Acetylcholinesterase in Goldfish Muscles

STUDIES ON SOME SUBSTRATES AND INHIBITORS

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In an earlier paper (Lundin, 1958) it was pointed out that the cholinesterase activity in muscles of goldfish (*Carassius auratus*) and guppy (*Lebistes reticulatus*) is due to an acetylcholinesterase. Working with goldfish muscles the author has obtained a high proportion of acetylcholinesterase activity in the supernatant after centrifuging the homogenized muscles. This preparation of acetylcholinesterase has been found to be very stable and is convenient for various purposes, e.g. routine tests of organic phosphorus compounds or kinetic enzyme studies. Thus it seemed to be of interest to give a detailed account of the results on which is based the statement that goldfish muscles contain acetylcholinesterase. It is well known (e.g. Augustinsson, 1949) that substrate and inhibitor relationships of acetylcholinesterases from different sources differ. A study of these relationships of the goldfish-enzyme preparation to some important substrates and inhibitors as compared with those of some other well-known sources has therefore been undertaken.

METHODS AND MATERIALS

Enzyme activities were measured by Tammelin's (1953) modification of Michel's (1949) electrometric method. The activities are expressed as μm-moles of acid liberated ml.$^{-1}$ min.$^{-1}$, after correction for the spontaneous hydrolysis of the substrates as measured in individual blanks in the presence of suitable concentrations of the denatured (heated to 100°) enzymes. The highest and lowest values obtained for the spontaneous hydrolysis are given in Table 1.

Buffers used

A. A buffer of the following composition and of ionic strength 0·25 was found to be the best to extract the acetylcholinesterase from the muscles of goldfish: 0·01 M-sodium barbital, 0·002 M-KH_2PO_4, 0·228 M-KCl. 0·1 N-HCl was added to obtain pH 8·2. The pH was measured with a pH meter, PHM 3 from Radiometer, Copenhagen, against a standard buffer at pH 7 (P-H Tamm, Altuna, Sweden).

B. Activity measurements of the fish enzyme preparations were carried out in a buffer of the same composition as *A*, except that the concentration of KCl was 0·3 M.

C. Erythrocyte and plasma cholinesterase activities were measured in a buffer of twice the concentration of *B*.

Sources of cholinesterases

From goldfish. Freeze-dried body muscles from goldfish were homogenized in buffer *A*. The homogenate was centrifuged for 15 min. at 1500 *g* and the supernatant was dialysed for 24 hr. against distilled water and freeze-dried once more. This preparation gave very uniform and stable activities when redissolved in buffer *A* (10 mg./ml.). The solution was diluted 30 times with buffer *B* before use.

From electric ray. A freeze-dried preparation of the electric organ of electric ray (*Torpedo ocellata*) was homogenized in *B* (0·3 g./100 ml.) and was centrifuged for 1 min. at about 1000 *g*. The almost clear supernatant was used.

From erythrocytes. Fresh, heparinized human blood was centrifuged for 15 min. at 1500 *g* and the plasma discarded. The remaining erythrocytes were washed three times with 0·9% NaCl and diluted first to the volume of the original blood and then five times with water (Augustinsson, 1957).

From human serum. A purified preparation, Cohn's fraction IV-6 (Kabi AB, Stockholm), containing butyrylcholinesterase was used, 100 mg. being dissolved in 300 ml. of buffer *C*.

Substrates and inhibitors

Substrates. The following substrates were used: acetylcholine iodide, propionylcholine iodide, acetyl-β-methylcholine iodide and butyrylcholine iodide, all prepared according to Tammelin (1957*a*); triacetin (Hopkin and Williams Ltd.).

The final concentrations in the reaction vessels are given in the Figures. In the inhibitor experiments acetylcholine iodide was the substrate at p*S* 2·2.

