# PROTECTION OF TRUE CHOLINESTERASE AGAINST DI*ISO*PROPYL FLUOROPHOSPHONATE BY BUTYRYLCHOLINE

by

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## INTRODUCTION

Organic phosphorus compounds with anti cholinesterase properties are coming into prominence for clinical use and as insecticides. Consequently the hasard of accidental intoxication has increased considerably, urging for the search of suitable antidotes.

From a number of compounds investigated KOELLE[1] found that eserine, prostigmine and carbamylcholine showed marked protection of cholinesterase against irreversible inactivation by di*iso*propyl fluorophosphonate* *in vitro*. He suggested that the protective property of these drugs did not depend on the potency of anti-cholinesterase activity alone but also on the ability to compete with DFP for a specific active group of the cholinesterase molecule. KOSTER found that eserine had a protective effect against DFP *in vivo* when administered to cats[2].

In previous experiments we had found that butyrylcholine was a specific inhibitor for true cholinesterase[3, 4]. It seemed reasonable to assume that this inhibition would be caused by competition for an active group and that therefore protection against DFP and other irreversible inhibitors may be afforded by a mechanism similar to the protection by eserine etc.

The purpose of the experiments to be described was first to assess the reversible character of the inhibition by butyrylcholine and then to investigate whether protection *in vitro* and *in vivo* against DFP could be demonstrated.

## EXPERIMENTAL METHODS

Cholinesterase was estimated using a continuous titration method[5]. Instead of using bromthymolblue as an indicator the titrations were carried out potentiometrically with a direct reading pH-meter at pH 7.4. The temperature was kept at 24° C.

The enzyme preparations were made from ox nucleus caudatus. Acetylcholine was used as substrate in a final concentration of 0.01 *M*.

Enzyme and inhibitors were incubated together for a period of 10–15 min at room temperature in a volume of 2.0 ml, acetylcholine was then added, the volume brought up to 10 ml with distilled water and the activity determined.

* Abbreviated DFP.

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## EXPERIMENTAL RESULTS

### I. *Reversibility of butyrylcholine inhibition*

A few experiments were carried out to show the reversible character of the inhibition of the enzyme by butyrylcholine. For this purpose butyrylcholine in a concentration of 0.05 *M* was incubated with true cholinesterase in a volume of 2.0 ml for a period of 15 min, the mixture was then dialysed for varying periods against distilled water at 0° C.

The results of a typical experiment are reproduced in Table I. It will be seen that total inhibition is effected by the butyrylcholine concentration used. Reversal was complete after a dialysis period of several hours.

TABLE I

REVERSIBILITY OF ChE INHIBITION BY BUTYRYLCHOLINE

| Time of dialysis | % inhibition |
|---|---|
| 0 | 100 |
| 15 min | 53 |
| 3 h | 27.5 |
| 4½ h | 15.5 |
| 18 h | 0 |

### II. *Protection of cholinesterase by butyrylcholine against inactivation by DFP*

#### a. *Dialysis experiments*

In these experiments true cholinesterase preparations were incubated with DFP for a period of 10 min. The controls were then immediately dialysed whereas the experimental mixtures were incubated for a further 10 min with various concentrations of butyrylcholine before dialysis. So the influence of butyrylcholine was studied on the inhibition of DFP occurring in the period after the first 10 min of incubation. Dialysis was carried out at 0°C in distilled water during 24 hours (a period sufficient for the reversion of any inhibition caused by butyrylcholine alone, according to Table I). Activities were determined both before and after dialysis and expressed in percentage inhibition of the untreated enzyme resp. before and after dialysis. The difference between percentage inhibition after dialysis of mixtures originally containing butyrylcholine + DFP, and percentage inhibition before dialysis of the mixture containing DFP alone, was compared with the difference in percentage inhibition between the mixture containing DFP before and after dialysis, and was expressed as percentage protection.

Two representative experiments are summarized in Table II.

Experiment *a* of the Table shows that protection is afforded by concentrations of butyrylcholine down to 0.05 *M*.

In experiment *b* the protection is even more striking, 0.05 *M* giving more than 100% protection. These protection figures of experiment *b* far above 100% suggest that even originally bound DFP may have been displaced. More careful control of the results, however, showed that this displacement is probably spurious.

It was found that the spontaneous inactivation of the enzyme on dialysis was far less when butyrylcholine was originally present. The enzyme appeared to be protected by butyrylcholine against spontaneous inactivation during dialysis.

