this experiment such dilution would involve a redistribution of the poison with fewer active enzyme groups blocked than in the original undiluted system. In other words the degree of inhibition would decrease after dilution.

In the first series dilution was effected by a simple washing out procedure. A brain suspension was incubated with half its volume of physostigmine $4 \cdot 10^{-7}$ M for half an hour. A portion of this mixture was washed with distilled water. The cholinesterase activities towards acetylcholine before and after washing were compared; they were found to be the same for both suspensions. The degree of inhibition had not been changed by washing, suggesting that no actual reversal can be accomplished by simple washing. This state of affaires is fortunate in so far as it proves, that the way of preparation of

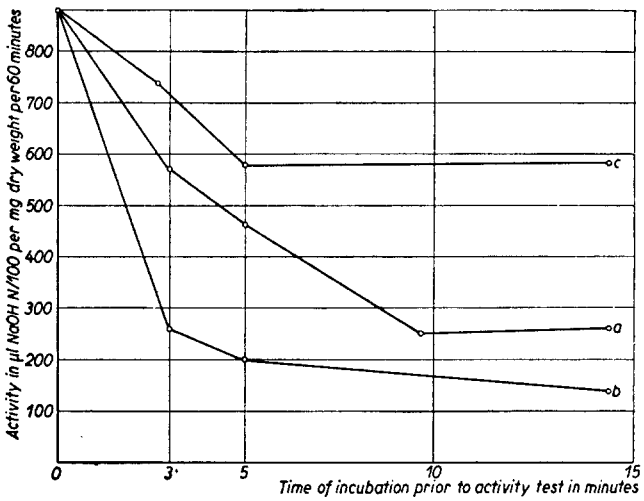

Fig. 6. Reversibility of the physostigmine inhibition of true cholinesterase by dilution. Preparation used was cholinesterase prepared from ox nucleus caudatus.
a. Diluted immediately before initiating test.
b. Undiluted.
c. Diluted from the beginning of the incubation period.

brain cholinesterase described, including washing, does probably reflect the original state of inhibition *in vivo*. This method of preparation is therefore suitable for the estimation of the activity of brain cholinesterase of physostigmine treated animals.

The next series of dilution experiments was carried out fundamentally according to a method described by NACHMANSOHN[13]. 0.1 ml ox nucleus caudatus suspension was incubated with physostigmine (0.1 ml $2 \cdot 10^{-6}$ M). Samples of this mixture were taken out after various periods of incubation and either diluted with water to 10 ml (series a) and then tested for activity, or tested without changing the concentration of physostigmine by making up to the required test volume with a physostigmine solution of calculated concentration (series b). The values so obtained were compared with values from control mixtures from test solutions, which had been incubating during the same periods, but had been diluted with water from the very beginning of the incubation period (series c). The concentration of physostigmine in this series c was the same as

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as the *final* concentration (after dilution) of the first series (series a). All tests were carried out using acetylcholine as a substrate.

By comparing the results of the activity tests of series a with those of series c, it was possible to gain an impression regarding the irreversible portion of the inhibition in series a; complete reversal ought to bring the values for series a back to those of series c. The difference between a and c represents the degree of irreversibility. In the same way the difference between series a and b represents the degree of reversibility. The results are summarized in Fig. 6.

The graph shows, that series a and c practically coincide after the first ten minutes. This indicates that practically no reversal of the inhibition occurs after an incubation period of as short as ten minutes by a dilution of 1 in 50.

DISCUSSION

No reversal could ever be demonstrated as a result of addition of amechol (competitive reversal). It is demonstrated that total reversal of the inhibition of true cholinesterase towards acetylcholine occurs after 4 hours dialysis. During the first two hours of dialysis, however, no reversal is demonstrable.

The latter fact is also borne out by dilution experiments, which always involved periods of less than one hour and never showed any reversibility.

In a previous communication[11] it was reported that the inhibition of cholinesterase by physostigmine was maximal after five to ten minutes of incubation when acetylcholine was used as a substrate, bnt needed a longer period to become maximal when tested against amechol. Further it was shown that the degree of inhibition by an equal physostigmine concentration for the same enzyme preparation was larger towards amechol than towards acetylcholine. It was concluded that two different types of inhibition were involved, either on two different enzyme groups or on the same group, but then affecting that group in a qualitatively different way. As a third possibility it was suggested that the difference in behaviour of the enzyme inhibitor complex towards both substrates might be due to the ability of acetylcholine to dissociate linkages which were unaffected by amechol: some linkages may be detachable by acetylcholine; others, only formed in the first five to ten minutes of incubation, not. This last possibility is rendered unlikely by our present experiments. It would be expected that acetylcholine would have the more difficulty to restore linkages, the higher the concentration of physostigmine. That this is not the case is proved by the similarity of the activities in series a and b of the dilution experiments. If in both cases detachment of linkages is supposed to take place, this detachment would have to be equal in spite of higher concentrations of physostigmine in series b than in series a. Moreover it was shown in a separate experiment not reported, that the degree of inhibition after incubation of enzyme and inhibitor (for periods of 20 minutes) towards acetylcholine was not affected by varying the concentration of acetylcholine from 0.005 to 0.04 M. Obviously, if dissociation of linkages were involved, some effect of the concentration of acetylcholine were to be expected.