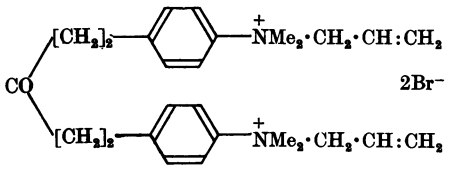
Table 1. *Highest and lowest values for the spontaneous hydrolysis of the different substrates used with the different inactivated enzyme preparations*

p*S* = − log (molar concn. of substrate).

Substrate	Source of enzyme	Spontaneous hydrolysis (μm-moles/ml./min.) at p*S* 1·3	p*S* 1·6	p*S* 1·8	p*S* 2·2	p*S* 2·8
Acetylcholine	Goldfish	10·0	8·0	6·0	5·0	3·0
Butyrylcholine	Erythrocytes	2·0	1·5	1·0	0·5	0·5

Inhibitors. The following inhibitors were used: eserine (physostigmine salicylate, T. and H. Smith Ltd., London; Sarin (*iso*propyl methylphosphonofluoridate); B & W 284C51 (I) mipafox (*NN'*-di*iso*propylphosphorodiamidic fluoride); DFP (di*iso*propylphosphorofluoridate); all these were prepared according to L.-E. Tammelin & L. G. Fagerlind (unpublished work); MEPtCh (Tammelin, 1957*b*):

$$Me{\cdot}PO(OEt){\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}\overset{+}{N}Me_3I^-$$



B & W 284 C51 (I)

The final concentrations in the reaction vessels before adding the substrate are given. The times of incubation with the inhibitors were chosen so that maximum inhibition was obtained.

Composition of reaction mixtures. The reaction mixture before adding the substrate consisted of equal volumes of one of the buffers mentioned above containing the enzyme preparation, and water or inhibitor solution. The volume of the substrate solution added was 10% of the volume of this reaction mixture.

RESULTS

In Figs. 1–4 the results of the experiments on substrate specificities are shown. These experiments were performed on acetylcholinesterase preparations from goldfish muscle, electric organ of electric ray and erythrocytes and on a purified butyrylcholinesterase preparation from human serum.

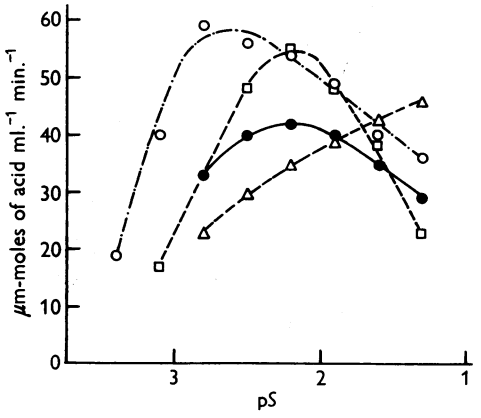


Fig. 1. Activity–p*S* curves for the enzymic hydrolysis of acetylcholine by the following cholinesterase preparations: goldfish muscles (●); electric organ of *Torpedo ocellata* (○); erythrocytes (□) (acetylcholinesterases); human serum (purified) (△) (butyrylcholinesterase).

In Fig. 1 are shown the results with acetylcholine. All the acetylcholinesterase preparations give the well-known 'bell-shaped' activity–p*S* curves. The maximum activities are obtained at slightly different values of p*S*. In the region of substrate concentration investigated the p*S* curve obtained with the butyrylcholinesterase preparation from serum conforms with the well-known 'S-shaped' curve (e.g. Augustinsson, 1949). It is observed that a maximum activity of about 20 μmoles of acetylcholine hydrolysed/mg. of dry

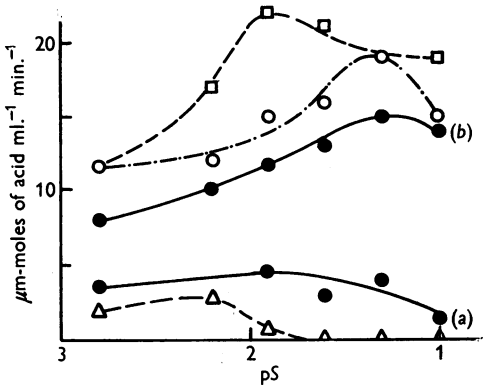


Fig. 2. Activity–p*S* curves for the enzymic hydrolysis of acetyl-β-methylcholine. (For explanation of symbols see Fig. 1.) Curve (*b*) was obtained by using twice the enzyme concentration used for (*a*).