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TABLE II*

PROTECTION OF CHOLINESTERASE BY BUTYRYLCHOLINE

| Experiment a | % inh. before dialysis | % inh. after dialysis | | % protection | |
|---|---|---|---|---|---|
| | | before corr. | after corr. | before corr. | after corr. |
| 1 | 2 | 3 | 4 | 5 | 6 |
| DFP ** | 19.0 | 48.4 | | | |
| DFP + but.chol. 0.2 *M* | 89.7 | 23.1 | 24.1 | 86.0 | 82.5 |
| DFP + but.chol. 0.15 *M* | 86.3 | 23.1 | 29.0 | 86.0 | 66.0 |
| DFP + but.chol. 0.1 *M* | 85.3 | 25.9 | 34.0 | 76.5 | 49.0 |
| DFP + but.chol. 0.05 *M* | 78.0 | 27.4 | 37.2 | 71.5 | 38.0 |
| Experiment b | | | | | |
| DFP | 37.1 | 55.8 | | | |
| DFP + but.chol. 0.2 *M* | 89.4 | 34.4 | 44.0 | 100 | 57.6 |
| DFP + but.chol. 0.15 *M* | 89.6 | 28.0 | 35.0 | > 100 | 100 |
| DFP + but.chol. 0.1 *M* | 89.4 | 26.5 | 35.5 | > 100 | 100 |
| DFP + but.chol. 0.05 *M* | 83.1 | 29.5 | 37.0 | > 100 | 100 |

Concentration DFP during incubation 1.3 to $1.8 \cdot 10^{-6}$ *M*.
* For reading see text.
** The concentrations given are the concentrations during the incubation period.

It therefore seemed reasonable to express the activities after dialysis of those mixtures where butyrylcholine was present as percentage of the activity of the enzyme after dialysis *in the presence of butyrylcholine* rather than as percentage of activity of enzyme dialysed alone. For example in the second line of experiment *b* the activity of the enzyme incubated with DFP + butyrylcholine 0.2 *M*, was 2781 µl 0.01 *N* NaOH /mg/h after dialysis. Before dialysis the activity of the enzyme without added inhibitors was 6450 and after dialysis 4300. After incubation with butyrylcholine alone the activity was 5050 after dialysis. From these data the percentage inhibition after dialysis is found as 33.4% when compared with the enzyme alone, and 44% when compared with the enzyme dialysed after incubation with butyrylcholine. The other figures were calculated in a similar way.

In this manner corrected values were obtained which are given in Table II in column 4 and 6. It will be seen that, when these corrections are applied reversal is no longer evident. At this stage therefore only a protective action of butyrylcholine has been proved.

b. *Acute experiments*

It was felt that in the dialysis experiments as described above no fair impression was given of the protecting properties of butyrylcholine. This is due to the lack of knowledge of the concentrations of butyrylcholine and DFP operating during dialysis. Initial protective effects may be spoilt later when the diffusion ratio of the two compounds would be unfavourable for butyrylcholine.

Therefore experiments avoiding the dialysis period were performed as follows:

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The enzyme was incubated with DFP and butyrylcholine in various concentrations for a period of 15 min. By comparing the inhibition obtained after DFP + butyrylcholine with that of DFP alone the percentage protection could be calculated. In order to calculate the protection the inhibition by butyrylcholine itself had to be accounted for. This inhibition had to be subtracted from the total inhibition in the presence of DFP in order to get the inhibition due to DFP. This value compared with the DFP inhibition gave the percentage protection.

Fig. 1 shows that when a constant concentration of DFP was used producing app. 50% inhibition complete protection was afforded at a butyrylcholine concentration of from 0.01 *M* upward.

For comparison a curve representing the inhibition of cholinesterase by butyrylcholine is also included. It will be seen that greater concentrations of butyrylcholine are required for inhibition than for protection.