It must therefore be concluded that only the first two of the above mentioned possibilities remain: the inhibition of true cholinesterase during incubation with physostigmine is a two-fold one. In either effects one and the same active group in a qualitatively different way, or two different groups are inhibited. Of these possibilities the former impresses as being rather artificial and the latter seems the more likely one.

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If this is the case true cholinesterase possesses two active groups: one of these is exclusively concerned with amechol hydrolysis, the other with acetylcholine and perhaps also with amechol hydrolysis. This state of affairs may also be expressed by the statement that true cholinesterase might consist of two different enzymes.

After about 5 minutes of contact of the enzyme with the inhibitor at room temperature the inhibition towards acetylcholine can no longer be reversed by dilution or dialysis. This condition of irreversibility lasts for a period of at least an hour, but reversal can be accomplished by dialysis during a period of 4 hours.

No competitive reversal by the addition of acetylcholine as a substrate was observed in any of the experiments; obviously, the inhibitions towards both substrates, acetylcholine and amechol, resemble each other in not being reversible by substrate competition.

It should be noticed that dialysis and dilution experiments were all carried out towards acetylcholine as a substrate. All conclusions concerning reversibility from these experiments are therefore restricted to that type of inhibition, which interferes with acetylcholine hydrolysis.

In the same way the conclusions regarding the enzyme kinetics should be understood to refer to the hydrolysis of amechol only.

Acknowledgement:

We are indebted to Prof. Dr S. E. DE JONGH for providing us room in his laboratory and for much help and advice.

SUMMARY

1. The kinetics of a true cholinesterase preparation from rat brain were studied. Acetyl-β-methylcholine (amechol) was used as a substrate. It was found that, at a constant concentration of enzyme, the reaction is of a unimolecular type when the concentration of substrate is lower than 0.01 M; when the substrate concentration rises above 0.01 M the reaction assumes a zero order character.

2. When the reaction velocity is determined in presence of physostigmine, it is found that a progressive inhibition takes place during the test, which is slowed down, but never reversed by the substrate amechol.

3. When the enzyme is incubated for varying periods, prior to the activity test, with the same low concentration of physostigmine, it is found that the degree of inhibition increases with the duration of the incubation period. It is found that also under these conditions, in presence of a low concentration of physostigmine, the inhibition is progressive and is not reversed by the substrate, when this is added after the incubation period. In these experiments amechol was used as a substrate.

4. Dialysis reverses the inhibition by physostigmine of true cholinesterase towards acetylcholine in 4 hours. The reversal of the inhibition of pseudo cholinesterase appears to proceed more slowly.

5. Washing out of rat brain suspensions previously inhibited with physostigmine does not cause reversal of the inhibition.

6. It is not possible to reverse part of the inhibition acutely by means of dilution after ten minutes of incubation.

7. It is concluded that no competitive reversal by substrate of physostigmine inhibition takes place, but that it is possible to reverse this inhibition by dialysis for several hours. Within shorter observation periods no reversal is demonstrable by dialysis or dilution using acetylcholine as a substrate except after very short periods of incubation.

8. It is suggested that, as a result of incubation with physostigmine, two types of inhibition of cholinesterase may occur simultaneously; one towards amechol, the other towards acetylcholine. The physostigmine inhibition probably affects two different active groups, both present in our preparation of true cholinesterase.

RÉSUMÉ

1. La cinétique d'une préparation de cholinestérase vraie, à partir de cerveau de rat, a été étudiée. Le substrat utilisé était l'acétyl-β-méthylcholine (amechol). On a constaté que, pour une

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concentration déterminée en enzyme, la réaction est monomoléculaire lorsque la concentration en substrat est inférieure à 0.01 M, et d'ordre zéro quand la concentration en substrat est supérieure à 0.01 M.