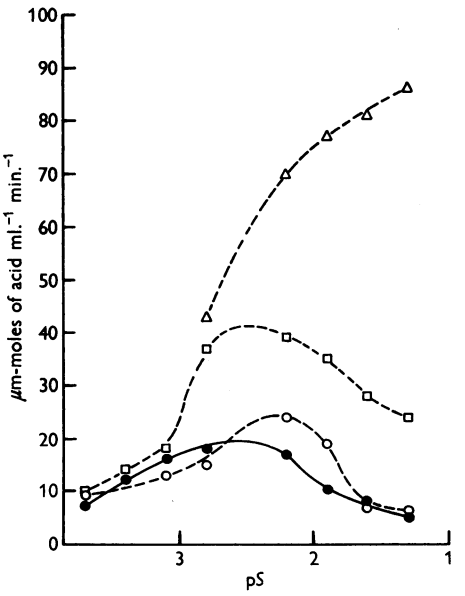


Fig. 3. Activity–p*S* curves for the enzymic hydrolysis of propionylcholine. (For explanation of symbols see Fig. 1.)

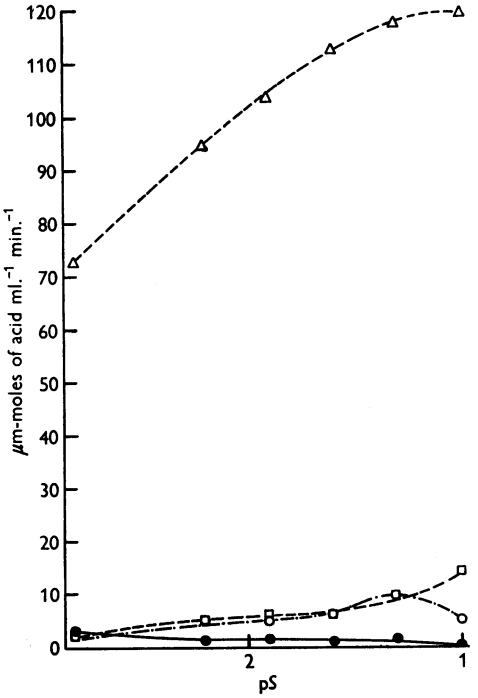


Fig. 4. Activity–pS curves for the enzymic hydrolysis of butyrylcholine. (For explanation of symbols see Fig. 1.)

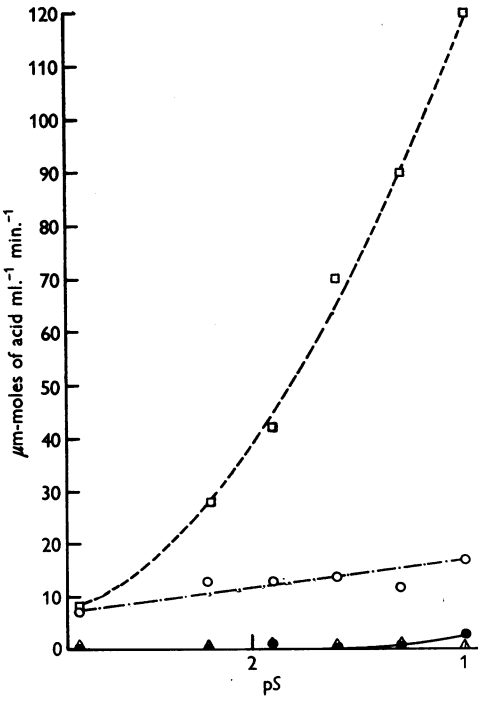


Fig. 5. Activity–pS curves for the enzymic hydrolysis of triacetin. (For explanation of symbols see Fig. 1.)

preparation/hr. is obtained with this preparation. This is in fairly good agreement with the value of 90 μmoles/mg./hr. obtained by Surgenor & Ellis (1954) with the same serum fraction (Cohn IV-6), particularly as the present preparation may have a reduced activity due to storage.