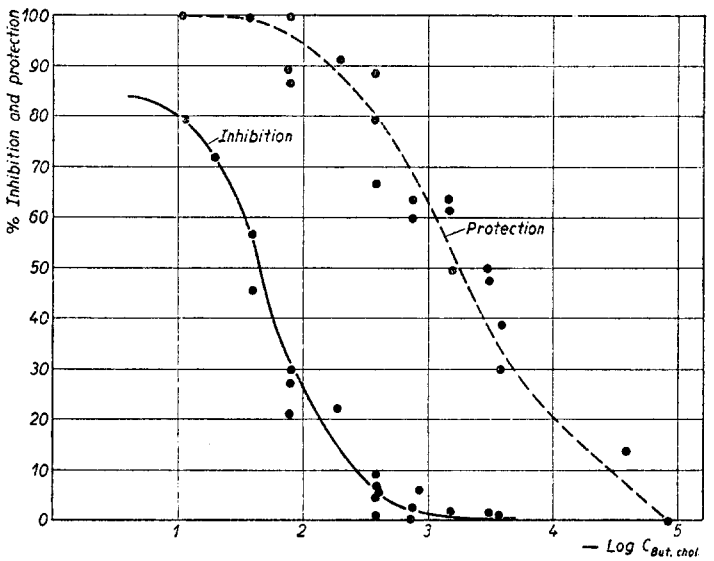


Fig. 1. Inhibition and protection of cholinesterase by butyrylcholine

About 30 times more butyrylcholine is needed for 50% inhibition than for 50% protection.

Assuming that for inhibition and for protection butyrylcholine is attached to the same enzyme groups this difference in concentration can be explained by the summation of the following two effects. The inhibition is measured in a volume of 10 ml and as no difference in pCt inhibition was found with and without previous incubation during 15 minutes in a volume of 2 ml, the inhibition as assessed under the conditions of the experiment reflects the number of active centers occupied by butyrylcholine.

In the protection experiment, however, butyrylcholine and DFP are incubated with the enzyme in a volume of 2 ml. Afterwards butyrylcholine and DFP are diluted 5 times for the activity determination. By this dilution the DFP reaches a concentration where it inhibits no longer. So the "active" concentration of butyrylcholine in the protection experiments is 5 times higher than that in the inhibition experiments which

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explains a shifting of the protection curve to concentrations 5 times lower than those found for the inhibition curve. A further difference is caused by the following. Acetylcholine displaces butyrylcholine from the enzyme surface. In the case of the inhibition experiments both acetylcholine and butyrylcholine are present. In the case of the protection experiments butyrylcholine and DFP, but not acetylcholine are present during the incubation period when butyrylcholine is protecting the enzyme against DFP.

To assess the extent of the difference it was necessary to find the butyrylcholine concentration giving for instance "50% inhibition" of the enzyme in the absence of acetylcholine.

This concentration was approximated by determination of the pCt inhibition of a number of butyrylcholine concentrations at various acetylcholine concentrations and extrapolation to zero acetylcholine concentration.

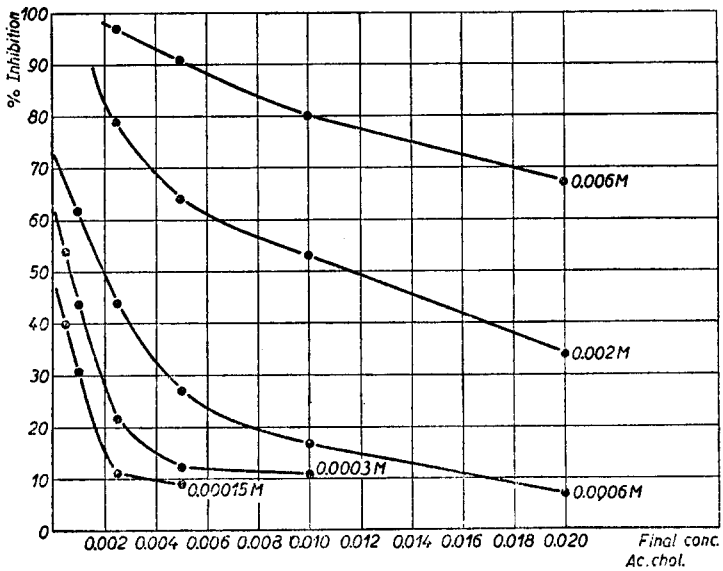


Fig. 2. Inhibition of true cholinesterase by a number of butyrylcholine concentrations (final molar concentrations) at varying acetylcholine concentrations

The results are shown in Fig. 2. A final concentration of 0.002 *M* butyrylcholine gives approximately 50% inhibition at an acetylcholine concentration of 0.01 *M* (the concentration used in the activity determinations). A final concentration of 0.00015 *M* gives approximately 50% inhibition on extrapolation to zero acetylcholine concentration.

So this effect accounts for a shift in concentrations of about 13 times. Taking into account the variation in the experiments of Fig. 1 and Fig. 2 a difference in concentration range of the order of that observed in the two experiments of Fig. 1 may be explained by the above-mentioned two phenomena.