2. Quand on mesure la vitesse de réaction en présence de physostigmine, on constate que ce corps exerce une inhibition croissante au cours de la mesure; cette inhibition est empêchée partiellement, mais jamais complètement par l'amechol, substrat de la réaction.

3. Quand on maintient l'enzyme, préalablement aux mesures d'activité, en présence d'une même concentration, faible, de physostigmine, on constate que l'importance de l'inhibition s'accroît avec la durée de la période d'incubation. On constate également que dans ces conditions, en présence d'une faible concentration de la physostigmine, l'inhibition est graduelle, et n'est plus suspendue par le substrat quand ce dernier est ajouté après la période d'incubation. Dans ces expériences, le substrat utilisé était l'amechol.

4. La dialyse supprime l'inhibition par la physostigmine de la cholinestérase vraie vis-à-vis de l'acétylcholine, en 4 heures. La suppression de l'inhibition de la pseudocholinestérase se fait plus lentement.

5. Le lavage de suspensions de cerveau de rat préalablement inhibées par la physostigmine ne supprime pas cette inhibition.

6. Une simple dilution ne permet pas de supprimer de façon rapide l'inhibition.

7. On conclut que la réversibilité de l'inhibition par la physostigmine, par une action de compétition du substrat, n'est pas possible, mais qu'il est possible de l'obtenir par une dialyse de plusieurs heures. Aucune réversibilité ne peut être observée au cours de dialyses faites pendant des temps courts, ou par dilution, en utilisant l'acétylcholine comme substrat.

8. Ces résultats montrent que deux types d'inhibition de la cholinestérase peuvent se manifester simultanément: l'un vis-à-vis de l'amechol, l'autre vis-à-vis de l'acétylcholine. L'inhibition par la physostigmine affecte probablement deux groupes actifs différents, tous deux présents dans la préparation de cholinestérase vraie étudiée ici.

ZUSAMMENFASSUNG

1. Die Kinetika eines Präparates wahrer Cholinesterase aus Rattenhirn wurden untersucht. Azetyl-β-methylcholin (Amechol) wurde als Substrat benutzt. Dabei wurde festgestellt, dass bei konstanter Enzymkonzentration die Reaktion monomolekular verläuft, wenn die Substratkonzentration geringer ist als 0.01 m; wenn die Substratkonzentration grösser wird als 0.01 m, wird der Reaktionscharakter von der nullten Ordnung.

2. Wenn die Reaktionsgeschwindigkeit bei Anwesenheit von Physostigmin bestimmt wird, wird gefunden, dass während des Versuches eine fortschreitende Hemmung stattfindet, die von dem Substrat Amechol zwar verlangsamt, aber nie aufgehoben wird.

3. Wenn das Enzym verschieden lange Perioden vor Beginn des Aktivitätstests mit derselben geringen Konzentration von Physostigmin inkubiert wird, dann zeigt sich, dass der Hemmungsgrad mit der Dauer der Inkubationsperiode zunimmt. Es wird festgestellt, dass auch unter diesen Bedingungen, bei Anwesenheit einer geringen Physostigminkonzentration, die Hemmung progressiv verläuft und durch das Substrat nicht umgekehrt wird, wenn es nach der Inkubationsperiode zugefügt wird. Bei diesen Experimenten wurde Amechol als Substrat benutzt.

4. Dialyse hebt die Physostigminhemmung der wahren Cholinesterase bei Azetylcholin als Substrat in 4 Stunden auf. Die Aufhebung der Hemmung von Pseudo-Cholinesterase scheint langsamer zu verlaufen.

5. Auswaschen von Rattenhirnsuspensionen, die vorher mit Physostigmin gehemmt worden waren, verursacht keine Aufhebung der Hemmung.

6. Es ist nicht möglich, die Hemmung unmittelbar durch Verdünnen teilweise aufzuheben.

7. Es wird gefolgert, dass durch das Substrat keine kompetitive Aufhebung der Physostigminhemmung erfolgt, dass es aber möglich ist, diese Hemmung durch mehrstündige Dialyse aufzuheben. Bei kürzeren Beobachtungsperioden ist bei Azetylcholin als Substrat keine Aufhebung durch Dialyse oder Verdünnungen nachzuweisen.

8. Es wird angenommen, dass infolge der Inkubation mit Physostigmin zwei Hemmungstypen der Cholinesterase gleichzeitig auftreten können; der eine für die Wirkung auf Amechol, der andere für Azetylcholin. Die Physostigminhemmung betrifft wahrscheinlich zwei verschiedene aktive Gruppen, die beide in unserem Präparat wahrer Cholinesterase anwesend sind.

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