Acetylcholinesterase from goldfish has a very low activity with acetyl-β-methylcholine ((*a*) in Fig. 2). If the enzyme concentration is doubled, however, demonstrable activity with this substrate is present ((*b*) in Fig. 2). The butyrylcholinesterase activity with this substrate was very low.

The same general results have been obtained for propionylcholine (Fig. 3) as for acetylcholine although the relatively much higher activity of the butyrylcholinesterase preparation is to be noticed. This preparation has a high activity with butyrylcholine (Fig. 4). The acetylcholinesterase preparations from erythrocytes and electric ray have traces of activity with butyrylcholine, but that from goldfish has virtually none. Triacetin (Fig. 5) is rapidly hydrolysed by the erythrocyte preparation, slowly by the ray preparation, and very slowly by the goldfish and the serum preparations. As to the effect of the inhibitors on the three different acetylcholinesterase preparations the following results were obtained. The two non-selective inhibitors Sarin and eserine inhibit the enzymic activities of the three preparations within the same characteristic ranges of concentrations (Fig. 6). In Figs. 7 and 8 the values obtained with four selective

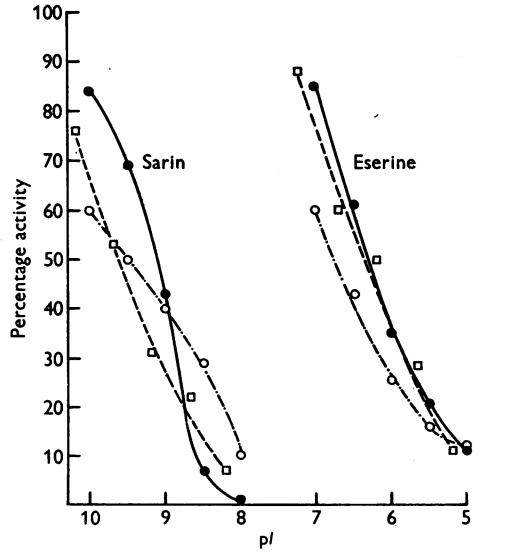


Fig. 6. Inhibiting action of eserine and Sarin on the following acetylcholinesterase preparations: goldfish muscles (●); electric organ of *Torpedo ocellata* (○); erythrocytes (□). Substrate, acetylcholine.

inhibitors have been plotted: B & W 284C51 and MEPtCh are known to be selective for acetylcholinesterase (Austin & Berry, 1953; Tammelin, 1958*b*), and mipafox and DFP for butyrylcholinesterase (Aldridge, 1953).

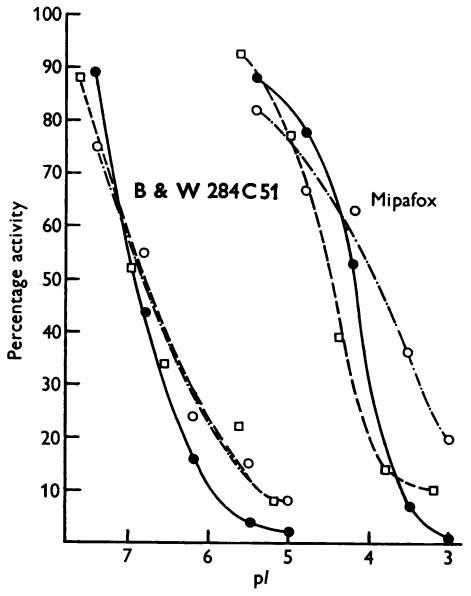


Fig. 7. Inhibiting action of B & W 284 C51 and mipafox on acetylcholinesterase preparations (see legend of Fig. 6). Substrate, acetylcholine.