Fig. 3 shows the protective effect of butyrylcholine when the DFP concentration is varied. It will be seen that complete protection against low DFP concentrations may be effected by butyrylcholine concentrations down to 0.0025 *M*. Butyrylcholine concentrations of 0.005 *M* and higher practically prevent any inactivation of the enzyme by DFP.

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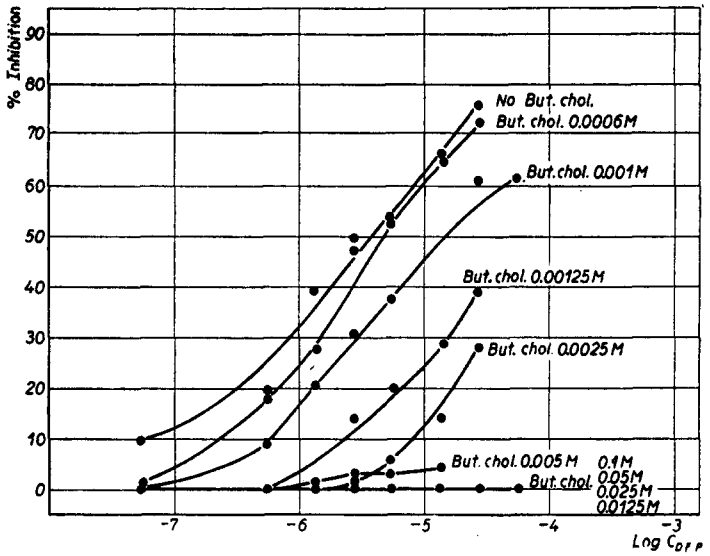


Fig. 3. Protection of cholinesterase by butyrylcholine against di*iso*propyl fluorophosphonate

## III. *In vivo experiments*

Butyrylcholine given subcutaneously to rats, even in high concentrations (over 15 mg/kg), does not produce many symptoms. The reason is that most of it is rapidly broken down by the pseudo-cholinesterase of the blood and does not reach the tissues.

Given 5–20 min before a lethal dose of DFP (5 mg/kg subc.) it does not protect the animals. On the contrary doses of butyrylcholine varying between 5 and 50 mg/kg caused death within 30 min, whereas the controls usually survived an hour or more. The curious phenomenon was observed that death occurred after the development of a complete paralytic picture widely different from the typical DFP symptoms in the controls.

Butyrylcholine has no favourable influence either when it is given shortly after 5 mg/kg of DFP subcutaneously. Again paralysis occurred with doses of butyrylcholine over 5 mg/kg.

## IV. *Experiments on isolated frog muscle*

The experiments described in the last section suggested that perhaps some curare-like effect may be exerted by butyrylcholine.

To investigate the effect of butyrylcholine on the neuromuscular receptor mechanism the isolated frog rectus muscle preparation of GADDUM[6] was used.

While CHANG AND GADDUM[7] found that butyrylcholine was about equally active as acetylcholine on the frog rectus we found that butyrylcholine was more than twice as effective as acetylcholine on a molar basis (Fig. 4). Moreover the effect was far more persistent. The relaxation after the contraction in Fig. 4 was effected by three successive washings with frog Ringer followed by an addition of eserinized frog Ringer and mechanical relaxation of the muscle to the zero line. After this the behaviour of the muscle was studied for 1½ min and the described washing procedure repeated, whereupon the muscle could be used for the next contraction. It will be seen that one series

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of washings is always sufficient to produce total or almost total relaxation after acetylcholine but by no means after butyrylcholine. This "sticking" of butyrylcholine to the receptor corresponds in a curious way to its adherence to true cholinesterase *in vitro* and suggests a connection between the two events (see discussion).

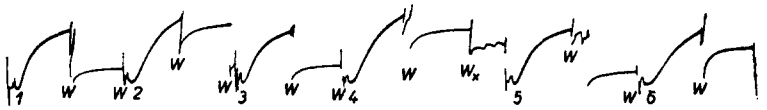


Fig. 4. Effect of acetylcholine and butyrylcholine on isolated frog muscle.
1: 4 $\gamma$ Ac.chol.; 2: 2 $\gamma$ But.chol.; 3: 4 $\gamma$ Ac.chol.;
4: 2 $\gamma$ But.chol.; 5: 6 $\gamma$ Ac.chol.; 6: 2 $\gamma$ But.chol.
W: washing and relaxation.
At $W_x$ the drum was left running during the washing.

## DISCUSSION

Butyrylcholine was found to inhibit true cholinesterase in a reversible way. The reversal of inhibition by dialysis was even more readily accomplished than that of eserine[5]. The chemical structure of butyrylcholine strongly suggests that the inhibition is of a competitive nature. This was also demonstrated by the fact that the inhibition could be partly released by raising the substrate (ac.chol.) concentration.

Also according to expectation butyrylcholine was found to be a very effective *in vitro* protector of cholinesterase against DFP. Reversal of existing DFP inhibition could never be demonstrated.

Concentrations of butyrylcholine sufficient to produce a certain degree of protection are lower than those required to give the same amount of inhibition. This seeming discrepancy may be explained by assuming that during incubation some of the active groups may be actually occupied by butyrylcholine, thus preventing the settling down of DFP molecules. These groups become free again later during the activity test by the addition of the substrate (ac.chol.) and by dilution.

On the isolated frog muscle butyrylcholine proved to be a more potent contractor substance than acetylcholine. The occasional observation was made that the frog muscle was reluctant to relax on washing out after a butyrylcholine contraction. This reluctancy never occurred after treatment with acetylcholine in the concentrations used.

This adherence of butyrylcholine to the receptor is a curious parallel to its clinging to true cholinesterase and adds another item to an admittedly not very convincing list which may suggest an identity of the cholinesterase molecule (or of a combination of such molecules) with the acetylcholine receptor in neuromuscular transmission. In this speculation the negative group occurring in the true cholinesterase molecule postulated by several authors is thought to be connected with its transmittor function. The hydrolysing group would be concerned with the hydrolysis of the acetylcholine securing the reversibility of the transmittor-receptor combination. If this hypothesis is true the clinical picture of intoxication by DFP-like compounds would not be governed by the accumulation of acetylcholine but by the sluggishness of the release of acetylcholine from its complex with the receptor.

No preventive or curative action *in vivo* against DFP intoxication in rats could be effected by butyrylcholine. Often the clinical symptoms in rats poisoned with DFP and treated with butyrylcholine are very strongly paralytic in character. However, no

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paralytic effect could be observed on isolated frog rectus muscles treated with butyrylcholine. No satisfactory explanation can be offered but it may be pointed out that similar paralytic effects are observed when massive doses of acetylcholine are poured into skeletal muscle of intact animals. Acetylcholine in high amounts may to some extent be comparable in its action to the "persistent" butyrylcholine.


## SUMMARY

1. Butyrylcholine was shown to be a reversible, probably competitive, inhibitor of true cholinesterase.
2. It prevented the irreversible inactivation of this enzyme by DFP. The degree of protection depended on the concentrations of DFP and butyrylcholine used.
3. On the isolated frog muscle butyrylcholine was more effective in producing contraction than acetylcholine.
4. Butyrylcholine given to rats before or after lethal subcutaneous doses of DFP had no favourable effect on the lethality of rats poisoned by DFP.

## RÉSUMÉ

1. Nous avons montré que la butyrylcholine était un inhibiteur réversible, probablement par compétition, de la cholinestérase vraie.
2. Elle empêchait la destruction irréversible de l'activité de cette enzyme par le DFP, le degré de protection dépendant des concentrations de DFP et de butyrylcholine employées.
3. La butyrylcholine était plus active que l'acétylcholine en produisant la contraction du muscle isolé de grenouille.
4. Lorsque des rats empoisonnés par l'injection subcutané de doses mortelles de DFP recevaient, avant ou après cette injection, de la butyrylcholine, ce traitement ne diminuait pas la mortalité des rats.

## ZUSAMMENFASSUNG

1. Es wurde gezeigt, dass Butyrylcholin wahre Cholinesterase reversibel hemmt, wahrscheinlich indem es an seiner Stelle reagiert.
2. Ausserdem verhinderte Butyrylcholin den irreversibelen Aktivitätsverlust dieses Enzyms durch DFP. Hierbei hing die ausgeübte Schutzwirkung von der DFP- und der Butyrylcholinkonzentrationen ab.
3. Butyrylcholin rief die Kontraktion des Froschmuskels energischer hervor als Acetylcholin.
4. Butyrylcholinbehandlung von Ratten vor oder nach subcutaner Injektion einer tödlichen Dosis von DFP, verminderte die Giftwirkung nicht.


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Received June 15th, 1950