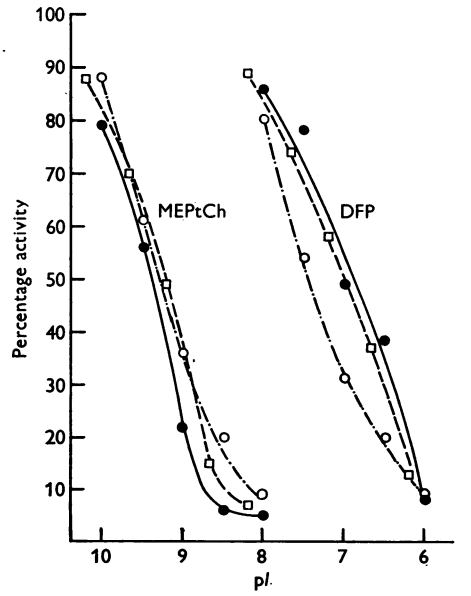


Fig. 8. Inhibiting action of MEPtCh and DFP on acetylcholinesterase preparations (see legend of Fig. 6). Substrate, acetylcholine.

The two selective acetylcholinesterase inhibitors give similar curves with all the three enzyme preparations. Both of the inhibitors selective for butyrylcholinesterase also inhibit all three acetylcholinesterase preparations at about the same concentrations. The effective concentration ranges for the acetylcholinesterase and the butyrylcholinesterase inhibitors are different.

DISCUSSION

The reason for choosing two different modifications of Michel's (1949) buffer for the determinations of the enzymic activities is that it was considered an advantage to use the standard methods for erythrocyte and serum cholinesterase already available in this laboratory. The only difference between the buffers of importance for this work is their different buffer capacity. As pointed out under Methods and Materials it is necessary to correct the activity values obtained for the spontaneous hydrolysis of the substrates. This hydrolysis is certainly rapid at the higher substrate concentrations and may cause considerable errors if disregarded (see Table 1). Hydrolysis measured at the lower concentrations, in the presence of inactivated enzyme, though small, was still too high to be accounted for by spontaneous hydrolysis (e.g. Tammelin, 1958*a*). Since at the time a plausible explanation for this was lacking it was considered better to use blanks with inactivated (heated to 100°) enzymes for every substrate and enzyme preparation instead of calculating the appropriate values.

One aim of this investigation was to confirm the classification of the cholinesterase activity of body muscles of goldfish as an acetylcholinesterase and to compare it with the acetylcholinesterase preparations from some well-known sources—erythrocytes and electric organ. The following relationships, as given among others by Augustinsson (1957), were considered suitable for investigation: (1) the form of the substrate concentration–activity curves; (2) the influence of different inhibitors, both reversible and irreversible, selective and unselective.

The results obtained with the different substrates make it clear that the goldfish preparation shows properties very similar to those obtained by the two other recognized sources of acetylcholinesterase. The preparation differs from the one containing butyrylcholinesterase. It is clear that when butyrylcholine, which may be regarded as a specific substrate for butyrylcholinesterase, is used little or no activity is obtained with the acetylcholinesterase preparations. The optimum substrate concentrations for the three preparations differ according to the source of enzyme and the substrate. This is well known (e.g. Augustinsson, 1949),

and does not modify the conclusion that acetylcholinesterase is present.

Of those tested here the only preparation splitting triacetin vigorously was that of the erythrocytes. This was to be expected, since acetylcholinesterase from erythrocytes does hydrolyse triacetin; furthermore, the preparation was not free from ali-esterases (Whittaker, 1951). Augustinsson (1949) has obtained a considerable activity with this substrate in preparations from both electric organ of electric eel (*Electrophorus electricus*) and muscles of guppy (*Lebistes reticulatus*). Acetylcholinesterase preparations from different fishes thus seem to differ in this respect.

An esterase is considered to be a cholinesterase if it is inhibited by 10^{-5}M-physostigmine (Augustinsson, 1957). This is true for the acetylcholine-hydrolysing activity of the goldfish preparation, as well as for those of electric ray and erythrocytes (Fig. 6). B & W 284C51 has been used as a selective inhibitor of acetylcholinesterase by several authors (e.g. Austin & Berry, 1953; Holmstedt, 1957). Recently, Tammelin (1958*b*) has shown methylethoxyphosphorylthiocholine to be a selective inhibitor of acetylcholinesterase. On the other hand, mipafox and DFP are known to be selective inhibitors of butyrylcholinesterase, their pI_{50} values [$-\log$ (concn. of inhibitor)] being between 7 and 8 (Holmstedt, 1957).

The results given in Figs. 7 and 8 show conformity between the three preparations tested. This is particularly clear for the two selective inhibitors of acetylcholinesterase. Also when treated with the selective inhibitors of butyrylcholinesterase the three preparations behave rather similarly. None is inhibited at significantly lower concentrations of the inhibitors than any of the others, the pI_{50} values being about 4 (mipafox) and 7 (DFP), instead of 7 and 8 as for butyrylcholinesterase. It thus seems reasonably well established that the extract from body muscles of goldfish contained acetylcholinesterase activity. Its substrate and inhibitor relations agree with those of the recognized acetylcholinesterase preparations from the electric organ of the electric ray and from erythrocytes.

SUMMARY

An extract from body muscles of goldfish (*Carassius auratus*) has been shown to contain acetylcholinesterase with the same substrate and inhibitor relationships as acetylcholinesterase preparations from human erythrocytes and from electric organ of electric ray (*Torpedo ocellata*).

My sincere thanks are due to the chief of this Institute, Professor G. Ljunggren, for his kind interest in my work, and to Professor P. E. Lindahl, Institute of Zoophysiology, University of Uppsala, for his valuable criticism. The skilful technical assistance of Mrs Kerstin Sjöström and Miss Ingegerd Brunnhagen is gratefully acknowledged.

REFERENCES

Aldridge, W. J. (1953). *Biochem. J.* **53**, 62.
Augustinsson, K.-B. (1949). *Arch. Biochem.* **23**, 111.
Augustinsson, K.-B. (1957). In *Methods of Biochemical Analysis*, vol. 5. Ed. by Glick, G. New York: Interscience Publ.
Austin, L. & Berry, W. K. (1953). *Biochem. J.* **54**, 695.
Holmstedt, B. (1957). *Acta physiol. scand.* **40**, 322.
Lundin, S. J. (1958). *Experientia*, **14**, 131.
Michel, H. O. (1949). *J. Lab. clin. Med.* **34**, 1564.
Surgenor, D. M. & Ellis, D. (1954). *J. Amer. chem. Soc.* **76**, 6049.
Tammelin, L.-E. (1953). *Scand. J. clin. & Lab. Invest.* **5**, 267.
Tammelin, L.-E. (1957*a*). *Acta chem. scand.* **11**, 487.
Tammelin, L.-E. (1957*b*). *Acta chem. scand.* **11**, 1340.
Tammelin, L.-E. (1958*a*). *Svensk kem. Tidsk.* **70**, 157.
Tammelin, L.-E. (1958*b*). *Ark. Kemi*, **12**, 287.
Whittaker, V. P. (1951). *Physiol. Rev.* **31**, 312.

An Unusual Property of Thyroxine and Other Iodophenols

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In the course of the work on enzymic deiodination of thyroid hormones, L-thyroxine and 3:5:3′-triiodo-L-thyronine, it was necessary to determine whether, and if so to what extent, spontaneous deiodination might occur. In some of the preliminary control experiments, which consisted of incubating thyroxine in enzyme-free aqueous buffer solutions, a surprising observation was made: when a solution of ^{131}I-labelled thyroxine in an aqueous organic solvent was diluted with a relatively large volume of aqueous buffer, there occurred virtually instantaneously a loss of radioactivity originally present in thyroxine. But later, mild shaking of the solution, without any other treatment whatsoever, produced a gradual reappearance of the lost radioactivity as thyroxine.

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